# Getting around the roundworms: Identifying knowledge gaps and research priorities for the ascarids

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## Abstract

The ascarids are a large group of parasitic nematodes that infect a wide range of animal species. In humans, they cause neglected diseases of poverty; many animal parasites also cause zoonotic infections in people. Control measures include hygiene and anthelmintic treatments, but they are not always appropriate or effective and this creates a continuing need to search for better ways to reduce the human, welfare and economic costs of these infections. To this end, Le Studium Institute of Advanced Studies organized a two-day conference to identify major gaps in our understanding of ascarid parasites with a view to setting research priorities that would allow for improved control. The participants identified several key areas for future focus, comprising of advances in genomic analysis and the use of model organisms, especially *Caenorhabditis elegans*, a more thorough appreciation of the complexity of host-parasite (and parasite-parasite) communications, a search for novel anthelmintic drugs and the development of effective vaccines. The participants agreed to try and

maintain informal links in the future that could form the basis for collaborative projects, and to co-operate to organize future meetings and workshops to promote ascarid research.

## Abbreviations

| | |
|---|---|
| **5-HT** | 5-hydroxy tryptamine (serotonin) |
| **Ach** | acetylcholine |
| **AP** | action potential |
| **ASABF** | Ascaris suum antibacterial factor |
| **BAL** | bronchoalveolar lavage |
| **ESP** | excretory/secretory products |
| **EV** | extracellular vesicles |
| **GABA** | gamma amino butyric acid |
| **GluCl** | glutamate-gated chloride channel |
| **miRNA** | microRNA |
| **MPLA** | monophosphoryl lipid A |
| **nAChR** | nicotinic acetylcholine receptors |
| **PBMC** | peripheral blood mononuclear cells |
| **PI** | post infection |
| **piRNA** | Piwi-interacting RNA |
| **RMP** | resting membrane potential |
| **siRNA** | small interfering RNA |

## 1. Introduction

The ascarids are a group of large parasitic nematodes of considerable medical and veterinary importance, causing serious problems for humans and their domestic animals, including poultry; the significance of some important members of this group is summarized in Table 1. Important genera within this group include *Ascaris, Parascaris, Toxocara, Toxascaris, Ascaridia, Baylisascaris, Heterakis* and *Anisakis*. *Ascaris lumbricoides* is one of the most important parasitic nematodes infecting humans and several of the others, such as *Toxocara* and *Baylisascaris*, cause serious zoonotic infections. At the end of 2021, the Loire Valley Institute for Advanced Studies, 'Le Studium', organized a conference entitled 'New Approaches to get Around Roundworms', which had the stated aim of identifying the gaps in our current knowledge of ascarid parasites that are hampering our efforts to control them. This chapter is a result of discussions held during that conference, where several general themes emerged. These include the central importance of 'omics' to virtually all aspects of ascarid research, the continued central role for the understanding of nematode physiology and

**Table 1** Some ascarid nematodes of medical and veterinary importance.

| Parasite | Definitive hosts | Zoonotic potential | Paratenic hosts | Notes |
|---|---|---|---|---|
| *Ascaris lumbricoides* | Humans Small intestine | Cross-infection between humans and pigs possible. | Earthworms Possibly chickens and dogs? | An estimated 807 million–1.2 billion people in the world are infected. Heavy infections can obstruct the bowel. |
| *Ascaris suum* | Swine Small intestine | Cross-infection between pigs and humans possible. | Earthworms Possibly chicken and dogs? | Eggs can survive extreme conditions. |
| *Ascaridia* spp. | *A. galli* – chickens. *A. dissimilis* – turkeys Small intestine | None | Earthworms | Heavy infection can cause anorexia, diarrhea, stunted growth, listlessness, a change in behaviour, and enteritis. These are non-migrating ascarids. |
| *Baylisascaris procyonis* | Raccoons Small intestine | Can infect humans and other mammals, including dogs. | Many mammals and birds | Very severe neurological symptoms possible. Seriousness of symptoms depends on number of eggs ingested. |

| | | | | |
|---|---|---|---|---|
| *Parascaris* spp. | Horses Small intestine | None | None | *Parascaris equorum, P. univalens* and *P. trivalens* are morphologically almost identical but can be distinguished by chromosome number. |
| *Anisakis* spp. | Marine mammals Stomach | Can infect humans who eat uncooked infected fish. | Crustacea, fish, squid | Symptoms include abdominal pain, nausea, vomiting, abdominal distention, diarrhea, blood and mucous in stool, and mild fever. Non-migrating |
| *Toxocara* spp. | *T. canis* – canids *T. cati* – felines Small Intestine *T. vitulorum* – water buffalos, cattle and other *Bos* spp., Small intestine | Humans infected by eating infected paratenic hosts or ingesting eggs. None | Small mammals such as mice and other rodents None | Larvae produce various ophthalmologic lesions. Transmammary and transplacental (*T. canis*) transmission. Uptake of infective eggs with L3 mostly leads to somatic migration. Transmammary transmission from cows to calves leads to hepato-tracheal migration and patent infection. Infection of cattle occurs predominantly when kept together with buffaloes or on pastures contaminated by them. |

(*continued*)

**Table 1** Some ascarid nematodes of medical and veterinary importance. (*cont'd*)

| Parasite | Definitive hosts | Zoonotic potential | Paratenic hosts | Notes |
|---|---|---|---|---|
| *Toxascaris leonina* | Canine and feline carnivores Small intestine | Humans rarely infected by eating infected paratenic hosts or ingesting eggs. | Small mammals such as mice and other rodents | Important parasite of foxes. Can be found in both feline and canine hosts. Less pathogenic than *Toxocara* spp. Larvae mostly directly settle in the intestine but hepato-tracheal migration also occurs. |
| *Heterakis* spp. | Birds Caecum | None | Earthworms | *H. gallinarum* is the most well-known species, and transmits the protozoan parasite, *Histomonas meleagridis* in birds. Non-migrating |

pharmacology in efforts to develop new control methods and the optimal utilization of existing ones – with a particular emphasis on the effects of drugs on the nematode gut – and the need for a much better appreciation of parasite-host interaction, which are crucial for pathology and vaccine development. There was also agreement that studies on the 'model' nematode, *Caenorhabditis elegans*, will continue to inform and complement studies on the parasites themselves. Of the parasites in this group, *Ascaris* spp. are the most studied and arguably the best understood, and that is reflected both in this chapter and in the literature (Holland, 2013). However, we hope to encourage additional research on the 'even-more neglected' members of the grouping. Recent excellent reviews of *Toxocara* and equine ascarids (Bowman, 2020; Cain and Neilsen, 2022), along with *Trichuris* spp., a soil-transmitted helminth that is often considered alongside ascarids in terms of biology and control (Hubbard et al., 2023) should complement and extend the ideas presented here.

This review follows the same organization as the meeting (https://www.lestudium-ias.com/events/new-approaches-get-around-roundworms) with six sections covering some major themes; these are based on the keynote presentations given in each of these sections. There is also a final section that summarizes some of the other, shorter, presentations that were given and picks out some of the key ideas that emerged during the lively final session of the meeting. This conference aimed to be the start of a series of follow-up meetings and the establishment of an international One Health-based scientific network focussing on multi-disciplinary research and educational activities concerning ascarids entitled 'The Ascarid Research and Training Initiative (ARTI, see also Box 1). It is hoped that this network will make a useful contribution to the fight against neglected tropical diseases (Forbes et al., 2023), and to increasing the sustainability of livestock farming.

## 2. An introduction to *Ascaris* physiology and pharmacology – highlighting opportunities for anthelmintic discovery

Adult *Ascaris* worms are impressive looking parasites (Fig. 1). The adult females of the species *Ascaris lumbricoides* and *Ascaris suum* are typically ~30 cm long with a body diameter of up to 1 cm, and they make vigorous swimming motions that enable them to maintain their position in the host intestine. This large size, coupled with a relatively abundant source of

**Box 1 The Ascarid Research and Training Initiative.**

Scope:

- Fundamental as well as applied research related to ascarids, irrespective of host species
- Training concerning methods, tools and approaches addressing all aspects of ascarid infections

Aims:

- Foster scientific, multi-disciplinary collaborations
- Contribute to new knowledge to the versatile consequences of ascarid infections
- Development of new approaches to combat the negative impact of ascarid infections in both humans and animals

Approach:

- Establishment of an open network for all researchers and scientists studying ascarid related research topics
- Organize scientific meetings promoting scientific exchange and the development of multi-disciplinary research collaborations
- Highlighting the relevance of ascarid research towards international research organizations

Contact and current ARTI facilitators: For information about future ARTI activities please contact one of the following.

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material, typically from pig abattoirs, has meant that our knowledge of the physiology and pharmacology of these parasites is more advanced than for many other parasitic nematodes. In this section we provide a brief historical perspective of physiological and pharmacological studies of these globally important animals and consider the outstanding gaps in our knowledge that need to be addressed if more effective ways of control are to be developed.

The body wall muscle and motor nervous system are arguably the best understood systems in *Ascaris* and relatively the easiest to investigate. The relationship between the body wall muscle cells and the neurones in the cord was first studied by Rosenbluth who showed that the muscles had arms which extend to one of the two nerve cords, dorsal or ventral, and where the motor neurones make their synaptic connections to control locomotion (Rosenbluth, 1965). This organization of the neuromuscular system typical for all nematodes and is distinct from that of nearly all other metazoans and underpins the coordinated contraction and relaxation of the

**Fig. 1** Live *Ascaris suum* collected from the packing plant.

body wall muscle in the dorsal and ventral planes of the animal to permit a sinusoidal swimming motion. The physiology and pharmacology of this process has been investigated using an organ bath system in which strips of the worm are excised from the intact animal, suspended in an organ bath system in physiological saline and attached to a force transducer. The muscle strip is contracted by acetylcholine (ACh) (Del Castillo et al., 1963) and relaxed by gamma-aminobutyric acid (GABA) (Del Castillo et al., 1964a). As the muscle cell bags are large, 200 μm in diameter, intracellular recordings can be made and voltage and current-clamp performed using two electrodes (Martin, 1980). The first intracellular recordings were made by Del Castillo et al. (1964b). who showed the muscles had a low resting membrane potential (RMP) and can generate action potentials (APs) (Del Castillo et al., 1967). Unusually, the RMP of approximately −30 mV is based on a high resting chloride conductance. The muscle cell arms are connected by gap junctions and act as a syncytium, contracting and relaxing in a co-ordinated fashion. The processes underlying the function of this syncytium are poorly understood and certainly worthy of investigation given the obvious importance of the system to motility. The muscle APs have an amplitude of 40–50 mV, with a duration of 500 ms. Intracellular

recordings have also been made from the motor neurone processes innervating the muscle cells, which also have low, −30 mV RMPs (Walrond et al., 1985). Unlike the muscle cells, and unusually for a motor nervous system in comparison to other metazoans, there is no evidence for classic all-or-none APs in the excitatory or inhibitory motor neurones. However, the ventral and dorsal inhibitory neurones do have slow, low amplitude oscillations in membrane potential indicative of the presence of voltage-gated ion channels (Cowden et al., 1989). The input of the excitatory and inhibitory motor neurones to the body wall musculature has been mapped by stimulating the motoneuron processes and recording from muscle cells (Walrond et al., 1985). Together with the evidence that these neurones have a high input resistance (Davis and Stretton, 1989a), it has been concluded that signalling in the motor nervous system is passive with low amplitude signals conducted without decrement along the long neuronal processes, resulting in graded synaptic transmission (Davis and Stretton, 1989b).

The central nervous system is distributed in anterior and posterior ganglia. The anterior and posterior central neurones have been mapped by Goldschmidt (1908), who identified 298 neurones and a very simple nervous system in terms of its neuroanatomy. The central neurones have few arborizations and appear to have physiological characteristics similar to the motor neurones in the nerve cords. For example, retrovesicular ganglion neurones have spontaneous rhythmic activity, 10 mV in amplitude, but do not generate full APs and have a low RMP, −30 mV (Holden-Dye and Walker, 1994). The neurones are of a relatively good size in terms of the technicality of achieving intracellular recordings. However, once impaled the neurones do not have a large resting membrane potential nor do they exhibit full action potentials making it difficult to determine when good intracellular access has been obtained. For this reason, there have been relatively few intracellular recordings from central neurones which represents a large gap in our knowledge.

The reproductive system has not been studied in depth, but it is possible to dissect the *vagina vera*, the spontaneously active distal portion of the ovijector, and mount it in an organ bath in the same way as a muscle strip and record responses to various compounds (Fellowes et al., 2000). For example, ACh induced a powerful contraction of the *vagina vera* while both GABA and glutamate inhibited motility but 5-hydroxytryptamine (5-HT or serotonin) had no consistent effect. Further investigation of this system has the potential to identify new approaches to control that limit fecundity of the parasite.

The gastrointestinal system consists of the pharynx and the intestine. Although the intestinal receptors have not been well characterized, they are currently under investigation and are discussed in Section 3. The pharynx has been better characterized. It was first studied in detail by Mapes (1966) who described its structure and function. The pharynx is composed of radial muscle that contracts to open the lumen, creating a negative pressure that sucks in fluid from the external medium. Once the fluid has been sucked in, the radial muscle relaxes, and the lumen snaps shut. This forces the contents of the pharyngeal lumen into the intestine against the hydrostatic pressure of the worm. It is important that the relaxation is fast as the internal hydrostatic pressure of the worm is high. The worm must overcome this pressure in order to feed. This rapid relaxation of the radial muscles is achieved by the unusual hyperpolarizing action potentials in pharyngeal muscle, first described by Del Castillo et al. (1964b), which enable the worm to overcome high hydrostatic pressure and force the contents of the pharynx into the intestine. The ionic features of these APs, and their potential molecular determinants, have been characterized using the pharynx of the free-living nematode *C. elegans* as a model system (Franks et al., 2006). This also has a rapid relaxation of pharyngeal muscle to force the contents into the intestine. These APs are underpinned by an unusual $K^+$ channel, EXP-2, which is vital for survival (Davis et al., 1999).

