


# A historical and systematic overview of *Ascaris* vaccine development

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## Review Article

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## Abstract

Ascariasis is the most prevalent helminth infection in the world and leads to significant, life-long morbidity, particularly in young children. Current efforts to control and eradicate ascariasis in endemic regions have been met with significant challenges including high-rates of re-infection and potential development of anthelmintic drug resistance. Vaccines against ascariasis are a key tool that could break the transmission cycle and lead to disease eradication globally. Evolution of the *Ascaris* vaccine pipeline has progressed, however no vaccine product has been brought to human clinical trials to date. Advancement in recombinant protein technology may provide the first step in generating an *Ascaris* vaccine as well as a pan-helminthic vaccine ready for human trials. However, several roadblocks remain and investment in new technologies will be important to develop a successful human *Ascaris* vaccine that is critically needed to prevent significant morbidity in *Ascaris*-endemic regions around the world.

## Introduction

*Ascaris lumbricoides* is the most prevalent helminth causing human disease, recording nearly 500 million cases a year in subtropical and tropical regions globally (Prevention, 2015; Disease, 2020) (Fig. 1). Ascariasis is an infection of poverty with high prevalence in low- and middle-income countries (LMICs) in Africa, Latin America, South East Asia and South Asia (Disease, 2020). However, ascariasis has also been reported in poverty-stricken regions within high-income countries (HICs) including the United States.

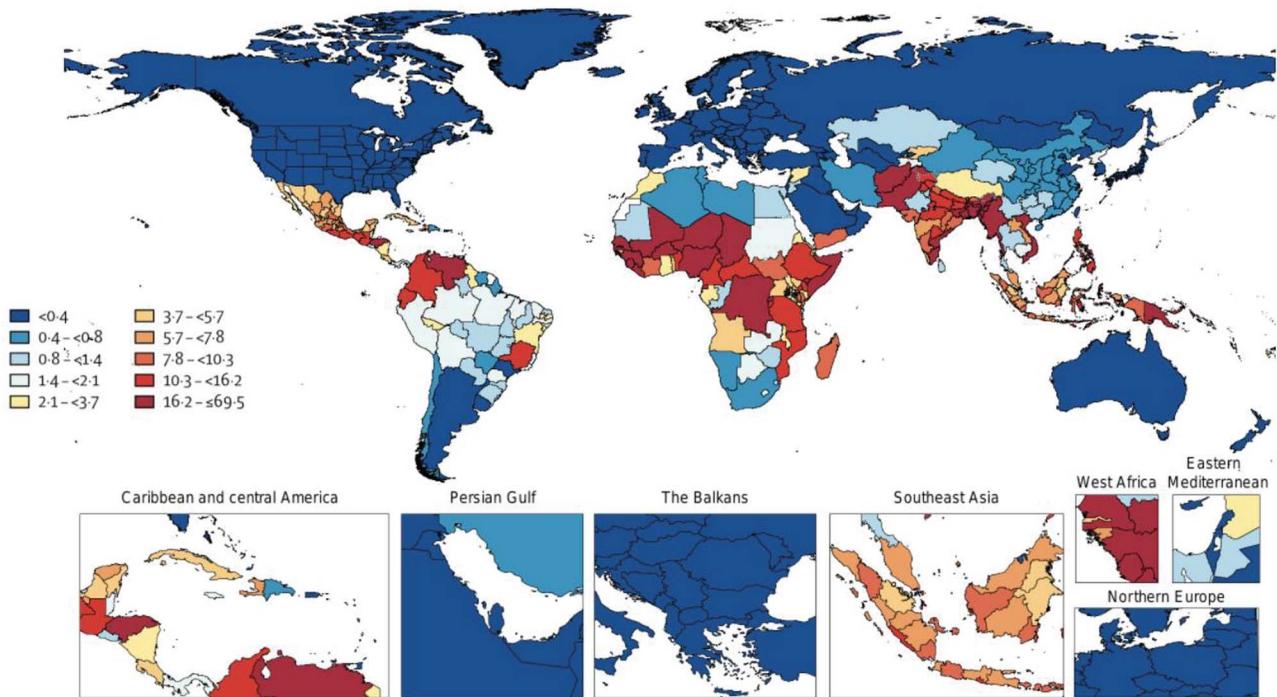
Historic reports have cited prevalence as high as 49.4% in school-aged children in the Southeast United States. However, in more recent years, the lack of robust epidemiologic monitoring within HICs has limited the current understanding of on-going transmission in these regions (Starr and Montgomery, 2011). Similar to other helminth infections, *Ascaris* spp. infects people living in extreme poverty, with insufficient waste management and inadequate sanitation measures, which allows for ubiquitous contamination of the environment with infectious *Ascaris* spp. eggs. Subsequent infection leads to long-term morbidity, which limits school achievement and job productivity, leading to a cycle of poverty within the community. The social and economic consequences of ascariasis at both the individual and community-level can be devastating.

Children harbour the highest disease prevalence and the highest disease burden of *Ascaris* infection (Disease, 2020). *Ascaris* burden peaks in pre-school and school-aged children before gradually tapering off into adulthood. The reasons behind the predilection for children remain largely unknown but may reflect both behavioural adaptations as well as acquired immunity as children age into adulthood (Weatherhead and Hotez, 2015). Immunity to *Ascaris* occurs after repeated infection; however, this immunity is only partial and does not prevent against re-infection leading to persistent, but reduced disease burden in adulthood (Colombo and Grecis, 2020). Approximately 2090 deaths occur annually as a result of ascariasis globally, with the highest death rates occurring in children aged 1–4 years old (Disease, 2020). Despite the lower mortality rates compared to other infectious pathogens, ascariasis causes significant morbidity, which can be measured by disability-adjusted life years (DALYs). Ascariasis alone accounts for over 750 000 DALYs a year, with disability peaking in children 5–9 years old (Disease, 2020) (Fig. 2).