The pharmacology of the *Ascaris* nervous system has particular significance from the perspective of anthelmintics as most drugs that are used to treat infections act on receptors that regulate vital behaviours such as locomotion and feeding. *Ascaris* uses the same classical neurotransmitters found in vertebrates, viz, ACh, GABA, glutamate, 5-HT, dopamine, octopamine and tryptamine (Walker et al., 2000). ACh and GABA act as neuromuscular transmitters while 5-HT has a wide range of physiological roles, viz, feeding where it stimulates the pharynx, involved in regulating metabolism (Donahue et al., 1981), locomotion (Trim et al., 2001) and egg laying (Fellowes et al., 2000). In *Ascaris* G-Protein Coupled Receptors (GPCR) have arguably been less well explored than ligand-gated ion channels, likely because the latter have long been recognized as anthelmintic targets (Martin, 1993). There is a wealth of information on nicotinic ACh receptors (Holden-Dye et al., 2013). One specific *Ascaris* nicotinic ACh receptor that has attracted recent interest is EAT-2. First characterized in *C. elegans* (McKay et al., 2004), it is found in pharyngeal muscle and is an important regulator of feeding (Choudhary et al., 2020). This receptor is distinctive and different from all other excitatory nicotinic ACh receptor agonist binding subunits in that it lacks a signature sequence of vicinal cysteines

in the agonist binding region and requires an auxiliary protein, EAT-18, to function. Such unique features are compelling when considering the potential of EAT-2 as a new anthelmintic target. There is also a very large group of neuropeptide transmitters, many of which are FMRFamide-like. The first neuropeptide identified in *Ascaris* was AF1 for Ascaris FMRF-amide-like neuropeptide 1 (Cowden et al., 1989). These peptides are surprisingly numerous and can have profound effects on the physiology of the worm (Mousley et al., 2010). The role of the neuropeptides in regulating neuromuscular physiology in *Ascaris* has arguably been the most thoroughly explored. There is evidence that AF1, AF2, PF1, PF2 and PF4 play roles in modulating the function of both ACh and GABA, providing insights into new anthelmintic strategies yet to be realized (Maule et al., 1996). Neuropeptides can act both locally at the synapse and over a long distance as neurohormones (Jékely et al., 2018). The time courses of their direct effect may also differ from classical, small molecule transmitters. For example, PF4 hyperpolarizes body wall muscle and inhibits muscle APs but is more potent and longer lasting than GABA when applied at the same concentration (Holden-Dye et al., 1997), and this may act via a ligand-gated ion channel (Purcell et al., 2002). The striking diversity of the specific neuropeptide sequences and their lack of evolutionary conservation, combined with their key physiological roles, has led to interest in their receptors to define novel pharmacophores for anthelmintic drugs (Greenwood et al., 2005). This goal has yet to be achieved but holds exciting potential for new resistance breaking therapies with a good toxicology profile. Similarly, neurotransmitter receptors that are constrained to the invertebrate phyla, or even nematode phylum, present an attractive route to new anthelmintic targets. An excellent exemplar of this is ivermectin, which targets a neurotransmitter receptor for glutamate, the glutamate-gated chloride channel, GluCl, which is not found in vertebrates (Cully et al., 1994). A systematic phylogenetic analysis of ligand-gated ion channels belonging to the CYS-loop family has identified further receptors that are not found in mammalian i.e. host nervous systems (Jones and Sattelle, 2008). One of these is a chloride channel that is gated by 5-HT (serotonin), first identified in *C. elegans* as MOD-1 (Ranganathan et al., 2000). MOD-1 is of particular interest at is appears to be restricted to the Nematoda. There are other channels of interest in this regard, dopamine-gated chloride channels, for example (Rao et al., 2009), which have yet to be fully explored.

Overall, there is still an abundance of physiology and pharmacology that is under-explained in *Ascaris*. For example, there is relatively little known about the sensory systems – olfactory, gustatory and mechanosensory – that are key regulators of the animal's behaviour. Arguably, for the adult, which inhabits a

relatively constant environment in the intestine of the host, such sensory cues are less important. But at key stages of development, for example during larval migration, these would be critical to survival. This could be further explored in terms of proprioception and chemoreception, leveraging what is known about these systems in *C. elegans* as a starting point. The hydrostatic skeleton is important in locomotory behaviour and there is a lack of information on how it is generated and maintained although there has been some interesting speculation that graded transmission at the body wall neuromuscular junction may be important (Davis and Stretton, 1989b). As indicated above, most pharmacological investigations in *Ascaris* have focused on ligand-gated ion channels and GPCRs have been less extensively studied. This may be because their physiological roles are more modulatory rather than direct and therefore anthelmintics targeting GPCRs might be predicted to have a less overt effect on *Ascaris* viability. Nonetheless, these are major drug targets in human medicine and consequently there is a wealth of chemistry that could be deployed from the perspective of drug repurposing. Here again, using the model system *C. elegans* to provide insight would facilitate this goal by providing the means to identify receptors that have a vital role in survival. Nearly all of the studies cited have used adult worms, in particular those of *A. suum*, as these are readily available and their large size facilitates the physiology experiments. As result we know rather less about the physiology of the larval stages, which have been used in in vitro studies on anthelmintics (e.g. Rew et al., 1986) and how this relates to possible differences in drug sensitivity between adults and larvae. Such differences might also reflect drug access to these stages, especially during migration. The physiology of ascarid eggs, which are extremely resistant to environmental degradation, is also imperfectly understood.

## 3. Ascaris physiology, drug action, and the gut-drug axis

Ideally, control and prevention of ascarid parasite infections uses effective sanitation and hygiene to break the life cycle of these parasites, but this is not practical in some resource-poor settings for humans nor for many domestic animals. In the absence of effective sanitation or vaccines (see Section 7), anthelmintic drugs are the only practical option for prevention and treatment.

There are three main classes of anthelmintic drugs: the benzimidazoles, the cholinergic drugs, and the macrocyclic lactones (Table 2). There is also

**Table 2** Some common benzimidazole anthelmintics, the cholinergic ligand anthelmintics, the macrocyclic lactone anthelmintics and the miscellaneous anthelmintics along with their classic target sites.

| Benzimidazole anthelmintics (β-tubulin antagonists) | Cholinergic anthelmintics (nAChR ligands) | Macrocyclic lactone anthelmintics (GluCl positive allosteric modulators) | Miscellaneous anthelmintics |
|---|---|---|---|
| Thiabendazole | Levamisole (agonist) | Ivermectin (glycone) | Emodepside (SLO-1K channel activation) |
| Albendazole | Pyrantel (agonist) | Abamectin (glycone) | Piperazine (GABA agonist) |
| Flubendazole | Oxantel (agonist) | Doramectin (glycone) | Dichlorvos, diazinon (organophosphates) |
| Fenbendazole | Morantel (agonist) | Eprinomectin (glycone) | |
| Oxibendazole | Monepantel (nAChR agonist) | Selamectin (glycone) | |
| Oxfendazole | Derquantel (antagonist) | Moxidectin (aglycone) | |
| Febantel (pro-drug) | | Milbemycin (aglycone) | |

a group of miscellaneous anthelmintics, including compounds such as emodepside. The regular use of this limited number of anthelmintic drugs has given rise to the development of resistance in nematode parasites of domestic animals, including some ascarids (Lyons et al., 2008; Collins et al., 2019, 2022; Nielsen, 2022). There is also evidence of reduced anthelmintic efficacy in parasites of humans (Krücken et al., 2017).

Thus, there is an unmet need to develop novel resistance-busting therapeutic approaches, including seeking out new anthelmintics with different modes of action than the existing anthelmintics. However, since 2000 only three new anthelmintic compounds have been introduced to the

animal, but not the human, market: emodepside, a cyclic octadepsipeptide; monepantel, an aminoacetonitrile; and derquantel, a spiroindole. Despite the clear need for anthelmintics with a novel mode of action, development is slow, in part, because of the cost of development for new drugs: US$ 2.5 billion for a human drug (DiMasi et al., 2016) and US$100 million for a veterinary one (Yarborough, 2016). We describe three research directions that we believe will support the development new anthelmintics and novel therapeutic approaches.

### 3.1 Use in silico methods to predict good drug targets and drug chemotypes to support the identification of new anthelmintics: target identification, repurposing drugs, docking studies with lead chemical structures.

Nematode parasite '-omics' have advanced dramatically over the last 10 years facilitating the development of bioinformatic target identification and in silico screening. These methods support the identification of novel targets unique to nematode parasites and can support drug target screening based on mechanisms of action of the putative drug (Consortium, 2019; Atkinson et al., 2021). The identification of 'chokepoint' enzymes in metabolic pathways, which are vital to survival of nematode parasites because there are no other metabolic routes, has been used for identification of novel therapeutic agents for cancer and other infectious diseases. The same approach can and has been used to identify molecules with in vivo broad-spectrum anthelmintic activity (Tyagi et al., 2019). The approach involves a multi-step process to assign 'Enzyme Commission' priority numbers to proteomes (drug targets) of interest (Consortium, 2019) using the programs KAAS, PRIAM, DETECTv2 and BREDA. Subsequently, the program ChEMBL can be used to identify potential drugs for the targets. It is important to select from these possible drug candidates, 'druggable' compounds that are likely to have suitable pharmacokinetic properties for a useful drug and that are non-toxic. There is also a need to check that the nematode parasite target selected does not have significant sequence similarity to a human or mouse protein with a structure in the protein data base (PDB) to reduce the potential toxicity (checked with the program PAINS) of the selected drug.

This approach, for example, suggests that ASU_10965 (Endonuclease 4: DNA polymerase delta catalytic subunit) is the putative drug target of bithionol (Trade name: Lorothidol) and that trimethoprim and dexibuprofen will interact with this drug target in *Ascaris suum* and could be of

therapeutic benefit. This approach can provide some good starting pointers for drug development. Nonetheless, any of the compounds selected in this way will still need to be tested with phenotypic screens on the potential parasite and in animal models (Tyagi et al., 2019).

Compounds like trimethoprim that have an established anti-microbial use and have completed phase IV drug trials, if they are found to have good anthelmintic action, can be repurposed or repositioned as anthelmintics at a fraction of the development costs. However, commercialization of repurposed drugs has less chance of attracting industrial investment because they are only patentable with 'method of treatment' claims but not as 'composition of matter claims': repurposed patents are also more difficult to enforce and therefore less valuable.

### 3.2 Increase our knowledge of the details of the modes of action of existing anthelmintics and the mechanisms of resistance: use state of the art methods to study the physiology and pharmacology of different *Ascaris* tissues including intestine, uterus and reproductive system, ovary, and pharynx

The mode actions of the three main classes of anthelmintics, the benzimidazoles, the cholinergic anthelmintics and macrocyclic lactones, are understood at a modest level but details of their physiological and pharmacological effects on individual ascarid parasite species and the tissues of their reproductive tract, ovaries, muscle, intestine, excretory/secretory system and pharynx are not sufficiently resolved. Mechanism of resistance to the cholinergic anthelmintics and macrocyclic lactones are also not well resolved.

The benzimidazole anthelmintics inhibit tubulin polymerization by binding to β-tubulin (isotype I). In many nematodes, mutations in β-tubulin such as F200Y, F167Y, G198Y, E198A, E198I, E198K, E198T, E198stop and Q134H (Chaudhry et al., 2015; Dilks et al., 2021) are associated with resistance to this class of anthelmintic. Some of these polymorphisms are present in ascarids (Martin et al., 2021), but they have not been confirmed to be relevant to drug resistance. The effects of mebendazole are seen on the intestine of *Ascaris suum* after six hours of oral administration to the host pigs (Borgers and De Nollin, 1975). The enterocytes of the intestine are damaged and show a loss of secretory granules in the terminal web and formation of autophagic vacuoles and loss of cytoplasmic microtubules. Benzimidazoles, in addition to damaging the

intestine leading to starvation of the parasite, also affect egg production of the parasites. They reduce egg production and development and inhibit cell division and production of parasite eggs. Separated ascarid tissues including the reproductive tract, the cells of the ovary and testis, body muscle cells, the intestine and pharynx have not received detailed physiological and pharmacological examination to determine the time course of the onset of and the sensitivity to the benzimidazoles; we do not know which are the most sensitive of these tissues.

The cholinergic anthelmintics act on the nematode parasite neuromuscular system with the agonists, levamisole, pyrantel, morantel, and oxantel (Martin et al., 1996; Martin, 1997) opening nicotinic acetylcholine receptor (nAChR) channels to depolarize nerves and muscles, resulting in a spastic contraction. In contrast, derquantel acts as a selective nematode nAChR antagonist (Robertson et al., 2002) and monepantel is a non-competitive antagonist (Abongwa et al., 2018). The nAChR channels are made up of five subunits which are encoded by up to 27 different genes in nematodes, though the exact repertoire varies greatly between species. The nAChR genes are expressed differently in different nematode species and tissues, giving rise to multiple receptor subtypes. For example, on *A. suum* body-wall muscle there are *N-, L-,* and *B*-subtypes which are preferentially selective for the agonists nicotine, levamisole and bephenium, respectively (Qian et al., 2006). An additional complication is that the sensitivity to an anthelmintic and expression of the receptor subunit RNA is not necessarily constant and can decrease after exposure to the drug. The sensitivity to cholinergic anthelmintics can be plastic (Kashyap et al., 2021), varying with prior exposure to anthelmintics. We do not yet understand the mechanisms involved in the changes in sensitivity (plasticity). This is an important process to investigate because such mechanisms can contribute to the development of resistance. We currently also do not know how the different tissues of the different ascarid species vary in their sensitivities to the different cholinergic anthelmintics and if the reproductive tract is more sensitive than the muscle or the intestine to the effects of levamisole. The species differences are also important: oxantel is not very potent on *A. suum* muscle (Dale and Martin, 1995), but it is potent on *Trichuris suis* ACR-16 receptors (Hansen et al., 2021). Clearly, we need more information about the different sensitivities of the ascarid parasites to the cholinergic anthelmintics. Resistance to pyrantel has been reported in *Parascaris* spp. (Martin et al., 2018), but we do not have a clear understanding of the mechanism of resistance in any of the different ascarid species. It may involve loss or mutation of one or more of the

nAChR genes, including elevated expression of splice variants as described in other parasitic nematodes (Courtot et al., 2023), or it may involve increased desensitization and stress response genes (Feder and Hofmann, 1999). Changes in the subunit composition of recombinant *A. suum* nAChR have been reported to change their sensitivity to cholinergic anthelmintics in vitro (Williamson et al., 2009).

The macrocyclic lactones are positive allosteric modulators of inhibitory GluCl ion-channels that are found in invertebrates. In *C. elegans* there are six genes that code for the GluCl channel subunits: *avr-14*, *avr-15*, *glc-1*, *glc-2*, *glc-3*, and *glc-4* (Cully et al., 1994; Cully et al., 1996; Dent et al., 1997; Vassilatis et al., 1997; Dent et al., 2000; Horoszok et al., 2001; Li et al., 2014; Ballesteros et al., 2016) but the gene family is divergent in parasitic nematodes and varies between parasitic nematodes. AVR-14, GLC-2, GLC3 and GLC-4 are the most conserved GluCl subunits throughout the Nematoda phylum (O'Halloran, 2022) and are present in *A. suum* and *Parascaris spp*. Except for GLC-4, all of *C. elegans* subunits can form functional homomeric channels when expressed in *Xenopus laevis* oocytes. When the *C. elegans* subunits were studied under two-micropipette voltage-clamp, all the homomeric channels were ivermectin-sensitive, except for those formed by Cel-GLC-2. However, we have a limited number of studies of expressed nematode parasite GluCls. Interestingly, expression of AVR-14, GLC-2, GLC3 and GLC-4 from *P. univalens* has been studied and may provide a useful guide for ascarids (Lamassiaude et al., 2021, 2022). Pun-GLC-2 and Pun-GLC-3 can assemble into heteromeric receptors that generate strong currents but Pun-GLC-2/Pun-GLC-4 and Pun-GLC-3/GLC-4 produce small or no currents, suggesting that they do not readily assemble to produce viable receptors, at least in the *Xenopus* oocyte. The Pun-AVR-14B/Pun-GLC-2 heterodimer is sensitive to ivermectin, moxidectin, abamectin, doramectin, emamectin, eprinomectin and selamectin. Interestingly, the Pun-GLC-2/Pun-GLC-3 heterodimer is more sensitive to the agonist, glutamate, than the Pun-GLC-3 homomer; the heterodimer is not directly activated by ivermectin or the other macrocyclic lactones alone although ivermectin did potentiate the effects of glutamate on this heterodimer.

We now have some very interesting observations about heteromeric dimers of the GluCl subunits when expressed in *Xenopus* oocytes. We know that there are species variations but how do the macrocyclic lactones exert their broad-spectrum therapeutic effect? Which tissues in the different nematode parasite are affected by the macrocyclic lactones and what

is the relative contribution of the different GluCl heteromeric combinations of subunits? To address these questions studies employing RNAscope in situ hybridization assays (McHugh et al., 2020) and spatial transcriptomics (Ståhl et al., 2016; Asp et al., 2020) will provide cellular and subcellular distributions of the expression of the different genes in the different tissues of the nematode parasites, which should shed light on these unresolved issues.

Macrocyclic lactone resistance has been reported in *Parascaris spp.* (Boersema et al., 2002; Lyons et al., 2008) infecting foals. Responses to different classes of anthelmintics, including macrocyclic lactones, of live worms collected at the abattoir were reported to show changes in phase I metabolic pathway short-chain dehydrogenase/reductase superfamily, flavin containing monooxygenase superfamily and cytochrome P450-family, and the membrane transporters major facilitator superfamily and solute carrier superfamily (Dube et al., 2022). These studies indicate that metabolism and excretion of macrocyclic lactones needs further study in ascarids and their association with macrocyclic lactone resistance. Recent observations (Doyle et al., 2022; Laing et al., 2022) suggest that the transcription factor, *cky-1,* and protein kinases may be involved in regulation of excitation and sensitivity to ivermectin is associated with resistance in *Haemonchus contortus*. It will therefore be of great interest to determine if this gene also plays a part in macrocyclic lactone resistance in ascarids.

### 3.3 Develop rational synergistic anthelmintic combinations that delay resistance using improved knowledge of the mode of drug actions and recognizing the intestine as a focus of drug metabolism, excretion, and site of action: the Gut-Drug Axis

We have commented above on the slow development of new anthelmintics, despite the unmet need for these new drugs. We also know that continued monotherapy with an anthelmintic drug can give rise to the development of resistance quite quickly – it's a Darwinian process – with the nematode parasite subjected to a severe selection pressure so that only resistant individuals survive. Here we comment on the MISER (**m**ultiple **i**ndependent **s**ites-of-action **e**vading **r**esistance) principle to slow the development of resistance:

If an anthelmintic has a single target protein with a single gene with a recessive anthelmintic resistance allele and only a fraction, *f1r*, of a

particular parasite population carries that allele, then the probability of seeing resistance in the diplotype population would be $f1r^2$ in the absence of any selective pressure. The use of a second anthelmintic that acts on a different target protein with a single gene with a recessive anthelmintic resistance allele with a fraction, *f2r*, of the population carrying a single copy of that gene, then if the anthelmintics act independently then the probability of seeing resistance would be: $f1r^2$ x $f2r^2$, a lower probability. This is the MISER concept: combinations of anthelmintics that do not have antagonistic effects on each other are anticipated to see a lower probability of resistance. The description above is oversimplified with only one target protein being involved in the resistance to each anthelmintic. Ascarid parasites have a 42–700 mega-base genome and are capable of different types and levels of resistance in addition to drug target mutations producing resistance. Contributing factors that require further investigation in ascarids include: increased drug metabolism; reduced entry and/or excretion of the anthelmintic drug; increase in the stress responses and homeostatic plasticity (Kashyap et al., 2021).

The ascarid intestine (Fig. 2) is a major feature of a transverse section of the nematode body. The ascarid intestine carries out vital functions that include: (1) digestion and nutrient absorption (Behm, 2002; Munn and Munn, 2002); (2) pH regulation via apical membranes V-type ATPases (Rosa et al., 2015); (3) storage of lipids (Mullaney et al., 2010); (4) innate immunity (Yin et al., 2008; Pukkila-Worley and Ausubel, 2012); (5) secretion of bactericidal peptides (Jasmer et al., 2015); (6) drug metabolism with cytochrome P450 enzymes and UDP transferases (Laing et al., 2015) and; (7) excretion by organic-anion-transporter (OAT-1), P-glycoprotein- and MDR-transporters into the lumen of the intestine (Yin et al., 2008; Rosa et al., 2015; Chelladurai and Brewer, 2019). Damage to the cells of the intestine will adversely affect these vital functions.

The intestine is an important site of action of anthelmintic drugs in ascarids. The ascarid intestines are sensitive to the actions of the benzimidazole anthelmintics (Borgers and De Nollin, 1975). Intestinal tubulin is a target for the chemotherapeutic action of mebendazole in parasitic nematodes (Köhler and Bachmann, 1981), as well as levamisole (McHugh et al., 2020), and Cry5B, a pore-forming protein produced by the soil bacterium *Bacillus thuringiensis* (Hu et al., 2010; Urban et al., 2013). The development of macrocyclic lactone resistance, in part, appears to involve increased exclusion from the parasite mediated by P-glycoproteins

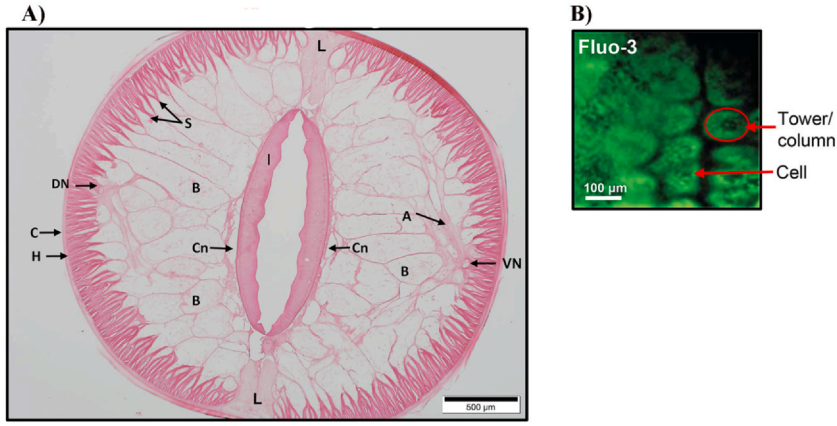


**Fig. 2** (A) Transverse section of female *Ascaris suum* in the anterior region of the worm. I: intestine of the worm. Cn: Canaliculi – canals for perienteric fluid. B: Bag region of the muscle cell. A: arm region of the muscle cells. S: spindle region of the muscle cells. L: lateral line. DN: Dorsal nerve cord. VN: Ventral region of the nerve cord. C: Cuticle. H: Hypodermis. (B) Whole mount preparation of the *Ascaris* intestine stained with the calcium dye, Fluo-3 for detection of calcium responses to anthelmintics. Notice that some of the cells (enterocytes) of the intestine are raised into folds that form towers or columns. *Both panels modified from McHugh, M., Williams, P., Verma, S., Powell-Coffman, J.A., Robertson, A.P., Martin R.J., 2020. Cholinergic receptors on intestine cells of Ascaris suum and activation of nAChRs by levamisole. Int. J. Parasitol. Drugs Drug. Resist. 13, 38–50, under Creative Commons CC-BY-NC-ND.*

(Jannsen et al., 2013), some of which are found in the intestine. For example, *Peq-pgp-11* and *Peg-pgp-16* mRNA can be visualized in *Parascaris* intestine (Chelladurai and Brewer, 2019).

We suggest that the study of the functional effects of anthelmintic actions and interactions on the intestine is important for understanding anthelmintic interactions, and these interactions have been relatively neglected in favour of drug actions on the nervous system. Using calcium fluorescence to study the effects of cholinergic anthelmintics (Fig. 2), it can be seen that levamisole produces a rapid rise in intracellular calcium levels in intestinal cells due to stimulation of the nAChRs present on these cells (McHugh et al., 2020). Given the vital function that the intestine of the nematode performs, more research on the action of anthelmintics and combinations of anthelmintics, such as benzimidazoles plus cholinergic anthelmintics or Cry5B, may help us to design more effective anthelmintic combinations if we can identify synergistic interactions.

## 4. Host-environmental interactions of *Ascaris*

Ascarid parasites cause complex diseases due to the intricate lifecycles of the nematodes, which can include extensive body migrations of the larvae, which may be incomplete in accidental hosts. For example, after being hatched from the eggs, *Ascaris* larvae migrate from the intestine to the liver, further to the lung and via the pharynx back to the intestine, where they develop to adult worms and reside in the small intestine. During the comprehensive body migration, *Ascaris* larvae encounter different host tissues and thus different host environments. Effectively, during the different phases of the infection, the parasites engage in multilateral interactions within their host: L3 and adult worms interplay with the host bacterial environment in the gut, host gut nutrients directly and indirectly impact the *Ascaris* infection, and *Ascaris* interacts with host cells, both immune cells and non-immune cells, at the host barrier. However, there are also inter- and intra-species interactions between ascarids and other parasites. Some ascarids have paratenic hosts during their life cycle and must therefore have specific interactions with these hosts in addition to those with their definitive hosts. Some of these paratenic hosts are invertebrates and other poorly studied species, and details of these interactions are scarce; invertebrates may also disperse eggs without acting as genuine paratenic hosts (the exact definition of 'paratenic host' caused some debate amongst the contributors to this chapter). Parasites have also been found in unusual locations within the definitive host (Freeman et al., 2022); the frequency of such events is completely unknown. This section will therefore concentrate on the interactions of *Ascaris* spp. with their mammalian hosts in the context of the conventional life-cycle as a starting point for the extension of such studies into other ascarid species.

In recent years a lot of evidence has appeared that indicates a dominant role for the excretory/secretory products (ESP) of the worms, and within the ESP, the extracellular vesicles and metabolites, to act upon the host environment. Thus, ESP are hypothesized to influence host tissues, immune and non-immune cells as well as the gut microbial environment. In this section, we emphasize the different multilateral interactions of *Ascaris* with its host environment and extract from it the outstanding questions for future research (Fig. 3).

### 4.1 Mediators of *Ascaris* interaction with its host: excretory/secretory products, and extracellular vesicles

*Ascaris* releases many molecules into its environment, which are predicted to be essential for host-parasite interaction but also for interaction with

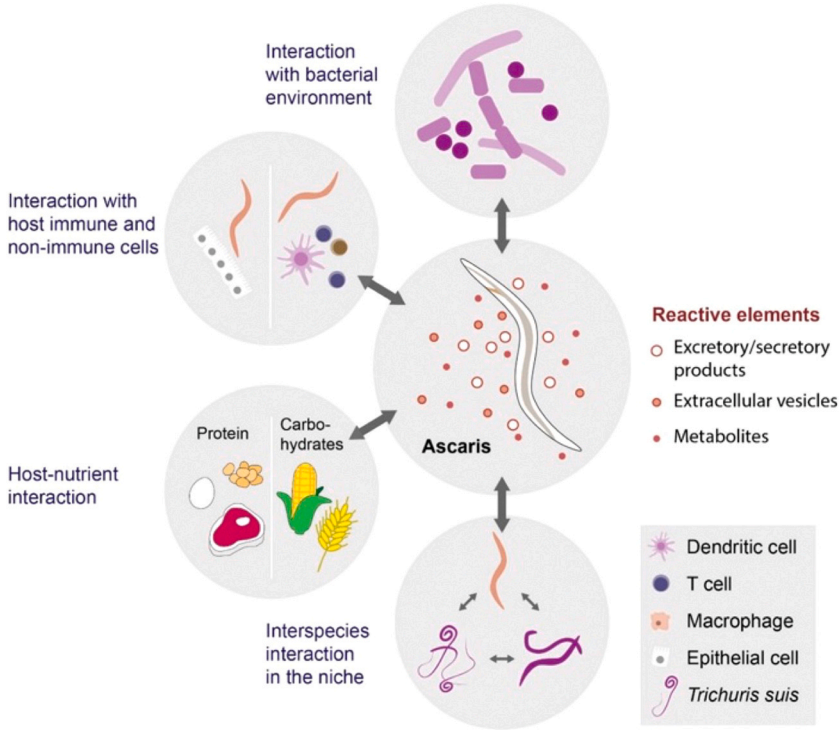


**Fig. 3** Overview of host environmental interactions of *Ascaris*. *Ascaris* shows a complex life cycle in which they encounter different environmental challenges. These multilateral interactions include the interwork with the bacterial gut microbiota, the confrontation with host immune and non-immune cells, direct and indirect exploitation of host nutrients and interspecies communication. Reactive elements mediating these interactions are excretory/secretory products of the worms as well as extracellular vesicles and worm metabolites.

other parasites and the microbiome. The helminth/helminth and helminth/microbiome interactions are dealt with in detail elsewhere in this Chapter; therefore, the focus here is the interaction between *Ascaris* released molecules and its host. ESP are molecules released from the cuticle, the intestine and excretory/secretory organs. Proteomic analysis has identified mainly stage-specific proteins in the ESP from infective L3 (17 proteins), lung L3 (31 proteins) and intestinal L4 (43 proteins) larvae, but 14–3–3-like protein and serpin-like protein were released from all stages (Wang et al., 2013). 14–3–3-like proteins regulate multiple pathways by binding to signalling molecules, and serpins are a large class of protease inhibitors that can inhibit target enzymes and are involved in a number of

functions including complement activation and inflammation. From the adult stage, Hansen et al. (2019) identified 129 proteins in the ESP, including several kinases, oxidases, peptidases and proteinases where the latter may function in food degradation and host immune modulation. Immune modulatory effects of ESP have been demonstrated for multiple helminths (Johnston et al., 2017; Laan et al., 2017; Leroux et al., 2018), but the majority of studies with *Ascaris* have focused on *Ascaris* body fluid (ABF), the biological relevance of which in an infection biology context is controversial, despite ABF showing potent immune regulatory effects on multiple cell types (Dall et al., 2022; Midttun et al., 2018). However, some studies have identified immune modulatory effects of individual proteins that whilst commonly present in ABF (Coronado et al., 2017, 2019; Oshiro et al., 2006) are also present in larval ESP, including PAS-1, shown to inhibit LPS mediated pro-inflammatory cytokine production and T cell responses to bystander antigens via induction of IL-10 (Antunes et al., 2015; Oshiro et al., 2006).