The high rate of disability, particularly in young children, highlights the critical need for novel interventions to prevent life-long health consequences in *Ascaris* endemic regions.

## Pathogenesis

*Ascaris* spp. is transmitted from human-to-human through the fecal–oral transmission route. Embryonated eggs found in contaminated environments (i.e. soil and homes) are ingested. Infective stage 3 larvae (L3) hatch from the eggs, penetrate the intestinal mucosa and enter the circulatory system. The larvae are carried *via* host blood from the liver to the lungs.



**Fig. 1.** Geographic distribution of *Ascaris*-associated DALYs per 100 000 from the Global Burden of Disease Collaborative Network, 2019. Institute for Health Metrics and Evaluation (IHME), Seattle, United States, 2020 ([http://www.healthdata.org/results/gbd\\_summaries/2019/ascariasis-level-4-cause](http://www.healthdata.org/results/gbd_summaries/2019/ascariasis-level-4-cause)).

While in the lungs, the larvae undergo considerable growth and alter their transcriptomic signature. Subsequently, the lung L3 reach the airways, ascend the bronchotracheal tree, are swallowed and enter back into the host small intestines completing the larval migratory cycle, as fully developed L4 stages. The larvae develop into large macroscopic adult worms, measuring up to 35 cm in length, within the intestinal lumen (Prevention, 2015). Adult worms subsequently live for 1–2 years within the host. Although adult female worms shed up to 400 000 eggs per day into the host feces, the eggs must embryonate within, preferably, moist, warm soil prior to becoming infectious (Prevention, 2015). The dependency on this soil stage requirement for completion of the life cycle is why ascariasis, in addition to trichuriasis and hookworm infection, is termed a soil-transmitted helminth (STH).

*Ascaris suum*, the porcine roundworm, can also cause human infection. The life cycle of both *A. lumbricoides* and *A. suum* can be completed in both humans and pigs. Both organisms can be transmitted from human to human, without direct pig exposure (Da Silva Alves *et al.*, 2016). As a result, outbreaks of *A. suum* infection in humans have occurred globally, including in HICs (Miller *et al.*, 2015). *Ascaris lumbricoides* and *A. suum* are morphologically indistinguishable with minimal genetic variance leading to an on-going debate if they are indeed separate species (Leles *et al.*, 2012; Zhou *et al.*, 2020). Sequencing of the mitochondrial genomes as well as miRNA profiles have shown a high degree of relatedness between *A. lumbricoides* and *A. suum* (Liu *et al.*, 2012; Shao *et al.*, 2014). Both *A. lumbricoides* and *A. suum* share an A and T nucleotide bias in their genomes with most genetic differences leading to synonymous mutations (Liu *et al.*, 2012; Da Silva Alves *et al.*, 2016). Gene sequencing demonstrates tight phylogenetic clustering, further suggesting shared ancestry (Liu *et al.*, 2012). While *A. lumbricoides* and *A. suum* may be the same organism, at minimum they both represent significant zoonotic potential. As such, elimination strategies should be formulated with a ‘One Health’ approach, targeting human and animal reservoirs to impede transmission between human

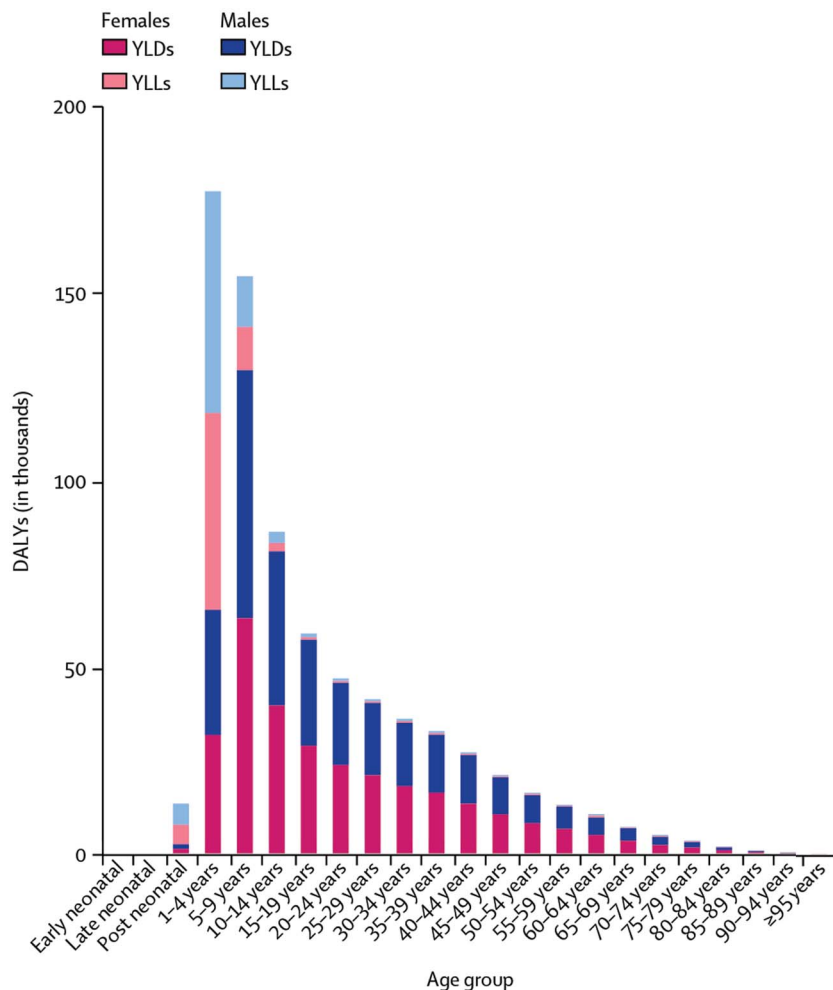
and animal populations (Betson and Stothard, 2016; Da Silva Alves *et al.*, 2016).

### Clinical manifestations

Ascariasis can cause a spectrum of diseases from asymptomatic to severe malnutrition, impairment in cognitive development and growth restriction (Ezeamama *et al.*, 2005; Rajagopal *et al.*, 2014; Pabalan *et al.*, 2018). Morbidity is associated with the acute larval migration stage as well as the chronic adult gastrointestinal stage. During acute larval migration through the lungs, *Ascaris* larvae stimulate an eosinophilic pneumonitis termed Loeffler’s syndrome. Long-term consequences of larval migration through the lungs include asthma and potentially chronic lung disease. Animal studies have shown that mice infected with *Ascaris* spp. can have prolonged allergic airway phenotypes (Nogueira *et al.*, 2016; Gazzinelli-Guimaraes and Nutman, 2018; Weatherhead *et al.*, 2018). Furthermore, wheezing episodes in *Ascaris* endemic regions are commonly attributed to allergic sensitization to *Ascaris* (Chico *et al.*, 2019). *Ascaris* infection may also contribute to long-term lung disease, exacerbating underlying lung pathology and aggravating pulmonary dysfunction. However, more information is needed to clearly understand the long-term consequences of larval migration through the host lung tissue (Oliveira *et al.*, 2019). Chronic intestinal ascariasis is associated with gastrointestinal symptoms including nausea, vomiting and diarrhoea. Individuals with high worm burden, particularly young children, are at high-risk of intestinal obstruction and intestinal perforation (de Silva *et al.*, 1997; Mishra *et al.*, 2008). Additionally, adult intestinal worms can move into the biliary ductal system causing hepatobiliary disease, including pancreatitis and cholecystitis (Jourdan *et al.*, 2018).

### Current management strategies

The significant impact on childhood morbidity and life-long chronic disease has led to the development of global partnerships



**Fig. 2.** Years of life lost (YLL) and years of healthy life lost to disability (YLD) from ascariasis globally from the Global Burden of Disease Collaborative Network, 2019. Institute for Health Metrics and Evaluation (IHME), Seattle, United States, 2020 ([http://www.healthdata.org/results/gbd\\_summaries/2019/ascariasis-level-4-cause](http://www.healthdata.org/results/gbd_summaries/2019/ascariasis-level-4-cause)).

to reduce *Ascaris* worm burden and to target disease control and elimination. Introduction of preventive chemotherapy through mass drug administration (MDA) programmes provides regular, repeated administration of a single-dose tablet of albendazole or mebendazole (benzimidazoles) to high-risk groups based on local disease prevalence. The goal of MDA programmes is to reduce worm burden in order to reduce morbidity (Weatherhead *et al.*, 2017; Weatherhead, 2019). Preventive chemotherapy is provided on an annual or biannual basis in young children (12–23 months of age), pre-school-aged children (24–59 months of age), school-aged children and women of child-bearing age living in endemic regions with prevalence >20% (World Health Organization, 2017). Regular preventive chemotherapy, known as deworming, has been associated with an increase in weight and height in children (World Health Organization, 2017). Expansion of preventive chemotherapy MDA programmes to provide therapy more frequently and/or to a broader range of the community may provide additional power to interrupt transmission dynamics in endemic regions. However, frequent and widespread use of anthelmintic drugs is associated with the development of anthelmintic resistance (Farrell *et al.*, 2018). Mutations in the beta-tubulin isotype-1 gene have been shown to induce resistance in many nematodes, including *Ascaris*, to benzimidazole anthelmintic therapy. Although the frequency of this mutation for *Ascaris* spp. currently remains low in humans, heightened anthelmintic pressure in a community can enhance resistance development (Furtado *et al.*, 2019). Thus, MDA scale-up strategies to eliminate *Ascaris* are likely to create additional hurdles. Introduction of water,

sanitation and hygiene (WASH) programmes may also benefit *Ascaris* control efforts. Implementation of sanitation standards, frequent handwashing policies and access to clean water can significantly reduce *Ascaris* infection within endemic communities (Strunz *et al.*, 2014). As a result, WASH programmes continue to play a critical role in developing sustainable control efforts. However, the effects of WASH programmes are not immediate and require long-term funding (Vaz Nery *et al.*, 2019). The implementation of MDA policies and WASH programmes has led to a modest reduction in disease burden and overall morbidity globally over the past decade. From 2010 to 2019, DALYs associated with ascariasis decreased by 17.4% (Disease, 2020). Despite the reduction of disease burden, control and eradication is unlikely to be achieved using MDA and WASH programmes alone.

*Ascaris* eggs are hardy, difficult to eradicate and remain viable in the environment for years (Jourdan *et al.*, 2018). People living in endemic regions are repeatedly exposed to *Ascaris* eggs directly from their environment, leading to re-infection. Individuals commonly become re-infected as early as 3 months post treatment. By 12 months post-treatment there is a 95% re-infection rate in communities with high *Ascaris* prevalence, providing further evidence that the current eradication strategies are insufficient (Jia *et al.*, 2012). In the setting of high rates of re-infection and the potential for drug resistance development, preventive and/or therapeutic vaccine technologies will be essential for disease eradication. To date, *Ascaris* vaccine progression has been limited, but new platforms to identify vaccine targets may provide innovative avenues in vaccine discovery.