The ESP has recently been shown to contain extracellular vesicles (EVs) (Borup et al., 2022; Hansen et al., 2019; Minkler et al., 2022). EVs are membrane-enclosed nanoparticles released from cells from organisms of all three domains of life that can traffic bioactive lipids, proteins and nucleic acids between cells, both on short, and long distances, as a lipid bilayer protects the cargo. Whilst showing similarities to mammalian EVs, some unique properties of helminth EVs have been identified, and therefore it has been speculated that EVs may be important for host-helminth interaction (Boysen et al., 2020; Buck et al., 2014; Coakley et al., 2017; Simbari et al., 2016; Whitehead et al., 2020; Zakeri et al., 2018). Indeed, Hansen et al. (2019), characterized the protein and microRNA content of *A. suum* EVs released from the L3, L4 and adult stages and found a rich arsenal of molecules that may be important for establishment and survival of *A. suum* in the pig host. In silico micro (mi) RNA target prediction identified potential host immune targets, but functional studies are lacking to confirm these predictions (Hansen et al., 2019). More recently, Minkler et al. (2022) have identified the presence of circular RNAs in the EVs of *A. suum* that may act as 'miRNA sponges', like circular RNAs in the human host, thereby negating the effects of host miRNA whilst delivering its own immune modulatory miRNAs. Borup et al. (2022) found that the EVs could downregulate LPS-induced pro-inflammatory responses in human PMBCs while the EV-depleted ESP had no such effect suggesting a crucial role of EVs in host-parasite interaction for *A. suum* in contrast to that

shown for ESP of other nematodes. It remains to be determined whether the infection niche of *Ascaris* in the intestinal lumen, compared to other helminths that are located in close contact with the host epithelia, may be a determinant of the increased immune modulatory effects of *Ascaris* EVs compared to EV-free ESP (Borup et al., 2022).

## 4.2 *Ascaris* – host-microbiome interaction

Following a body migration phase, *Ascaris* settle in the jejunum of their hosts where they develop into sexually mature adults, which may survive for more than a year (Olsen et al., 1958). Interactions between *Ascaris* and microbes could therefore be beneficial or detrimental to the parasite and/or the host. Though most of its life is spent in a microbial environment within the intestine, many questions remain regarding interactions between microbes and *Ascaris* in particular.

Recently, several studies have documented alterations in the host microbiota during *Ascaris* infection in pigs (Wang et al., 2019; Williams et al., 2017) and humans (Kupritz et al., 2021). In the pig, Williams et al. (2017) reported increased diversity in the host colonic microbiota at 14 days post-infection, shortly after *Ascaris* returns to the intestine after its hepato-tracheal migration (Williams et al., 2017). In contrast, during the adult-stage of the infection, Wang et al. (2019) reported reduced microbial diversity in the colon at 54 days post-infection, while Midha et al. observed reduced diversity at the site of infection in the jejunum at 56 days post-infection (Midha et al., 2022). Enrichment of specific taxa in the *Ascaris*-infected gut was also observed across different studies. *Lactobacillus, Megasphaera, Prevotella* and *Succinivibrio* all appear to be enriched in the porcine colon (Wang et al., 2019; Williams et al., 2017). Interestingly, *Prevotella* and *Succinivibrio* spp. Were also found to be strongly associated with *Ascaris* infection in humans (Kupritz et al., 2021). Several studies have reported expansion of lactobacilli in different murine helminth infection models (reviewed in Midha et al., 2020); Reynolds et al. (2014) demonstrated that a *Lactobacillus* species promotes itself and infection with *Heligmosomoides polygyrus*. Whether the species enriched in the host gut during *Ascaris* infection have a similar commensal relationship is yet to be determined. Additionally, the metabolomic consequences of *Ascaris* infection have thus far not been reported.

In addition to living within the host gut microbiota, *Ascaris* itself has an intestine which is colonized by microbes (Hsu et al., 1986; Nalin and McLaughlin, 1976; Shahkolahi and Donahue, 1993). Bacteria can be

cultured from the intestine of adult *Ascaris* (Hsu et al., 1986), even after the worms have been exposed to antibiotics for several days (Shahkolahi and Donahue, 1993). *Ascaris lumbricoides* expelled by human cholera patients were found to be colonized by *Vibrio cholerae* (Nalin and McLaughlin, 1976). Thus, whether *Ascaris* may serve as a reservoir for pathogens or drug-resistant microbes within the host needs to be explored. More recently, Midha et al. (2022) characterized the microbiome of adult *A. suum* and found that *Ascaris* selectively acquires its microbiome from the pool of available microbes in the small intestine of its host. Taken together, these findings demonstrate that the intestine of *Ascaris* is itself a unique niche for microbes within the host intestine. How the parasite-associated microbiota interacts with the host and other microbiota remains to be determined. Additionally, how manipulation of the host microbiome impacts worm fitness is currently unknown. Finally, how the *Ascaris* microbiome changes across different life stages is also unknown, as all studies to date have focused on adult worms.

Although our understanding of the direct impact of microbes on *Ascaris* biology is limited, numerous studies on other nematode species indicate that a multitude of interactions are likely to take place. Studies in *Trichuris muris* and *T. suis* have demonstrated a clear role in bacterial-mediated egg hatching (Hayes et al., 2010; Vejzagić et al., 2015). Studies in the free-living nematode *C. elegans* have shown that microbes may further benefit nematodes by providing nutrients (Yilmaz and Walhout, 2014), helping to protect against infection by other microbes (Rafaluk-Mohr et al., 2018), and promoting fecundity (Pike et al., 2019). In addition to beneficial effects, microbes may also be harmful to worms. *C. elegans* is subject to numerous infections, including human and porcine intestinal pathogens such as *Salmonella enterica* (Cohen and Troemel, 2015). A bacterial protein from *Bacillus thuringiensis*, Cry5B, has demonstrated anthelmintic activity against *A. suum* (Urban et al., 2013). Thus, there are ample opportunities for studying which microbes are beneficial or harmful for *Ascaris*.

*Ascaris* and other nematodes have evolved antimicrobial strategies to deal with infectious challenges in their environments. The ESP of the intestinal life-stages of *A. suum* (L4 and adults) contain numerous proteins and peptides with known or predicted antimicrobial activity, including antimicrobial peptides from the *A. suum* antibacterial factor (ASABF) and cecropin families, lysozymes, and various lectin domain-containing proteins (Midha et al., 2018). Collectively, the ESP inhibit bacterial growth, disrupt bacterial biofilm formation, and neutralize bacteria via agglutination

(Midha et al., 2018). Similar effects have been demonstrated for the ESP of *H. polygyrus* (Rausch et al., 2018). Interestingly, ASABFs and cecropins are induced upon bacterial challenge of adult *A. suum* (Pillai et al., 2003, 2005). The contribution of these antimicrobial factors to modulating the microbiota of *Ascaris* and the *Ascaris*-infected host remains to be elucidated.

Our understanding of the interactions between *Ascaris,* its own microbiota, and the host microbiota are only beginning to be deciphered. Importantly, our current understanding is primarily limited to bacteria, and future studies should also include interactions with viruses, fungi and other eukaryotic microbes. Elucidating these complex relationships has the potential to unveil novel therapeutic strategies while deepening our appreciation for the impact of *Ascaris*-microbe interactions on host physiology and ongoing coevolution occurring within the gut.

### 4.3 *Ascaris* – host-immune and -non-immune cell interactions

The lack of an ascarid helminth with tractable model organism hosts, such as mice, combined with the inability of *A. lumbricoides* to complete the infection cycle in these widely used model organisms has hampered the determination of *Ascaris* effects on host cellular responses. Much of our knowledge to date has been gleaned from human field/clinical studies and pigs infected with the closely related *A. suum*, that is notably capable of establishing patent infection in humans (Nejsum et al., 2012). Therefore, when combined with the similarities in pig and human physiology, particularly intestinal physiology, *A. suum* infection of pigs provides an excellent animal model for studying *A. lumbricoides* infection biology whilst being an economically significant helminth in its own right (Thamsborg et al., 2013).

From studies in pigs, there is a clear effect on host cell populations in the main sites of infection, liver, lung and intestinal tissue where a pronounced eosinophilia is observed upon migration of larvae and contact with worms in the intestine (Dawson et al., 2005, 2009; Williams et al., 2017). The observed influx of granulocytes in the lungs is consistent with the common presentation of eosinophilic pneumonia (Loeffler's syndrome) in *A. lumbricoides* infected humans (Else et al., 2020). Increased levels of intraepithelial T cells are also detected in pig intestine post-infection (PI) with both eosinophilia and T cell (CD3 +) levels coinciding with parasite control and expulsion (Masure et al., 2013a,b; Williams et al., 2017). Bulk jejunal transcription profiles were suggestive of a cytotoxic T cell phenotype in Masure et al. (2013), but direct characterization was not performed,

therefore the subtype of T cells expanded during *A. suum* infection is unknown. Whilst the self-cure mechanism commonly referred to as "weep and sweep" is present during *A. suum* infection in pigs and results in expulsion of most worms 17–21 days PI (Roepstorff et al., 1997), the molecular mechanisms of this are less well defined for *Ascaris* than nematodes of mice (Gerbe et al., 2016; Howitt et al., 2016). Of note, increases in intestinal macrophages, *A. suum* specific antibodies and hepato-tracheal migration are not required for *A. suum* self-cure but oral infection with L3 or L4 larvae did show a role for L3 interactions with host intestinal immune responses in parasite clearance (Masure et al., 2013b).

Investigations into the effects of *A. suum* L3 on the porcine non-transformed epithelial cell line IPECJ2 showed minimal effects on transcription of genes commonly associated with the response to helminth infections, suggesting that, as for other helminths, initial recognition and initiation of Th2 immune responses are mediated by specialized cell types such as tuft cells (Ebner et al., 2018). In support of this, transcriptomic analysis of pig jejunum after infection with *A. suum* showed Gasdermin C as the most upregulated gene compared to non-infected controls (Midttun et al., 2018) consistent with recent work describing a role for this pore forming protein in the non-conventional release of IL-33, a key first line Th2 cytokine, in a Pou2f2 (tuft cell) dependent mechanism (Zhao et al., 2022). However, the role of tuft cell derived IL-25 interactions with type 2 innate lymphoid cells (ILC2s) (Von Moltke et al., 2016) remains to be fully elucidated for *Ascaris*.

Indeed, L3 but not L4 were capable of inducing eosinophil degranulation in the presence of infected pig serum (Masure et al., 2013b) and in vitro co-incubation of *A. suum* larvae with eosinophils and serum from infected pigs resulted in reduced mobility and viability of larvae (Coakley et al., 2020; Masure et al., 2013a). *Ascaris* antigens specifically induced histamine release from mast cells isolated from the intestines of *A. suum* infected pigs but not controls (Ashraf et al., 1988). Animals pre-exposed to *Ascaris*, and therefore possessing some immunity, showed increased numbers of eosinophils, goblet cells and mast cells in caecum of infected pigs suggesting a role for these cell types in pre-hepatic immunity to *Ascaris* upon challenge (Urban et al., 1988).

Consistent with the increased numbers of Th2 effector cells in pigs, controlled infection of human participants with *A. suum* showed Th2 skewed responses in infected person's PBMCs with an increase in IL-4 levels at days 66 and 88 post-infection compared to baseline (Da Silva et al., 2021). Similar results were shown in the serum of *A. lumbricoides* infected

children that had increased IL-4 and IL-5 levels compared to uninfected controls (Shalaby and Shalaby, 2016). An increased frequency of IL-4 and IL-5 secreting cells were observed in PBMCs from *A. lumbricoides* infected humans when stimulated with *Ascaris* antigens (Cooper et al., 2001). Although in this study polyclonal stimulation using mitogens did not show significant differences between infected humans and controls (Cooper et al., 2001), other studies showed significant reductions in Th1 cytokines in response to polyclonal stimulation of PBMCs from *A. lumbricoides* infected individuals compared to both endemic and non-endemic controls (Geiger et al., 2002). This is potentially the result of differences in infection biology of *A. suum* and *A. lumbricoides* (Deslyper et al., 2020), worm burden or time of analysis. Changes in peripheral cellularity were not present in human infections with *A. suum* and therefore it is likely that eosinophilia is restricted to local sites of infection (Silva et al., 2021), as in pigs.

In conclusion, whilst there are several gaps in our knowledge of the interactions between *Ascaris* and host-cells, it is clear that a Th2 driven response is present and involved in initial self-cure and also subsequent partial immunity to re-infection as postulated earlier (Jackson et al., 2004). However, the development of immunological reagents for porcine models will be crucial to elucidating molecular pathways of host recognition and clearance of *Ascaris* spp. infections in the coming years.

## 4.4 Parasite-parasite communication and parasite population regulation

*Ascaris* nematodes need to communicate with each other, for example, during mating. Can they also communicate in different ways to regulate their population or recognize their own kin, and does *Ascaris* communicate with other parasites species?

The primary expulsion of *A. suum* happens within the first month after infection, depending on the infection regime (Nejsum et al., 2009; Roepstorff and Murrell, 1997), and hereafter about 20% of the host population carries 80% of the worm load. Nejsum et al. (2009) have shown that 45% of this phenotypic variation in worm load can be attributed to host genetics (Nejsum et al., 2009). However, under continuous exposure, is *A. suum* also able to regulate its population to avoid crowding? Although infected individuals seem resistant to further infection with *Ascaris*, a phenomenon called concomitant immunity, reinfection occurs rapidly after anthelmintic treatment in humans and pigs, suggesting that removing the existing population allows a new cohort to establish. This reinfection may

relate to a relaxation in immunity, but as it happens within weeks (Boes et al., 1998), this observation may also suggest that the existing parasite population prevents new incoming larvae from establishing. Under continuous *A. suum* exposure, the population grew to maturity with time, but some immature larvae were still observed 14 weeks post first infection (Mejer and Roepstorff, 2006; Nejsum et al., 2009). These immature larvae may represent stunted worms but are more likely worms from new infections that cannot establish due to the existing adult population. In conclusion, a current population of worms does not prevent new incoming larvae from migrating to the liver and the lungs but may directly prevent them from establishing in the small intestine. In this way, crowding can be minimized which increases the existing population's fitness.

Several studies have shown that related *Ascaris* genotypes can be found in the same hosts (Anderson et al., 1995; Nejsum et al., 2005). This suggests that the host becomes infected by ingesting genetically related eggs as these may be clustered in the environment (for example by originating from the same faecal sample). Alternatively, *Ascaris* eggs may be randomly distributed in the environment, but different host genotypes select for specific *Ascaris* genotypes. In favour of the latter explanation, Nejsum et al. (2009) found that despite pigs being infected with the same share of *A. suum* eggs from four different females, the proportion of the offspring differed significantly between pig hosts. Interestingly, this difference increased with time post-infection. Remarkably, within a single host, related *Ascaris* genotypes aggregated along the intestinal tract. Whether this is due to direct worm communication and kin recognition allowing siblings to assemble or the different location in the intestine reflects different "fitness profiles" remains unknown.

Although *Ascaris* may regulate its population and recognize its kin, is there any communication with other parasite species? *Ascaris lumbricoides* has been found to be positively associated with *Trichuris trichiura* infection, while no association was found with hookworms (Lepper et al., 2018). Similar transmission routes may explain this for *T. trichiura* and *A. lumbricoides,* but it is interesting as the latter share the small intestine with hookworms, and some interaction, in this case, may have been expected. For *Ascaris* and *Giardia,* a negative association between the two parasites was found by Blackwell et al. (2013) but Hagel et al. (2011) found higher *Giardia* prevalence in people with moderate *Ascaris* infection compared to low infection level. However, even though exposure to infective parasite stages remains unknown in field studies, controlled experimental infections

can overcome this limitation. In two studies, low levels of interaction were observed in pigs co-infected with *Ascaris* and *Oesophagostomum dentatum* or *Metastrongylus apri* (Frontera et al., 2005; Helwigh et al., 1999). In contrast, *O. dentatum* was displaced from the proximal to a more distal location in the large intestine by *T. suis* (Petersen et al., 2014). Whether this is mediated by direct parasite-parasite communication or indirectly via the host immune response or changes in the microbiota, or all three, remains unknown but may mainly be immune-mediated as *T. suis* induces a strong Th2 response in contrast to *O. dentatum* (Andreasen et al., 2015).

How *Ascaris* may communicate with its own and other species remains elusive but may take place by direct contact, using pheromones, peptides and proteins, or exchange of EVs as shown for *C. elegans* (Wang et al., 2014). If we can decipher how *Ascaris* regulates its population or affect other helminth species, this may provide novel avenues for parasite control.

## 4.5 *Ascaris* – host nutrient interactions

*Ascaris* can interfere directly with the host's access to nutrients as it competes for nutrients with its host. For example, an individual *A. lumbricoides* adult worm consumes more than 100 mg of glucose per day (Castro and Fairbairn, 1969), which then is no longer at disposal for the host. In parallel, nematode infections in general prime alternatively activated macrophages to fight the worms; this response also repairs infection-induced damage. Both activities require energy. However, this kind of immune response to the nematodes also plays a key role in the impairment of glucose absorption by host enterocytes (Notari et al., 2014). Consequently, *Ascaris* infection also induces reduced systemic glucose availability for the host indirectly via the helminth-induced immune response. This leads to catabolic metabolism, muscle wasting and reduced growth of infected individuals. Therefore, *Ascaris* changes host access to nutrients both directly and indirectly. As such, the *Ascaris*-nutrient interaction is important in the parasite-host interaction especially when food availability and quality is limited.

Human ascariosis is mostly prevalent in tropical areas where the infrastructure for sanitation remains underdeveloped. Here, particularly children are vulnerable to acute and chronic *Ascaris* infections. Moreover, in endemic areas, *Ascaris* infection often co-exists with undernutrition in children living in poverty. *Ascaris* and malnutrition are both associated with reduced physical and cognitive development. Thus, *Ascaris* might exacerbate malnutrition. *Ascaris* infections in pigs equally affect livestock health

and their associated productivity. Thus, *Ascaris* infection impacts humans and their economy by affecting the pigs in endemic regions. Consequently, ascariosis is a One Health issue and requires similar control approaches in both humans and livestock. Chronically infected individuals and their livestock linger in a vicious cycle of poverty, and *Ascaris* infections therefore cause significant morbidity and economic devastation for such populations.

Malnutrition in humans is estimated by reduced height for age (being stunted) and reduced weight for age (being wasted). In *Ascaris*-endemic areas, micronutrient deficiencies are common such as iron deficiency, zinc and vitamin A deficiencies (Muriuki et al., 2020). However, although iron, vitamin A and zinc deficiencies have been linked to *Ascaris* infection in children, micronutrient supplementation intervention studies have revealed inconsistent or relatively small effects on *Ascaris* reinfection rates (Isah et al., 2020; Yap et al., 2014).

In the context of macronutrients (proteins, carbohydrates, and fibres), protein energy malnutrition plays an important role in *Ascaris*-endemic countries. The impact of protein energy malnutrition was studied in *Ascaris*-infected pigs. Feeding low protein energy led to an increased *Ascaris* worm burden, accompanied by higher faecal egg output (Pedersen et al., 2002). In addition, the low protein diet resulted in lower pig body weight gains, serum albumin, and haemoglobin. These effects were allied with diminished peripheral eosinophils and thus a compromised anti-*Ascaris* immune response (Pedersen et al., 2002). Similar effects have also been described for protein-malnourished mice infected with *H. polygyrus*, which had higher worm burdens, reduced local and systemic Th2 responses (IgE, eosinophilia and mast cell activity) and increased IFNγ-associated Th1 responses (Ing et al., 2000). Consistent with these data, dietary protein supplementation in rats infected with *Nippostrongylus brasiliensis* resulted in the opposite effects and caused genes related to Th2 immunity to be upregulated in the lung (Masuda et al., 2022).

Interestingly, prebiotic fibres such as inulin are described as being beneficial for gut barrier function by influencing the gut microbiota. The effects of dietary inulin have been studied in *Trichuris suis* infected pigs. Dietary inulin enhanced the effects of *T. suis* by modulating the microbiota composition toward an upregulation of *Prevotella* and a decrease in *Proteobacteria* towards a bacterial composition associated with reduced gut inflammation (Stolzenbach et al., 2020). Similarly, another study showed that inulin feeding to *T. suis*-infected pigs augmented the *T. suis*-induced

Th2 immune genes (IL13, IL5) in the colon while suppressing Th1-related genes (*IFNg, IL1A, IL8*) (Myhill et al., 2018).

Together, these data suggest a direct and indirect effect of nutrients on the *Ascaris* infection. This is further nicely demonstrated by bioactive plant secondary metabolites in ascariosis. An indirect effect is demonstrated by polyphenols in the modulation of an *A. suum* infection. Here, polyphenols were enriched in a diet for pigs which were subsequently inoculated with *A. suum*. No direct influence on the *A. suum* establishment could be detected but changes in the composition of the gut microbiota and a modulation of the host immune response via a significant increase in eosinophils in the duodenum, jejunum and ileum (Williams et al., 2017). Other plant secondary metabolites tested with *A. suum* in vitro and in vivo showed a direct effect (Williams et al., 2014, 2020). Here, condensed tannins, natural plant extracts, directly reduced the migratory ability and motility in vitro of *A. suum* larvae (Williams et al., 2014).

In conclusion, an *Ascaris* infection impacts the host gut microbiome and the host nutrient metabolism (Midha et al., 2021, 2018b; Myhill et al., 2020). The interaction between *Ascaris*, the bacterial microbiome and the host nutrients is direct or indirect and changes with the diet and probably other extrinsic factors. Whether specific nutrient supplementations can be used to interfere with an *Ascaris* infection and at the same time contribute to gut health needs further investigation. The implications of the *Ascaris*-host nutrient interaction for an infected individual such as a growing child or pig are multiple and include alterations in availability of nutritional resources, effects on immune reactivity, and influences on the microbiota composition and gut health.

Through the course of chronic ascariasis, *Ascaris* nematodes can inhabit the intestine for over a year. In this environment, they engage in many intricate interactions not only with each other and other parasites, but also with the gut microbiota, incoming nutrients and metabolites, as well as with host cells. These interactions are dominantly mediated by ESP produced by the worms. Each of these types of interactions have thus far been studied independently of one another, given the complexity of the system. Many questions remain, including which bacterial genera are important for *Ascaris* establishment in the gut, the metabolomic consequences of infection, the coordination of different cell types in immunity against *Ascaris,* the regulation of crowding and parasite burdens within the host, communication between parasitic nematodes, and the influence of dietary interventions in infection outcomes are some of the many areas ripe for

investigation. As more reagents are developed for the porcine system, and as 'omics' technologies are further developed, it will be possible to study these various factors on a 'systems' level.

## 5. The current scope and future perspectives of *Caenorhabditis elegans* as a tool for ascarid research

Much of our understanding of the basic biology of nematodes comes from the study of the free-living roundworm, *Caenorhabditis elegans*, and many of the insights from these studies are equally applicable to the ascarids. Nowhere is this statement more true than in our knowledge of anthelmintic resistance (Wit et al., 2021). Studies from the past three decades have defined the molecular mechanisms of resistance to benzimidazoles, macrocyclic lactones, and nicotinic acetylcholine agonists. These discoveries were enabled by the tractability of this species, including its genetics, laboratory culturing without the need for a host, and small genome. Within the last decade, *C. elegans* genetics has been transformed by CRISPR-Cas9 genome editing approaches (Frøkjaer-Jensen, 2013). These advantages make this free-living nematode species a powerful model to understand parasitic worms (Zamanian and Eriksen, 2016).

When investigations of anthelmintic resistance are combined with experiments using *C. elegans*, a cycle of discovery where advantages of both species can be leveraged to mitigate disadvantages of either species is possible (Wit et al., 2021). Targeted and whole-genome sequencing of benzimidazole-resistant parasitic nematode species have long identified mutant alleles in a beta-tubulin gene. Because experiments to specifically test these alleles are not possible in parasites, these discoveries have only been correlations between a genetic change and a resistance phenotype. Genome-editing experiments in *C. elegans* allow direct tests of this correlation to establish a causal relationship between the genotype and phenotype. In several studies, every known beta-tubulin allele detected in benzimidazole-resistant parasite species was created using CRISPR-Cas9 allele replacements (Dilks et al., 2020, 2021). These edited strains were resistant to benzimidazoles unlike the unedited parent genetic background, demonstrating a direct link between beta-tubulin mutant alleles and benzimidazole resistance. Because of the tractability of *C. elegans*, these genome-editing and resistance assays are quick, in one recent case, demonstrating resistance of a novel beta-tubulin allele identified in dog

hookworm only months before (Venkatesan et al., 2023). Genetic screens have also identified a wide range of mutant beta-tubulin alleles that might also be involved in parasite benzimidazole resistance (Pallatto et al., 2022). In ascarids refractory to benzimidazole treatments, investigators did not find canonical beta-tubulin resistance alleles (Krücken et al., 2017; Martin et al., 2021; Özben et al., 2022), suggesting that novel beta-tubulin alleles might be present or that other genes beyond beta-tubulin are involved in ascarid benzimidazole resistance. *Caenorhabditis elegans* offers a powerful toolkit to validate any newly discovered beta-tubulin alleles or novel resistance genes.

Just like much of what we know about parasitic nematodes and anthelmintic resistance comes from the study of *C. elegans*, nearly everything we know about *C. elegans* comes from the study of a single individual strain in the species. This situation is like stating that everything we know about human biology comes from the study of a single person in our species. Parasitic nematodes have high levels of genetic diversity, including ascarids (Doyle et al., 2018, 2020; Sallé et al., 2019; Ding et al., 2022; Eamsobhana et al., 2022; Chen et al., 2022), so this diversity must be incorporated into *C. elegans* studies to ensure that we understand natural differences across populations. In *C. elegans*, the common laboratory strain, N2, is adapted to that environment making it a powerful model organism (Sterken et al., 2015), but this adaptation means that it is far from a "natural" nematode. Recent studies of *C. elegans* wild diversity have described the worldwide population (Andersen et al., 2012; Cook et al., 2016, 2017; Lee et al., 2021), the natural niche for the species (Crombie et al., 2019, 2022), and how to use quantitative genetics to identify specific genes (Evans et al., 2021a; Andersen and Rockman, 2022). The *C. elegans* Natural Diversity resource enables future studies where researchers want to move beyond the laboratory-adapted reference strain. A survey of natural differences in the *C. elegans* beta-tubulin gene *ben-1* identified numerous missense, insertion, deletion, and structural variants (Hahnel et al., 2018), which were found most often in strains naturally resistant to benzimidazoles. These results suggest that *C. elegans* must encounter benzimidazoles in its natural environment so that survival depends on losing the susceptibility locus *ben-1*. Using a genome-wide association mapping, this study also found a novel benzimidazole locus on the X chromosome far from any known beta-tubulin gene, suggesting that natural diversity can also lead us to new benzimidazole resistance loci. Beyond benzimidazoles, *C. elegans* natural diversity has also been important for macrocyclic lactone resistance (Ghosh et al., 2012, Evans et al., 2021b). As the field begins to study

resistance to more anthelmintic compounds, *C. elegans* will undoubtedly contribute to our understanding of resistance mechanisms and mode of action of new anthelmintics.