### The complex *Ascaris*–host immune response interface as the basis for vaccine development

The host immune response to *Ascaris* is parasite developmental-stage specific, leading to either inflammation or immunomodulation. Larval migration through the host is an essential survival phase of *Ascaris*. Attempts to control and eradicate the larvae by the host are dependent on a coordinated innate and adaptive immune response. Antigens from the larvae or from their secreted products, excretory secretory (ES) products or extracellular vesicles (EVs) cause local damage during larval migration. The acute damage to host tissue induces release of alarmins, interleukin (IL)-25, thymic stromal lymphopoietin and IL-33, that promote activation and regulation of innate cells including neutrophils, eosinophils, type 2 innate lymphoid cells and alternatively activated macrophages as well as CD4<sup>+</sup> type 2 helper cells (Th2). This polarized type-2 immune response leads to high concentrations of IL-4, IL-5, IL-9 and IL-13 (Cooper *et al.*, 2000; Gazzinelli-Guimaraes and Nutman, 2018; Weatherhead *et al.*, 2020). Type-2 cytokines cause hypercontractility of smooth muscle, goblet cell hyperplasia, mucous production, influx of immune cells such as eosinophils and isotype switching to immunoglobulin E (IgE) that aids in parasite killing and expulsion in early infection. However, once larvae reach the intestinal lumen and develop into adult worms, they create an immunomodulatory environment. Adult *Ascaris* ES products contain immune mimicry markers such as transforming growth factor- $\beta$  that activate regulatory T cells (Treg), creating a more immune tolerate environment and allowing for chronic long-standing intestinal infection (Gazzinelli-Guimaraes and Nutman, 2018).

Ideally, the goal of an *Ascaris* vaccine development programme is to identify vaccine targets that mimic the host immune response elicited during natural infection while avoiding the long-term morbidity that occurs with natural infection. Currently, immune-mediated larval killing and/or expulsion mechanisms serve as the major immunologic basis for *Ascaris* vaccine development. *Ascaris* re-infection studies using experimental animal models have been instrumental in understanding stage-specific, immune-mediated larval killing mechanisms. There are likely two major host immune sites that contribute to the host immune barrier against ascariasis, the post-hepatic lungs and intestines and the pre-hepatic intestines, that could be targeted for vaccine development purposes (Magalhães *et al.*, 2021). The post-hepatic lungs serve as an immune barrier to larval migration by immune-mediated larval killing mechanisms. Using a mouse model, a partial protective immune response associated with larval killing can be developed with repeated infection, reducing the parasite burden in the lungs by 72–90% as well as reducing the associated clinical morbidity (Urban, Alizadeh and Romanowski, 1988; Masure *et al.*, 2013b; Nogueira *et al.*, 2016). Multiple exposures of mice to *A. suum* lead to a significant increase in cellularity in the lung tissue and airways, characterized by an increase in lymphocytes, M2-macrophages and eosinophils. Pulmonary eosinophils have specifically been associated with larval killing and prohibition of larval development in the lungs (Nogueira *et al.*, 2016; Gazzinelli-Guimaraes *et al.*, 2019). Additionally, Nogueira *et al.* (2016) demonstrated that pulmonary-associated protection generated by *Ascaris* re-infection was associated with a systemic mixed Th2/Th17 immune response. Additionally, the post-hepatic intestine also provides protective immunity relying on activation of gut motility to create a prohibitive environment for *Ascaris* larval to develop into adult worms. Using a pig model at the time which L4 are becoming established in the pig intestine and transitioning into adult worms, gastric transit time is significantly decreased leading to larval expulsion (Masure *et al.*, 2013b). Intestinal expulsion is likely driven by STAT-6

dependent upregulation of type-2 cytokines IL-4 and IL-13, leading to increased luminal fluids (weep) and muscle contractility (sweep). The weep and sweep mechanism makes the intestinal lumen an inhospitable environment for the helminth parasite to develop (Anthony *et al.*, 2007). Pre-hepatic immunity in the intestines can also develop after repeated infection. Masure *et al.* (2013a, 2013b) demonstrated that pigs continually exposed to infective *A. suum* eggs develop an almost sterilizing immunity (99.7% reduction in number of larvae) (Masure *et al.*, 2013a). The generated immunity was associated with eosinophilia, mastocytosis and goblet cell hyperplasia in the caecum suggesting the pre-hepatic intestinal mucosa provide a primary barrier to invading larvae. Targeting host immune-mediated larval killing and/or immune-mediated expulsion in animal models has provided significant insight into *Ascaris* vaccine development pathways. Identification and use of immunogenic *Ascaris* antigens for vaccine development that enhance larval killing and/or larvae expulsion will prevent the maturation of *Ascaris* larvae into adult worms and, in effect, prevent chronic infection.

### Animal models used for *Ascaris* vaccine development

Several animal models have been used to identify and evaluate *Ascaris* vaccine targets, including guinea pigs, rabbits, chicken (*Ascaridia galli*), mice, piglets and pigs. Although pigs are a natural host and are competent models for both the larval migration phase and chronic adult worm phase, their use in vaccine development is limited by the physical and financial challenges of working with and housing larger animals. As a result, mouse models have evolved as the primary *in vivo* animal system used in the development and evaluation of *Ascaris* vaccines. However, although mouse models do support the completion of the larval migration phase, the model does not support the development of adult intestinal worms, limiting its usefulness (Lewis *et al.*, 2005). Embryonated *Ascaris* eggs, classically *A. suum*, are given to mice by oral gavage and larval migration proceeds along the same pathway as described in humans but at an accelerated time scale; larval burden peaks in the mouse lungs at day 8 post infection and larvae are evacuated from the host on day 14 post infection (Gazzinelli-Guimaraes *et al.*, 2013). Susceptibility of mice to *Ascaris* eggs vary based on the genetic background of the mouse species. C57BL/6j, which generally have a polarized type-1 immune response, are more susceptible to *Ascaris* larval migration marked by high worm burdens in the lungs compared to other strains, including Balb/c and CBA mice, which are more resistant to larval migration (Lewis *et al.*, 2005, 2007). Resistant strains, however, have been found to have more prolific immune responses, particularly in the lungs, with elevated concentrations of neutrophils, lymphocytes and eosinophils and type-2 cytokines compared to susceptible strains (Lewis *et al.*, 2007). Mouse models remain the major mechanism for *Ascaris* vaccine development because of their ease of use and the ability to evaluate the impact of vaccines on the larval migration cycle, the over-arching goal of *Ascaris* vaccine development programmes.

### Historical review of *Ascaris* vaccine development and lead candidates

Decades of scientific literature indicate that efficient, protective immunity against nematodes, including parasites from the order Ascaridida, can be induced using extracts and suspensions of living and dead parasites (Sprent and Chen, 1949; Soulsby, 1957). However, the results of these studies are complicated by non-specific immunity and uncontrolled clinical consequences limiting their use. But, technological advancements in the field have allowed for expansion beyond whole extracts or parasites to

engage a more sophisticated, standardized and specific vaccine-induced immunity. A historical overview highlighting the parasite targets, vaccine types, animal models, protocols and major findings (Table 1) helps guide the discussion on how *Ascaris* vaccine technology has evolved and what could occur in the future (Fig. 3).

In the 1950s and early 1960s, crude extract from different developmental stages of *Ascaris* was used in vaccine pre-clinical trials as either a positive control for protection or as a tool to elucidate the immunological mechanisms of protective immunity (Gazzinelli-Guimarães *et al.*, 2018). Subsequently, standardized pre-clinical trials in guinea pig models demonstrated that immunizations with the crude extract of *Ascaris* eggs or larvae provide a 28–77% larval reduction in infectious challenge models, indicating a relative protection and potential use of *Ascaris* antigens as vaccine targets (Soulsby, 1957; Taffs, 1960). In the late 1970s and early 1980s, ultraviolet-attenuated (UV) parasite models, achieved by feeding pigs UV-irradiated fully embryonated eggs, were used to elicit protection. Tromba (1978) demonstrated that pigs that were fed eggs attenuated by short-wave UV radiation developed resistance to challenge infection. Corroboratively, Urban and Tromba (1982) showed that oral inoculation of pigs with 10 000 eggs irradiated by exposures of 150, 100 and 75  $\mu\text{W}\cdot\text{min cm}^2$  resulted in 88% protection. Furthermore, adjusting the number of UV treatments provided additional protection, reducing parasite burden by up to 94% (Urban and Tromba, 1984). These early studies using UV-attenuated eggs highlighted the fact that protective immunity against *Ascaris* could be induced. Following the use of UV-attenuated eggs, more specific *Ascaris*-derived products, such as larval stage ES products as well as purified fractions of the whole parasite, were evaluated (Urban, 1985; Frontera *et al.*, 2003). Studies found that specific *Ascaris* worm products could provide up to 88% protection, suggesting more targeted antigens could play a role in inducing protective immunity.

In the early 1990s and 2000s, advances in molecular biology and genomics provided a new perspective for *Ascaris* vaccine development: instead of using live-attenuated or irradiated parasites or their products, recombinant proteins could be used. To identify potential antigenic targets, *Ascaris* proteins were screened *in vitro* and the most promising antigens were selected to move into pre-clinical trials based on their reactivity to immune serum from infected pigs. Several immunogenic recombinant proteins identified from *A. suum*, including As14 (Tsuji *et al.*, 2001), As16 (Tsuji *et al.*, 2003; Wei *et al.*, 2017), As24 (Islam *et al.*, 2005b), As37 (Tsuji *et al.*, 2002; Versteeg *et al.*, 2020), enolase-1 (Chen *et al.*, 2012) and AsPPase (Islam *et al.*, 2005a, 2005b) have been expressed in different recombinant protein systems, and used in pre-clinical trials. Vaccine efficacy of these recombinant proteins in mouse models has varied from 38 to 77%. Alone, recombinant protein-based vaccines are poorly immunogenic. *Ascaris* recombinant proteins can induce an antigen-specific antibody response polarized towards a type-2 immune phenotype (elevated IgG1 levels), which is thought to be advantageous in the protection against helminths, however this response is weak. Coupling recombinant proteins with immunogenic adjuvants provide a more robust immune profile. Use of adjuvants such as alum (alhydrogel™), which promotes a type-2 immune response, enhances the magnitude and durability of immunity. Coupling *Ascaris* recombinant proteins with bacterial agents such as cholera toxin B (CTB), that classically induces a mixed type-1 and type-2 immune response, was shown to promote vigorous mucosal antigen-specific IgA production, high levels of IgG1 and lower levels of IgG2a in addition to a 64% reduction in L3 recovery from vaccinated mice (Tsuji *et al.*, 2001). Adjuvants are now essential components of recombinant vaccine

technology to generate a strong antigen-specific systemic immune response (Yamamoto *et al.*, 2001; Chen *et al.*, 2002). Varying dosages, intervals between doses and different adjuvants have all been found to have significant impact on recombinant protein candidate performance.

### Promising recombinant protein *Ascaris* vaccine targets: As14, As16 and As37

Tsuji *et al.* (2001) isolated cDNA encoding a 14-kDa antigen (As14) as well as a 16 kDa antigen (As16) from *A. suum* L3 larval stages. Both proteins are found in larvae and adult worms as well as in ES products and are under continued investigation as vaccine targets. To test the efficacy of As14 and As16, the proteins were expressed in an *Escherichia coli* system and coupled with CTB. The recombinant vaccine products were administered intranasally to mice and generated a 64% (As14) and 58% (As16) protection following challenge with *A. suum* in a mouse model (Tsuji *et al.*, 2001). Recombinant As16 coupled with CTB and administered to pigs was also partially protective against *A. suum* challenge (Tsuji *et al.*, 2004). Furthermore, Wei *et al.* (2017) demonstrated that rAs16 formulated with the adjuvant Montanide ISA720 or alum induced protection against *A. suum* in mice and was associated with a type-2-skewed immune response and stunted larval development. However, when rAs16 was formulated with monophosphoryl lipid A (MPLA) or Addavax, known for a more predominant type-1 immune polarization, vaccinated mice were not protected against *Ascaris* challenge infection (Wei *et al.*, 2017).