*Caenorhabditis elegans* is a clade V nematode related to strongyle parasitic nematodes (e.g., hookworms) (Blaxter, 1998), so findings in *C. elegans* are likely more easily translated to related nematodes. As clade III nematodes, ascarids have distinct life cycles, host biology, genome structures, and likely anthelmintic resistance loci. Ascarid genetics and genomics gives us the opportunity to discover aspects of their biology unique to this clade. Looking from *C. elegans* to ascarids, we can take some lessons from the past nearly fifty years of focused research on this tractable model species. The ease of *C. elegans* genetics has facilitated numerous discoveries not possible in any other species, making huge impacts on not just parasitic nematodes but also human biology.

To make ascarid genetics tractable with a goal to understand ascarid-specific anthelmintic resistance loci, we need to consider four different points: (1) the costs of the host species, (2) the methods to perform controlled crosses, (3) the quality of reference genomes, and (4) genome editing for validating candidate genes. First, although porcine hosts are similar to humans, the high costs make it difficult to scale genetic crosses. This point is not insurmountable, as genetic crosses in the sheep parasite *Haemonchus contortus* have been invaluable for the discovery of anthelmintic resistance loci (Sargison et al., 2018; Doyle et al., 2019). Other ascarid hosts, such as chickens, turkeys, mice, or rats, might offer more cost-effective and tractable hosts to enable genetic crosses of ascarids. Second, once a host-parasite species pairing is identified, methods for controlled crosses must be developed. Because of high genetic diversity, crosses of resistant and sensitive isolates would enable bulk-segregant approaches where anthelmintic selection is powerful (Burga et al., 2019). Identification of female and male individuals is possible for most ascarid species, but delivery of those individuals into the host for the remainder of the life cycle is more difficult. Random crossing followed by anthelmintic selection might be the most effective means of performing crosses in these species. Third, reference genomes need to be chromosomal (no gaps) for any ascarid species where genetics will be applied. It is difficult to identify loci using genetic mapping experiments with genomes that lack chromosome-level assemblies, as has been shown nicely in *H. contortus* mappings (Doyle et al., 2019). New long-read sequencing technologies combined with chromosomal-contact maps make genome assemblies more complete than

ever before. Fourth, once candidate loci are identified, they must be validated. In *C. elegans*, CRISPR-Cas9 genome editing made this process scalable. Delivery of the Cas9 and RNA machinery into embryos should be possible, but the genome edits will not occur in all cells (genetic mosaics). If the germline stem cells are edited, then strains can be created. Markers for genome editing at the cell level will likely need to be developed in ascarid models in parallel to delivery methods. Once these advancements are made, genetics and genomics in ascarids will become more tractable, enabling rapid advances in host-pathogen interactions, anthelmintic resistance, and clade III nematode biology.

## 6. Genomics – genomic/transcriptomic analyses of ascarids

Improved genomics technologies have immensely accelerated the amount of data on ascarids. For *Ascaris*, we saw the completion of the germline and somatic genomes, the accumulation of comprehensive mRNA and small RNA transcriptomes, as well as genome-wide histone and chromatin data (Wang, 2021). In contrast to the challenges in genetics-based studies, genomic research is relatively straightforward to carry out in *Ascaris* and most other ascarids, thanks to their large sizes and synchronized egg development (Wang and Davis, 2020). The long germline tissues allow dissecting of any stages of spermatogenesis and oogenesis. Large quantities of spermatids, oocytes, and fertilized eggs can also be easily obtained. The synchronized development of eggs enables the collection of discretely staged embryos and larvae. These features allow robust sampling and generation of various types of sequencing libraries in *Ascaris* (Wang et al., 2011, 2012, 2014, 2017, 2020; Kang et al., 2016). Similar genomic datasets have been or are being generated in other ascarids. These genomic resources are critical for future studies on the basic biology of these parasites, the host-parasite relationship, anthelmintic resistance, and novel drug target identification.

A fascinating feature of ascarids genome is programmed DNA elimination (PDE) (Estrom and Wang, 2023). In PDE, parts of the germline genome are lost during development (Fig. 4), resulting in a reduced somatic genome (Wang and Davis 2014b; Zagoskin and Wang, 2021). First discovered in the horse parasite *Parascaris* in 1880 s by Theodor Boveri, the nature of the eliminated sequences and DNA breaks was largely unknown

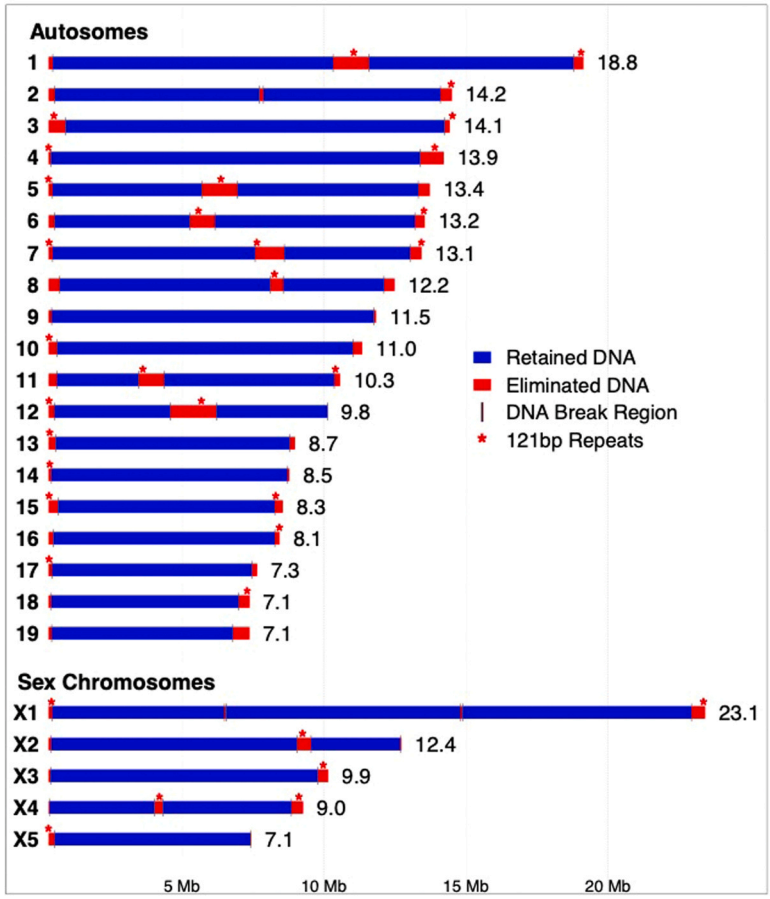


**Fig. 4** Genetic material lost during PDE. A linear presentation of Ascaris germline chromosomes. All chromosome ends and 12 internal regions are eliminated (shown in red). Many larger chromosomes have internally eliminated sequences which split them into smaller chromosomes in the soma. Eliminated regions containing 121-bp repeats are marked with a red asterisk. *Modified from Estrem, B., Wang, J., 2023. Programmed DNA elimination in the parasitic nematode Ascaris. PLoS Pathog. 19, e1011087 under Creative Commons CC-BY.*

for over 100 years. Recent large-scale genomic studies were carried out in *Ascaris* and *Parascaris* to understand the basic biology of PDE (Wang et al., 2012, 2017, 2020; Kang et al., 2016). These efforts also led to insights into early zygotic transcription and its contribution to animal development (Wang and Davis 2014a; Wang et al., 2014) and the flexibility of small RNA pathways in nematodes (Wang et al., 2011; Zagoskin et al., 2022).

In addition, genomic resources were built to identify potential drug targets (Jex et al., 2011; Zhu et al., 2015; International Helminth Genomes Consortium, 2019; Hu et al., 2020; Xie et al., 2021), used to study mechanisms of anthelmintic resistance (Gerhard et al., 2020; Roose et al., 2021), and worm control (Betson et al., 2014; Nielsen et al., 2014; Hansen et al., 2019; Pilotte et al., 2019; Easton et al., 2020). Below, we briefly review the genomes, transcriptomes, and epigenomes of ascarids. A more detailed description of ascarid genomics, including the history of genome research on *Ascaris*, can be found in a recent review (Wang, 2021).

## 6.1 Genomes

Ascarids have two genomes. The somatic genome is reduced from the germline genome after PDE in five independent cell lineages between the 4–16 cell stages (Wang and Davis 2014b; Streit et al., 2016). Comparative analyses suggest that 72 chromosomal break regions (CBRs) lead to the removal of all subtelomeric and telomeric sequences as well as some DNA in the middle of the chromosomes (Wang et al., 2020). Unlike the model hermaphroditic *C. elegans*, *Ascaris* is sexual dimorphic with separate males and females. However, the sex is determined using the same XO system as in *C. elegans*. In the *Ascaris* germline, cytological data indicates 24 chromosomes (19 autosomes and 5 sex chromosomes) become 36 somatic chromosomes (27 autosomes and 9 sex chromosomes) after PDE (Niedermaier and Moritz, 2000). This cytological observation is confirmed by chromosome conformation capture (Wang et al., 2020). Most ascarids undergo PDE (Estrem and Wang, 2023). While the number of germline chromosome varies in different ascarids (for example, *Parascaris univalens* has only one germline chromosome), the numbers of somatic autosomes and sex chromosomes after PDE are the same for all ascarids examined, suggesting PDE may function to restore the karyotypes of somatic chromosomes from drastically different germline chromosomes that may have arisen from fusion events during evolution (Simmons & Wang, unpublished).

The *Ascaris* germline genome is ~308 Mb, with 55 Mb (~18%) of DNA eliminated during PDE. The eliminated DNA consists largely of a 121 bp satellite repeat. However, 20 Mb of unique sequences encoding ~1000 germline-expressed genes are also eliminated (Wang et al., 2012, 2017, 2020), suggesting that PDE is an ultimate way of silencing germline genes by their permanent removal in the somatic cells. The CBRs and sequences

lost are the same in the five independent PDE lineages. This consistent loss of DNA suggests specific mechanisms are involved in *Ascaris* PDE.

## 6.2 Transcriptomes

Using comprehensive and stage-specific transcriptomes, the number of genes for the *Ascaris* germline genome is predicted to be 15,714 (Wang, 2021). More than 60,000 alternative spliced isoforms were identified across developmental stages. Many *Ascaris* genes are trans-spliced and some genes are in polycistronic transcripts (Davis, 1996; Blumenthal, 2012), making it difficult to precisely determine the gene models. Thus, manual examination and/or experimental data (*e.g.* RT-PCR) might be necessary to further validate the predicted gene models (Gerhard et al., 2020; Roose et al., 2021). Analysis of gene expression changes through *Ascaris* early development led to novel findings of gene regulation in early development of animal embryos (Wang et al., 2014; Wang and Davis 2014a). RNA-seq data revealed ~4000 *Ascaris* genes are transcribed before pronuclear fusion and in the 1–4 cell embryos. This large-scale early transcription is not seen in many model organisms, including *C. elegans*, which has almost identical cell lineages but where major transcription occurs after ~100 cells. This comparative analysis indicates that organisms with the same developmental program can use different mechanisms of gene regulation, post-transcriptional in *C. elegans* vs. transcriptional in *Ascaris*, to drive early embryogenesis. The use of transcriptional control is attributed to the long cell cycle (15–20 hours) in *Ascaris* that allows ample time for the transcriptional machinery to make new RNAs (Wang et al., 2014; Wang and Davis 2014a). Thus, this rewiring of the gene regulation during embryogenesis could be viewed as an adaption to the much longer cell cycle associated with *Ascaris*.

## 6.3 Small RNAs

Small RNAs in nematodes play key and diverse regulatory roles. *C. elegans* has three major classes of small RNAs: microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting (piRNAs) (Billi et al., 2014; Almeida et al., 2019). These small RNAs are associated with at least 19 expressed Argonautes that interact with their targets (Seroussi et al., 2023). In *Ascaris*, a reduced number of 10 Argonautes are encoded in the genome (Zagoskin et al., 2022). Total small RNA sequencing identified 100 miRNAs (Wang et al., 2011). However, sequencing of the total small RNAs from various stages indicates that these miRNAs are only 5–10% of

the total small RNA population (Zagoskin et al., 2022). The majority of *Ascaris* small RNAs are 22–24 nt, starting with guanine (G) and have 5′-triphosphates (Zagoskin et al., 2022). These 22–24G-RNAs mainly target repeats and/or mRNAs. *Ascaris* also has a class of 26 G-RNAs with 5′-monophosphates. They are specifically expressed during male meiosis and only target testis-specific genes (Zagoskin et al., 2022). Surprisingly, *Ascaris* has lost Piwi-clade Argonautes, piRNAs, and other associated proteins (Wang et al., 2011; Zagoskin et al., 2022). This loss of the piRNA pathway may have been compensated with siRNAs associated with other WAGO Argonautes. Indeed, antibody immunoprecipitation and small RNA sequencing revealed Argonautes plasticity by changing WAGO targets between repeats and mRNAs during development (Zagoskin et al., 2022). Although initial total small RNA sequencing reveals no correlation between small RNAs and retained or eliminated DNA (Wang et al., 2011), antibody staining, ChIP-seq, and IP data indicate that specific WAGOs may be differentially deposited to the retained or eliminated DNA during PDE (Zagoskin et al., personal communication). Small RNAs are also known to contribute to host-parasite interactions and transgenerational epigenetic inheritance in other organisms (Rechavi and Lev, 2017; Cai et al., 2019). Whether small RNAs in ascarids also play similar roles remains to be determined.