The third candidate under investigation, 37 kDa protein (As37) isolated from *A. suum* infective eggs (Tsuji *et al.*, 2002) is a member of the Ig superfamily and contains three Ig domains. Pre-clinical trials in mice found that recombinant As37 formulated with AddaVax™ adjuvant provided 48.7% worm reduction after challenge with *A. suum* infective eggs, and the protection was associated with a mixed Th1/2-type immune response characterized by high levels of IL-4, IL-5, IL-10 and IL-13 as well as IgG1 and IgG2a antibodies (Versteeg *et al.*, 2020). Additionally, sequence alignment of As37 with its homologues in other nematodes ranged from 80 to 100% similarity. Although anti-As37 strongly recognized hookworm and *Trichuris muris* homologues, it did not recognize human tissue, suggesting As37 may be a candidate for a pan-helminth vaccine (Versteeg *et al.*, 2020).

Finally, As14, As16 and As37 have recently been used together as a multivalent epitope-based vaccine in pre-clinical trials against *Ascaris* infection (de Castro *et al.*, 2021). The most immunogenic epitopes from the three selected proteins were predicted using bioinformatic tools. The peptides with the highest prediction scores were then selected for the construction of a chimeric protein. Notably, mice immunized with this novel chimeric protein alone exhibited only a 42.9% reduction in larval burden after *Ascaris* challenge, while mice immunized with the chimeric protein plus MPLA lead to a 74% larval reduction in addition to higher IgG responses and reduced lung inflammation. These data suggest that adjuvanted chimeric proteins containing more than one epitope offer a novel approach to the development of *Ascaris* vaccines, opening the door for creating intricate vaccine targets that can provide a more robust, targeted protective immunity.

### Discussion and future directions

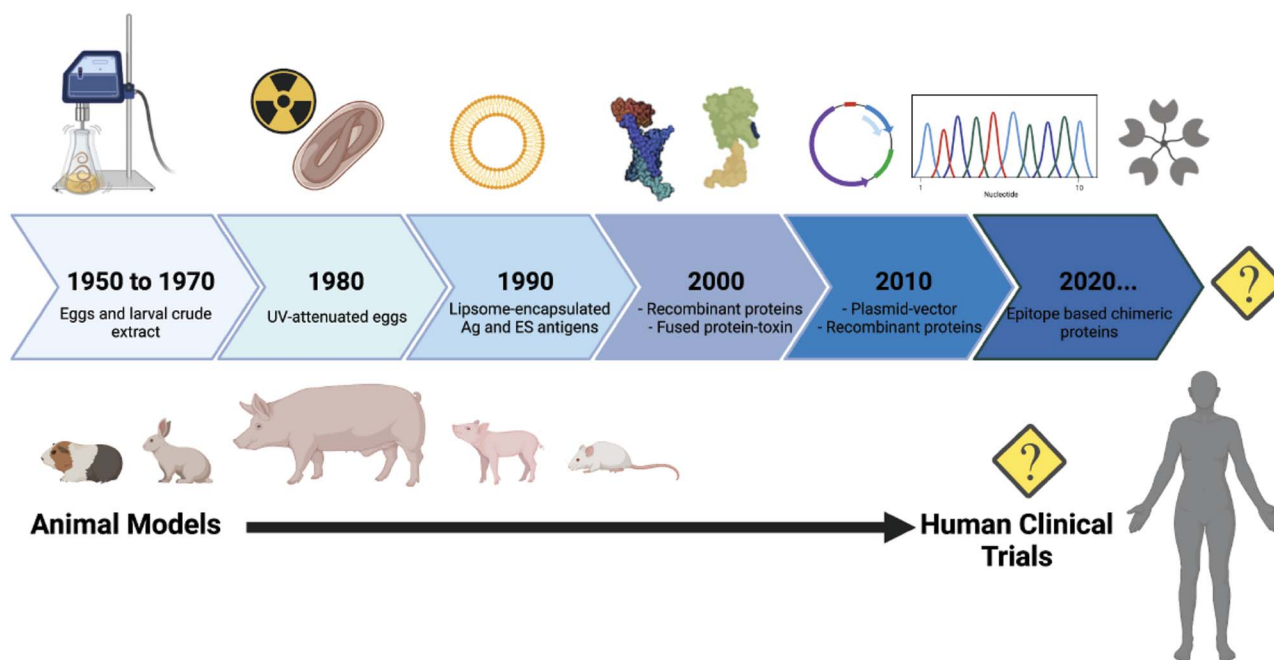
Although several vaccine candidates have been identified, developed and evaluated in pre-clinical animal trials, there is no vaccine candidate for *Ascaris* that has advanced into human clinical trials. Vaccine development requires significant time,

**Table 1.** Historical and systematic summary of the pre-clinical trials on *Ascaris* vaccine

Year	Vaccine type	Target	Model	Adjuvant	Protocol – route/doses	Immunogenicity	Efficacy*	Reference
1957	Extract	1) Infective eggs 2) ES larval	Guinea pigs	–	1) s.c./3 doses 2) s.c./6 doses	–	1) 50% 2) 28%	Soulsby (1957)
1960	Extract	L3 larval stage	Guinea pigs	–	i.v. – dose: 2000 L3	–	77%	Taffs (1960)
1962	Lyophilized <i>Ascaris</i> larvae	L2 larval stage	Rabbits	–	i.p. – 2.5 mg twice a week/3 weeks At 5th week: 2 doses of 5.0 mg w/ 3 days interval	Antibody titre ranging from 1:50 to 1: 256 000	–	Arean and Crandall (1962)
1978	UV-attenuated	Infective eggs	Pigs	–	p.o. – day 0 – 500 eggs at 150 ET/day 10 – 500 eggs at 100 ET/day 24 – 500 eggs at 75 ET	–	89.2%	Tromba (1978)
1982	UV-attenuated	Infective eggs	Pigs	–	p.o. – 3 successive weeks w/ 10 000 eggs – total exposures of 150, 100 and 75 $\mu$ W min cm <sup>-2</sup> respectively	↑ Peripheral blood lymphocytes	88%	Urban and Tromba (1982)
1984	UV-attenuated	Infective eggs	Pigs	–	p.o. – day 1 – 10 <sup>4</sup> eggs at 150 ET/day 7 – 10 <sup>4</sup> eggs at 100 ET/day 17 – 10 <sup>4</sup> eggs at 75 ET/day 31 – 10 <sup>4</sup> eggs at 75 ET/day 38 – 10 <sup>4</sup> eggs at 75 ET	–	94%	Urban and Tromba (1984)
1985	ES	3rd to 4th larval stage	Pigs	Alum	i.p. – day 1 – 1 mg of L3,4ESP (7 days in culture) + L3,4ESP (14 days in culture) + L3,4ESP (21 days in culture) p.o. – day 7 – 30 000 UV-irradiated eggs	↑ to EP, L2,3ESP (14 days in culture) and L3,4ESP (7 and 14 days in culture)	80%	Urban (1985)
1992	Liposome encapsulated	Adult crude antigen	Mice	Levamisole	s.c. – 100 mg of antigen in 2 doses (days 1 and 14)	Significant increase of antibody reactivity	88.9%	Lukes (1992)
1994	Cuticle extract	L2/L3 larvae and adult worm	Pigs	FIA + alum	i.m. – day 0 w/ 300 $\mu$ g of cuticle + FIA i.p. – days 7 and 15 w/ 300 $\mu$ g of cuticle + alum	↑ IgG at day 15 (more in adult cuticle) ↑ Cellular immunity at day 28	Adult cuticle: 44% L2/L3 cuticle: 49%	Hill et al. (1994)
2001	Recombinant protein + CTB	As14	BALB/c mice	CTB or FCA	i.n. – day 1 – 50 $\mu$ g of rAs14 + 20 $\mu$ g of CTB, day 21 – 30 $\mu$ g of rAs14 + 10 $\mu$ g of CTB, day 35 – 30 $\mu$ g of rAs14 + 10 $\mu$ g of CTB	↑ IgG; IgE (serum) ↑ IgG1; ↓ IgG2a, IgG2b, IgG3 ↑ Specific IgA mucosal	64%	Tsuji et al. (2001)
2001	Purified fractions	1) 97 kDa 2) 42 kDa 3) 14 kDa	Pigs	FIA	s.c. – 6 doses (days 0, 7, 14, 21, 28, 35) w/ 1 $\mu$ g of fraction/kg body weight	–	14 kDa and 42 kDa: 67–93%	Serrano et al. (2001)

2003	Purified fractions	1) 97 kDa 2) 42 kDa 3) 14 kDa	Pigs	–	s.c. – 6 doses (days 0, 7, 14, 21, 28, 35) w/ 1 $\mu$ g of fraction/kg body weight	↑ IgG1; IgG2 ↓ IgM	1) 49.5% 2) 77.1% 3) 88.2%	Frontera <i>et al.</i> (2003)
2003	Recombinant protein + CTB	As16	BALB/c mice	CTB or FCA	i.n. – day 1 – 25 $\mu$ g of rAs16 + 20 $\mu$ g of CTB day 21 – 15 $\mu$ g of rAs16 + 10 $\mu$ g of CTB day 35 – 15 $\mu$ g of rAs16 + 10 $\mu$ g of CTB	↑ IgG; IgE; IgG1; IgG2a, ↑ Mucosal IgA ↑ IFN- $\gamma$ ; IL-10; IL-2	58%	Tsuji <i>et al.</i> (2003)
2005	Recombinant enzyme	AsPPase	BALB/c mice	CytRx	s.c. – 3 doses (days 1, 21 and 35) – 50 $\mu$ g of rAsPPase + CytRx each dose	↑ IgG and IgG1, ↑ IL-10, ↑ IL-4, ↑ IFN- $\gamma$ , IL-2	71%	Islam <i>et al.</i> (2005a, 2005b)
2005	Recombinant protein	As24	BALB/c mice	FCA	s.c. – 3 doses (days 1, 21 and 35) – 50 $\mu$ g of rAs24 + FCA	↑ IgGs (IgG1 IgG2a; IgG2b, IgG3) ↑ IFN- $\gamma$ , ↑ IL-10, ↑ IL-4	58%	Islam <i>et al.</i> (2005b)
2009	Chimeric protein + CTB	As16	BALB/c mice	CT	p.o. – Tg rice seeds + CT weekly (1 g day <sup>-1</sup> ) for 7 weeks	↑ Specific serum IgG against As16	Significant reduction <sup>#</sup>	Matsumoto <i>et al.</i> (2009)
2011	Purified haemoglobin	AsHb	Piglets	QuilA	i.m. – 3 doses (days 0, 14 and 28) w/100 $\mu$ g of AsHb + 500 $\mu$ g of QuilA each	↑ AsHb-specific IgG and IgA	28.7%	Vlaminck <i>et al.</i> (2011)
2012	Crude extract	AsCE	Mice	FCA	i.m. – 3 doses on weeks 0, 2 and 4 w/100 $\mu$ g AsCE + FCA each	↑ IgG, ↑ cytokines (IFN- $\gamma$ , IL-2, IL-4 and IL-10), ↑ proliferation SI	88,62%	Chen <i>et al.</i> (2012)
2012	Plasmid vector	As-enol-1	Mice	–	i.m. – 3 doses on weeks 0, 2 and 4 w/100 $\mu$ g pVAX-Enol DNA each	↑ IgG, ↑ cytokines (IFN- $\gamma$ ; IL-2; IL-4 and IL-10), ↑ proliferation SI	61,13%	Chen <i>et al.</i> (2012)
2017	Recombinant protein	As16	BALB/c mice	Montanide ISA720	s.c. – 3 doses (days 1, 21 and 35) w/50 $\mu$ g rAs16 + ISA720 each dose	↑ Specific IgG1 and IgG2a, ↑ IL-2; IL-4; IL-5 and IL-10	36.7%	Wei <i>et al.</i> (2017)
2017	Recombinant protein	As16	BALB/c mice	Alum (Alhydrogel)	s.c. – 3 doses (days 1, 21, 35) w/ 25 $\mu$ g rAs16 + 200 $\mu$ g alum each dose	↑ Specific IgG1 and IgG2a, ↑ IL-5, ↑ IL-12 and GM-CSF	38.9%	Wei <i>et al.</i> (2017)
2018	Crude extracts	1) L3 larvae 2) Adults (AD) 3) AD cuticle	BALB/c mice	MPLA	s.c. – 3 doses (days 0, 10, 20) w/ 25 $\mu$ g of each antigen + 25 $\mu$ g MPLA	↑ IgG; IgG1; IgG3, ↑ IgE and IgA, ↑ IL-5; IL-10	1) 61% 2) 51% 3) 59%	Gazzinelli-Guimarães <i>et al.</i> (2018)
2020	Recombinant protein	As37	BALB/c mice	1) Alhydrogel 2) MPLA 3) AddaVax	s.c. – 3 doses (days 1, 21 and 35) w/25 $\mu$ g rAs37 + 200 $\mu$ g alum; 20 $\mu$ g MPLA or 50 $\mu$ L AddaVax	↑ IgG1; IgG2a ↑ IL-4; IL-5; IL-10; IL-13	1) 38.9% 2) 40.7% 3) 49.7%	Versteeg <i>et al.</i> (2020)
2021	Chimeric protein	As37, As16, As14	BALB/c mice	MPLA	s.c. – 3 doses (days 0, 10, 20) w/ 25 $\mu$ g chimeric protein + 25 $\mu$ g MPLA	↑ Specific IgG	73.54%	de Castro <i>et al.</i> (2021)
2021	Crude extract	Intestinal tract homogenate	BALB/c mice	FCA	i.p. – 3 doses (days 0, 10, 20) w/ 50 $\mu$ g of extract + 50 $\mu$ g FCA each dose	↑ IgG response only after infection	No significant <sup>#</sup>	Girasol <i>et al.</i> (2021)