## 6.4 Epigenomes

Many histone modification marks associated with active or repressed loci have also been mapped out in *Ascaris* genome. ChIP-seq data for several common modifications including active marks H3K4me3, H3K36me3 and H4K20me1, and repressive marks H3K27me3 and H3K9me3 are available in *Ascaris* (Wang et al., 2017, 2020). These data provide a glimpse of the *Ascaris* epigenome; they can also be used for annotation and facilitate other genomic analysis. In addition, chromatin accessibility in *Ascaris* was profiled using the Assay for Transposase-Accessible Chromatin using sequencing, ATAC-seq, approach (Wang et al., 2017). Often, chromatin accessible regions in *Ascaris* are enriched at the transcription start regions of active genes. Interestingly, *Ascaris* CBRs for DNA elimination also become more open before DNA elimination, coincident with the sites for new telomere addition (Wang et al., 2017, 2020). Mechanisms for the open chromatin at the CBRs, however, remain to be determined. Another epigenetic mark, the histone H3 variant CENP-A, dictates where centromeres are deposited (Black and Cleveland, 2011; Fukagawa and Earnshaw, 2014). Like *C. elegans* (Gassmann et al., 2012),

*Ascaris* has holocentric chromosomes with multiple centromeres distributed along the length of the chromosomes. *Ascaris* CENP-A ChIP-seq identified many enriched loci that are not associated with repeats (Kang et al., 2016). Instead, the CENP-A deposition is dynamic through gametogenesis and embryogenesis and is inversely related with transcription. Interestingly, *Ascaris* CENP-A is reduced in the eliminated regions before and during PDE. This loss of centromeres in the eliminated regions leads to the loss of sequences after the DNA breaks during PDE (Kang et al., 2016), providing a mechanism for selective loss of DNA during *Ascaris* PDE.

## 6.5 Comparative genomics

Besides *Ascaris*, many genomes and transcriptomes from other ascarids have been recently sequenced. The dog parasite *Toxocara canis* genome was used to identify potential new drug targets (Zhu et al., 2015). The genomes from the panda parasite *Baylisascaris schroederi* suggested mechanisms for its adaptation to sharp-edged bamboo enriched gut (Xie et al., 2021) and coevolution between host and parasites (Hu et al., 2020). The poultry parasites *Ascaridia dissimilis, Ascaridia galli* and *Heterakis gallinarum* genomes are being built to study anthelmintic resistance (Collins and Andersen, 2023). The germline and somatic genomes from the horse parasite *Parascaris* were also sequenced to provide a comparative analysis of PDE in ascarids (Wang et al., 2017). In addition, large-scale efforts using comparative genomics of 81 genomes of major parasitic and non-parasitic worms identified births of novel gene families and expansions related to host-parasite interactions; and in silico screening further identified and prioritized new potential drug targets and compounds for testing and combating parasitic worm infections (International Helminth Genomes Consortium, 2019).

## 6.6 Research priorities

The genomic resources for ascarids have provided a rich trove of datasets that are being used by the research community. Future studies on ascarids genomics need to continue using cutting-edge technologies, including long reads and Hi-C, to build high-quality reference genomes. Additional genomic approaches, including RNA-seq and ISO-seq, nascent transcription (PRO-seq), ribosome profiling and epigenomics (ChIP-seq and CUT&RUN) are needed to further annotate the genomes. The large size of these parasites also allows the collection of samples from various tissues and developmental stages, enabling the establishment of transcriptomic and

epigenomic atlases for ascarids (Wang, 2021). These analyses may reveal novel aspects of alternative splicing, trans-splicing, lncRNAs, small RNAs, repeats, and epigenetic features in ascarids that may provide potential novel drug targets. In addition, comparative genomics using completed chromosomal assemblies and comprehensive transcriptomes will delineate the evolution of nematode chromosomes (Carlton et al., 2022) and the evolutionary developmental biology of spermatogenesis, oogenesis, fertilization, embryogenesis and larval development. Furthermore, emerging technologies, such as single-cell genomics (Camp et al., 2019) and in situ sequencing for RNAs (Lee et al., 2014) and DNA (Payne et al., 2020) can also be used to study the heterogeneity and spatial organizations of chromosomes in the cells. These studies may reveal unique features in these parasites that are distinct from their hosts.

Ascarids are important parasites of human and animals of veterinary importance. High-quality genomic resources will enable future genome-wide studies on the epidemiology, population genomics, and metagenomics (Anderson et al., 2018; Bennuru et al., 2018; Doyle and Cotton, 2019; Wit et al., 2021). It will also facilitate studies on host-parasite interactions, anthelmintic resistance, and immune response of the infections. Thus, continuing efforts are needed to further establish, curate and improve the genomic resources. These resources need to be organized and made available with easy-to-use computational tools, genome browsers, and databases such as the WormBase ParaSite (Bolt et al., 2018).

## 7. Current challenges in vaccine research against ascarids and future perspectives

Currently, there are no licensed preventive vaccines for *Ascaris* infection and no vaccine antigens in clinical trials or advanced enough to expect clinical or field testing in the next years (Wong et al., 2023). Research is still exploratory and at the point of identifying *Ascaris* antigens that are safe, immunogenic and efficient against infection.

The rational for *Ascaris* vaccine research differs slightly from filarial, fluke or hookworm infections and is not primarily driven by high mortality, low rates of anthelmintic treatment efficiency or the emergence of drug resistance. Although anthelmintic drugs can effectively treat *Ascaris* infections in humans (Olliaro et al., 2022), they do not provide long lasting immunity, and reinfections are common in endemic areas (Zeleke et al., 2020; Zerdo et al., 2016).

Therefore, the development of a vaccine against *Ascaris* infections in humans is seen as an important strategy to achieve long-term control and eventual elimination of the parasite when used alongside mass drug administration (MDA) and improving public health and sanitation infrastructures (Hotez et al., 2016). The idea of developing a multivalent, pan-anthelmintic vaccine targeting all three intestinal parasites *A. lumbricoides*, *T. trichiura* and hookworms is a desirable and theoretically feasible goal, hampered by obvious differences in life cycle diversity, biology, and immune evasion strategies (Else et al., 2020) and the challenges of finding potent vaccine antigens.

Vaccine targets should ideally mimic the host immune response during natural infection and prevent infection. However, our current understanding of the protective mechanisms elicited during *Ascaris* infection is limited. Known host strategies to control *Ascaris* infections include intestinal expulsion mechanisms (Masure, Wang, et al., 2013) and immune-mediated-larval killing (Coakley et al., 2016) as described in further detail in Section 5.3. The life cycle of *Ascaris* gives rise to different host immune barriers that could be involved in host protection and potentially targeted by vaccination. In pigs immunized with a trickle infection scheme (100 *A. suum* eggs/daily/14 weeks) the intestinal barrier (pre-hepatic immunity) was associated with a mucosal eosinophilia, submucosal mast cells and goblet cell hyperplasia (Masure et al., 2013). Although being effective at the level of worm control, the immunological and physiological consequences of a high-level of gut eosinophilia, mast cell infiltration, and goblet cell hyperplasia will likely have a negative impact on host gut health and therefore a cost (pathology) – benefit (protection) assessment should take place in vaccine developing programs that target intestinal parasite expulsion. Immune mediated larval killing in the lung (post-hepatic immunity) is also considered an important protective mechanism. Here, repeated infections in a mouse model where *Ascaris* cannot complete its life cycle drive protective lung eosinophilia and a systemic and mixed Th2/Th17 against newly invading larvae (Nogueira et al., 2016). In addition, $CD4^+$ Th2-mediated eosinophil-dependent *Ascaris* larval killing was shown in house dust mite (HDM) sensitized mice (Gazzinelli-Guimaraes et al., 2019) underlining the importance of Th2 orchestrated anti-worm programs. *Ascaris* vaccines should ideally prevent the initial entry of invading larvae across the intestinal barrier, but also strategies targeting larval tissue migration in livers or lungs, larval maturation, or strategies reducing the fecundity of adults to interfere with transmission are conceivable.

Single vaccine targets like the aspartic protease of *Necator americanus*, Na-APR-1 (Pearson et al., 2010), that interfere with or neutralize an essential process in *Ascaris* development or viability have not yet been identified. Similar to other helminths where in most cases holistic approaches outcompete single subunit vaccine candidates, vaccine studies in pigs and mice demonstrated a clear difference in the level of protection achieved by holistic approaches such as ultraviolet (UV) irradiated *A. suum* eggs (Tromba, 1978; Urban and Tromba, 1982, 1984) and crude extracts (Urban and Romanowski, 1985; Lukes, 1992; Gazzinelli-Guimarães et al., 2018) versus single, recombinant protein vaccine targets such as As12, As14, As16, As24, As37, AsHb, AsEnol, AsPase (reviewed in Vlaminck and Geldhof, 2013; Gazzinelli-Guimarães et al., 2021; Wong et al., 2023). Notably, parasite reduction rates cannot be directly compared between pig and mouse models. While immunization studies in pigs allow for simple macroscopic examination of *Ascaris* worms directly in the intestine after challenge, in mouse models migrating larvae need to be quantified from bronchoalveolar lavage fluid (BAL) and/or lung-tissue extracted larvae, a process which is not yet standardized. A more detailed overview of *Ascaris* vaccine development, including the history of *Ascaris* vaccine research, can be found in a recent review (Gazzinelli-Guimarães et al., 2021).

In a recent study, immunization of BALB/c mice with crude extracts from *Ascaris* L3, adult worms or adult cuticles resulted in 61%, 51% and 59% reduction of lung parasite burdens compared to unvaccinated animals, respectively (Gazzinelli-Guimarães et al., 2018). In the same study, a transfer of protein A – purified total IgG from immunized into naïve animals resulted in comparable reduction rates of 64.5%, 65%, and 64% after challenge, suggesting a role for antibody-mediated protection in mice at the lung stage (Gazzinelli-Guimarães et al., 2018). Notably, BALB/c mice quickly develop protection against invading *Ascaris* L3 and repeated infections (3 doses of 1000 eggs at days 1, 21, and 35) result in almost sterile (99.8% larval reduction) immunity (Versteeg et al., 2020). The use of single, recombinant subunit vaccines such as the immunodominant As37 formulated with the adjuvant AddaVax™ reduced lung larval burden by 49.7% compared to PBS-immunized animals (Versteeg et al., 2020). In an attempt to develop a multi-valent, chimeric vaccine, linear B cell epitopes of the three immunogenic antigens As37, As16, and As14, were predicted and expressed as a chimeric protein. Immunizing BALB/c mice with the chimeric protein increased vaccine efficiency and resulted in lung larvae reduction of 74% when formulated with monophosphoryl lipid A (MPLA)

compared to PBS-immunized mice (De Castro et al., 2021). B-cell epitope prediction was also used by Gazzinelli-Guimarães et al. to define a multipeptide chimera vaccine based on conserved B-cell epitopes predicted from 17 common helminth proteomes combined with immune-serum reactivity to predicted and synthesized epitopes (Gazzinelli-Guimarães et al., 2021). The chimeric protein consisted of the 35 linear B-cell epitopes with the highest reactivity to *Ascaris*-immune sera and immunizing BALB/c mice with this construct formulated with MPLA resulted in lung larvae reduction of 50% compared to PBS.

The partial protection achieved with contemporary subunit vaccine antigens versus the historic success of UV-attenuated whole-parasite vaccines argues for some level of redundancy within helminth protein targets and strengthens the current view that "one-hit-is-not-enough" when developing vaccines against helminth. This, together with the current gaps in our understanding of the major mechanisms of protection, highlights the need for further promoting *Ascaris* vaccine research and the need for investigating novel vaccine antigen design and adjuvant platforms. The latter have been recently reviewed in the context of *Schistosoma* spp. and filarial worms (Perera and Ndao, 2021).

There are several studies that have predicted immunogenic *Ascaris* proteins which are yet to be tested and evaluated as vaccine candidates in relevant in vivo models. These include a study delineating human $CD4^+$ T cell epitopes of *A. suum* adult ESP for HLA-DRB1 * 07:01 or HLA-DRB1 * 15:01 by combining an in vitro antigen processing system coupled to quantitative proteomics of *Ascaris* ESP (Ebner et al., 2020), a bioinformatics in silico approach predicting a multimeric, codon-optimized multiepitope candidate that possesses B- and T cell epitopes for HLA-DRB1 * 07:01 and HLA-DRB1 * 15:01 MHC-II alleles (Kaur et al., 2021) and a reverse vaccinology approach using a pipeline that accounts for 27 different HLA alleles, sub-cellular protein location, T-cell and B-cell molecular binding, antigenicity, allergenicity and phylogenetic relationship resulting in the selection of four transmembrane domains as potential vaccine candidates (Evangelista et al., 2022).

These approaches indicate that promoting high-quality *Ascaris* genomes, proteomes, and transcriptomes as discussed earlier (Section 2.6) will enable the generation of numerous novel antigen repertoires for vaccine discovery. In parallel, recent technological advances in porcine immunology enable the identification and high-resolution phenotyping of (vaccine) antigen-specific CD4 + T cells in the pig as a natural host for

*A. suum* (Ebner et al., 2017). Profiling *Ascaris*-specific T cells in natural hosts will likely enhance our knowledge on the host-pathogen interaction and the immune response to vaccine antigens (Schmidt et al., 2020).

## 7.1 Research priorities

One of the reasons why many vaccine trials with recombinantly produced *Ascaris* antigens show only moderate protection compared to crude worm preparations or attenuated *A. suum* eggs could be the lack of species-specific native post-translational glycan modifications. As an example, native glycans on vaccine antigens of the intestinal parasite *Cooperia oncophora* were shown to be highly immunogenic and their absence impaired vaccine-induced protection significantly (González-Hernández et al., 2018). Incorrect or a lack of glycan modifications can have an effect on protein folding, immune cell activation, or availability of epitopes. The latter has been documented for *Ascaris* haemoglobin (AsHb), an immunogenic antigen used for serodiagnostics, where N-linked glycan epitopes form important human IgG4 antibody targets (Vlaminck et al., 2016).

The PNGase A-released N-glycans of adult A. suum consist of bi- and triantennary N-glycans, some modified by core α1,6-fucose and peripheral phosphorylcholine and the presence of paucimannosidic N-glycans, some carrying a core α1,3-fucose and oligomannosidic oligosaccharides (Pöltl et al., 2007). The role of glycans on Ascaris vaccine targets as well as glycan composition and glycan abundance of larval stages of A. suum need to be studied in further detail and protein expression systems should be explored that mirror native roundworm-specific glycosylation.

The level of antigenic variation between natural worm populations (e.g. between farms or states/countries) and polymorphisms is be an important consideration for selecting and evaluating potential vaccine candidates. The risk of genetic and antigenic variability to affect vaccine efficiency is even higher when single vaccine antigens are targeted rather than multiple epitopes from different proteins. This, independent of the actual level of genetic variance in ascarids (Nejsum et al., 2005), emphasizes the need to monitor polymorphisms across populations and the general need for continuous genomic efforts. Similarly, improving functional protein annotation and comparative analytics between Ascaris species and other STH as discussed in Chapter 2.6 will substantially impact validity and quality of future reverse vaccinology (RV) approaches. High-quality genomes and transcriptomes will also serve to better study Ascaris virulence-associated

genes that are differentially modulated as vaccine targets (Mohd-Shaharuddin et al., 2021).

RV offers a huge potential to the field of Ascaris vaccine research that we have just begun to explore. There are few published RV studies on ascarids that parasitize non-model organisms or hosts for which MHC haplotype or high-quality genomic information are available. While integration of 'multi-omics' technologies into vaccine discovery has made substantial steps during the SARS-Cov2 pandemic, the helminth field in particular would benefit from integrating (helminth) glycomics and orthologue mapping.

Expecting a large set of Ascaris T- and B-cell epitopes or proteins to be predicted as vaccine targets within the next years, we are still lacking standardized in vitro screening platforms for testing their immunogenicity for the many different hosts of ascarids (human, pigs, dogs, chicken). In turn, in vitro systems will need to be developed that allow for functionally testing whether vaccine targets within ascarids have a role in survival, virulence, or other essential processes. This will require the development of genome editing and mutation in germline stem cells of the worms as previously discussed in Chapter 6. The development and integration of 'multi-omics' of both host and parasite alongside finding solutions for Ascaris-specific challenges such as genome editing will certainly accelerate Ascaris vaccine research.

## 8. Discussions on important future topics in research on ascarids

Overarching discussions were used to identify the most pressing research topics to improve our understanding of ascarid biology but also to find new options to control these parasites, and these are summarized below (Table 3).

### 8.1 Ascarid physiology and drug action

The session on ascarid physiology and drug action particularly emphasized that improved in silico tools would be helpful to reliably identify promising target proteins and overcome the problem that in many cases, such as β-tubulins, parasite and host proteins show considerable similarity. Reliable in silico docking simulations of candidate compounds to such "druggable" targets are desirable to at least identify lead structures that can be further

**Table 3** Summary of research priorities.

| Theme | |
|---|---|
| **Physiology** | Sensory systems; olfactory, gustatory and mechanosensory that are key regulators of the animal's behaviour The hydrostatic skeleton; how it is generated and maintained. G-protein coupled receptors |
| **Drug action** | Use in silico methods to predict good drug targets and drug chemotypes to support the development of new anthelmintics: target identification, repurposing drugs, docking studies with lead chemical structures Increase our knowledge of the details of the modes of action of existing anthelmintics and the mechanisms of resistance: use state of the art methods to study the physiology and pharmacology of different ascarid tissues including intestine, uterus and reproductive system, ovary, and pharynx. Develop rational synergistic anthelmintic combinations that delay resistance using improved knowledge of the mode of drug actions and recognizing the intestine as a focus of drug metabolism, excretion, and site of action: the Gut-Drug Axis |
| **Host:parasite interactions** | Better understanding of the importance and mechanisms of extra-cellular vesicles in mediating inter-organism communication. The importance of parasite-parasite communication Impact of ascarid-nutrient and parasite-microbiome interactions |
| ***C. elegans* as a tool** | Development of methods for genetic crosses *C. elegans* offers a powerful toolkit to validate any newly discovered genes and alleles. Development of markers for genome editing |
| **Genomics** | Establishment, curation and improvement of high-quality genome resources for target species. Epigenomics |
| **Vaccines** | Combine 'multi-omics' to promote antigen identification. Target a combination of different antigens. Consider the effect of antigenic and genetic variation on vaccine efficacy Develop standardized in vitro screening platforms for functional testing of vaccine targets and testing host immunogenicity. Integrate glycomics and orthologue mapping |

modified by chemical modifications of lead compounds and their optimization and re-testing using appropriate systems allowing at least medium throughput. In comparison to high-throughput screening, such approaches would allow drug screening by academia while high-throughput screening requires huge compound libraries, highly optimized tests and considerable financial resources. As infections with ascarids are neglected tropical diseases (*A. lumbricoides*, larva migrans caused by *Toxocara* spp.), neglected diseases of poverty (*Toxocara* spp.) (Carlin and Tyungu, 2020; Holland et al., 2022) and often affect livestock production systems with low economic profit such as swine (*A. suum*) and poultry (*Ascaridia* spp. and *Heterakis gallinarum*) but also cattle (*Toxocara vitulorum*) in subsistence farming systems, such "low cost" approaches would allow substantial contributions by academic partners.

The cost aspect regarding the development of new drugs was also driving the discussion of using already licensed drugs for new purposes such as control of ascarids. This drug repurposing approach has been suggested for many NTD-relevant helminth parasites such as trematodes and nematodes including ascarids (Panic et al., 2014) in the last decades and might be an attractive option since studies regarding drug safety do not have to be repeated as long as the same dose range is used. A promising example with activity against gastrointestinal nematodes is nitazoxanide, which was previously known for its effects against protozoa and trematodes (Panic et al., 2014).

Combinations of drugs were in particular considered to be relevant to prevent evolution of drug resistance. As detailed above and well known from more rapidly evolving pathogens such as human immunodeficiency virus (Menéndez-Arias and Delgado, 2022), combinations of two or more drugs that have independent modes of action and no antagonistic effects strongly decrease the speed of resistance evolution. For the commercially available combination of two anthelmintics from different drug classes, i.e. the spiroindole derquantel with the macrocyclic lactone abamectin (Startect®), bioinformatic simulations have revealed that the combination can significantly delay evolution of drug resistance compared to sequential application or annual rotation of the same drugs (Learmount et al., 2012). Such combinations of drugs and their effects on the speed at which resistance evolves should be evaluated not only in silico but also experimentally and in controlled field work.

*Bacillus thuringiensis* subsp. *israelensis* bacteria have been widely used to manage populations of insects, in particular of mosquito larvae

(Zhang et al., 2017), but are also of interest regarding their cytotoxic effects on nematodes (Chalivendra, 2021) and their effect is well known to be due to the membrane pore forming effects of Cry proteins (Silva-Filha et al., 2021) as also demonstrated for *A. suum* in a mouse model (Hu et al., 2010). The effects of *Bacillus thuringiensis* Cry5B pore forming protein on *A. suum* intestinal cell $Ca^{2+}$ homeostasis were described in a talk and participants of the meetings discussed aspects of sustainability in terms of ecotoxicological effects such as toxic effects on dung-dwelling and -feeding insects as described before for macrocyclic lactones (Jacobs and Scholtz, 2015).

Monitoring for resistance against current anthelmintics should obviously continue, and it would helpful to have more information on the size of effective refugia of the ascarid parasites in the soil, especially in view of the longevity of fertilized eggs in this environment.

## 8.2 Host-parasite-interactions in ascarids

The discussion of this section was initially focusing on the question how the interaction between the host, its parasites and gut and tissue microbiota can be experimentally investigated since the strong interaction between these compartments results in simultaneous changes in both all three of them once one of the components is manipulated. This makes it difficult or even impossible to dissect the cascade of events and discriminate direct from indirect effects. Modulation of the host immune response by ESP including extracellular vesicles (Hansen et al., 2019) has indirect effects on the composition and diversity of the microbiota that are considerably changed during infection as revealed by *A. suum* infections in pigs (Springer et al., 2022). The changes in the composition of the microbiota again have effects on the host's immune response. Regarding the extracellular vesicles and their content of proteins and nucleic acids such as microRNAs that may target the host immune system it was also discussed whether they might function in communication between worms. In the context of new approaches to control ascarid infections it was further discussed if some bacteria in the microbiota can act as pathogens for the worm and if so, whether it might be possible to exploit this interaction. Other options might be viruses specific for ascarid nematodes to specifically interfere with the life cycle of the parasite. Other options to attach the parasite that were discussed centred around the idea to downregulate the innate immune system of *Ascaris* with the anticipated effect that this might lead to pathogenic effects of bacteria in the host microbiota.

## 8.3 Current scope and future perspectives of *C. elegans* as a tool for ascarid research

No tools for forward or reverse genetic manipulation of ascarid worms are available and even RNAi has only been described in a single publication in *A. suum* (McCoy et al., 2015) and this technique has never been taken up by other groups to functionally characterize ascarid genes. In the absence of powerful genetic tools, the idea to use the versatile *C. elegans* model is obvious as detailed in chapter 6. In the discussion, it was emphasized that the power of the *C. elegans* model decreases with the phylogenetic distance between the parasite and the model organism and thus *C. elegans* is better suited as a model for strongyle nematodes than for ascarids. The use of *C. elegans* to study the function of genes expressed as transgenes has been criticized recently stating that it tells us only something about the function the gene or expressed protein can have in the framework of *C. elegans* but not on its function in the organisms from which the gene was derived (Streit, 2022). In general, a kind of position paper "How to use *C. elegans* best in parasitic nematode research" was something that would be helpful to improve study design and data interpretation and make *C. elegans* as a model more accessible to parasitologists that have not yet worked with the model organism. This should include the recommendation on quantitative traits to characterize phenotypes, the preference of kinetic data in comparison to end-point recordings. Moreover, it should also emphasize that gain-of-function mutations can be as valuable as loss-of-function mutations when characterizing physiological pathways in nematodes. Although the well characterized Bristol N2 strain is typically best suited for analyses of gene function using parasite-derived transgenes, it was recommended to take more advantage of the high intraspecies variability of natural *C. elegans* strains in future research (Shaver et al., 2023). These strains offer the advantages that the high genetic diversity results in a phenotypic diversity which can be exploited in evolutionary experiments *e.g.* addressing the evolution of drug resistance. Moreover, some of these strains might show responses that are found in parasitic nematodes but are absent from N2, including responses to anthelmintic drugs that sometimes only occur at higher concentrations in N2 compared to parasitic nematodes.

## 8.4 Genomic and transcriptomic analyses of ascarids

In order to develop more powerful experimental tools in ascarids, high quality, telomere-to-telomere genome assemblies are required as they provide much better resolution power for forward genetic studies in

comparison to draft genomes as shown previously for *H. contortus* (Doyle et al., 2020). In comparison to other parasitic nematodes, the ascarids have the advantage that due to their size for several species tissue specific transcriptomic and proteomic data can be obtained. Good genomic and transcriptomic data should also cover multiple species and cover all different types of life cycles in the group such as a direct life cycle with tracheal migration in the definitive host (*Ascaris* spp., *Parascaris* spp.), sometimes with vertical transmission (*Toxocara vitulorum*), a life cycle that can include vertebrate paratenic hosts either with (*T. canis*, *T. cati*) or without (*Toxascaris leonina*) tracheal migration and vertical transmission, a life cycle that can include paratenic invertebrate hosts without tracheal migration in the definitive host (*Ascaridia* spp., *Heterakis* spp.), or complex life cycles involving multiple invertebrate and vertebrate paratenic hosts (*Anisakis* spp., *Pseudoterranova* spp.). A deeper understanding of the functional role of genes in ascarids would moreover require a system to mutagenize the genome (*e.g.* by exposure to ethyl methanesulfonate) and develop methods to perform outcrosses and backcrosses between isolates with different phenotypes. This would allow to map resistance loci as described before for *H. contortus* (Doyle et al., 2019, 2022; Laing et al., 2022) or to identify dominant phenotypes caused by gain-of-function dominant mutations in genes by deep genome and transcriptome sequencing of the offspring. It was proposed that *Ascaridia* spp. might be best suitable to establish this model as the costs of the poultry animal model are moderate and that isolates resistant to anthelmintic drugs are available (Collins and Andersen, In Press). However, the fact that these parasites do not undergo tracheal migration was considered by some discussants to be an advantage simplifying the model but a disadvantage preventing analysis of tissue migratory larval stages by others. The research community would furthermore highly benefit from an electronic database to exchange data and make tools such as reagents, strains and even mutant worms available to the community as has been demonstrated by the *C. elegans* community over decades.

## 8.5 Vaccines

The general discussion after the vaccine session focused on vaccination against *A. suum* in pigs. The use of hidden/concealed antigens was suggested to circumvent immune evasion mechanisms that might have evolved for antigens to which the host immune system is naturally exposed. This approach has been successfully used to develop a commercially

available vaccine against *H. contortus* using a midgut epithelial cell surface protein complex (Nisbet et al., 2016; Scarff et al., 2020). However, since natural immunity already requires multiple exposures to develop, and immunity to hidden antigens in a vaccine is not boosted by natural infections, it was expected that multiple boosters are required for a vaccine using hidden antigens. As alternative, anthelmintic treatments that kill migrating larvae and lead to exposure of the hidden antigens to the immune system was suggested. However, there was also considerable concern that boosting of vaccine-induced immune responses might lead to increased pathology such as increased size of milk spots.

## 9. Conclusions

The two-day event focusing on the biology of ascarid parasites demonstrated a broad research interest on this important group of parasites and diverse research activities, including applied aspects such as improved diagnosis to cutting edge research in the areas of genomics and interactions between hosts, parasites and microbiota. In the absence of available genetic tools for any ascarid species, there was a strong interest in *C. elegans* as a model – not only to address important questions in the model organism but also to learn from the *C. elegans* model and the researcher community to set up experimentally manipulatable ascarid models. A first step to set up such models could be the generation of high-quality genome and transcriptome data. Due to their large size, the ascarids even have a considerable advantage in comparison to other, more intensively investigated parasitic nematodes as transcriptome and proteome data could be more easily generated on the tissue level. The field of vaccine research would also benefit from excellent genome and transcriptome data to use bioinformatic approaches to identify potentially protective antigens with little intraspecies variability. Understanding of ascarid physiology and biochemistry would obviously benefit from characterization of the parasites' repertoire of neuronal receptors, xenobiotic metabolizing enzymes, and biochemical pathways. Genomics and transcriptomics are very powerful, easily accessible and quite cheap, and provide an obvious umbrella to bring together most other biomedical aspects of ascarids including diagnostics, epidemiology, physiology, biochemistry, host/pathogen/microbiota interaction and vaccinology. However, the meeting also revealed the urgent need for experimental models that allow forward and/or reverse genetic

manipulation and that *C. elegans* can be only a partial surrogate. Although development of such models requires to address and overcome multiple complex hurdles, all the above-mentioned fields in ascarid research would benefit from the establishment of such experimental models since they would allow manipulation of the parasite to observe the effects it causes on hosts, microbiota, drug effects and resistance, and many other practically relevant aspects.

The participants in this meeting, which mostly also represent members of the ARTI consortium (see above), agreed to maintain informal cooperation and links, including organizing meeting sessions and workshops to promote ascarid research, with a view to developing a network of formal and informal collaborations in future years. One example was the organization of an ascarid symposium at the ICOPA meeting in August 2022 in Copenhagen.

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