\*reduction of parasite burden; s.c., subcutaneous route; i.v., intravenous route; i.p., intraperitoneal route; i.m., intramuscular route; i.n., intranasal; p.o., *per os*; ES, excretory-secretory; EP, isolated from *Ascaris suum* eggs; L2,3ESP, L2 developing *in vitro* to L3; L3,4ESP, L3 developing *in vitro* to L4; <sup>#</sup>data not shown; CTB, cholera toxin B; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; CytRx, TiterMax Gold adjuvant; MPLA, monophosphoryl lipid A; CT, cholera.



**Fig. 3.** Timeline of *Ascaris* targets and animal models used in the *Ascaris* vaccine development process. To date there have been no vaccine candidates that have moved from pre-clinical animal models into human clinical trials. Created with [BioRender.com](https://www.biorender.com).

financial investment as well as political and community interest. As ascariasis is the most prevalent-neglected tropical disease worldwide and associated with significant morbidity, it should be a major global interest to develop a vaccine to prevent and/or control infection. However, this has not been the case and researchers have struggled to fund and gain public interest in this endeavour. As a result, increasing awareness of ascariasis and other neglected tropical diseases (NTDs), including in HICs, has been a focus of public health campaigns. In the United States, public health campaigns have led to the proposition of new legislation termed ‘STOP: Study, Treat, Observe and Prevent Neglected Disease of Poverty’ Act in 2019 which aimed, in part, to accelerate the development of new control tools such as vaccines against NTDs (Hotez and Booker, 2020). Increasing political and social recognition of these diseases globally may lead to future funding and support to aid in vaccine development, particularly a pan-helminthic vaccine.

There remain significant barriers to the development of an *Ascaris* vaccine. Despite decades of progress in antigen discovery, results of pre-clinical testing of vaccine candidates have largely been underwhelming (as outlined in Table 1). Thus, further investigation in novel vaccine targets remains a critical step in the vaccine development process. Additionally, helminths are complex, multicellular organisms that have an intricate, helminth-specific and developmental-stage specific relationship with the host immune system (Weatherhead *et al.*, 2020). Unfortunately, much of the helminth–host immune response interface remains unknown, which has limited discovery of new antigens and impeded the development of efficacious, directed and safe *Ascaris* vaccines. To date, most of the selected *Ascaris* antigens have been largely limited to worm or ES proteins, however undiscovered proteins may exist in other compartments such as in EVs. Further analysis of EVs, their role in communication with the host and development of immunity may provide insight into antigens of interest (Mekonnen *et al.*, 2018). Another limitation in *Ascaris* vaccine development is the difficulty in selecting an appropriate target to induce protective immunity that does not simultaneously elicit an IgE response or an autoimmune response in the human host. This requires down-selecting of proteins with

significant homology to common allergens and human proteins as well as down-selecting of proteins that induce strong IgE in natural infection. For example, pre-existing circulating hookworm-specific protein IgE from natural infection was found to result in urticarial reactions in individuals immunized with Na-ASP-2, a hookworm-specific protein previously tested in a human clinical trial (Diemert *et al.*, 2012). Furthermore, although adjuvanted recombinant proteins have risen as lead targets for *Ascaris* vaccine development, the induced immune response, even with currently available adjuvants, may be insufficient for *Ascaris* control or eradication efforts (Noon and Aroian, 2017). Identifying innovative adjuvant platforms that can enhance vaccine efficacy must be a priority moving forward if recombinant proteins remain the main stay in *Ascaris* vaccine development programmes.

Modern vaccine technologies that lead to the discovery of novel immunogenic antigens may accelerate the development of a human *Ascaris* vaccine in the near future. Rapid advancement in the fields of transcriptomics and proteomics have provided an algorithmic pipeline to predict *in silico* the most immunogenic and antigenic B- and T-cell epitopes from *Ascaris* parasites based on their molecular and structural features (de Castro *et al.*, 2021). With this technology, it has become possible to screen the entire parasite without the need to maintain the parasite life cycle *in vivo*. This approach in *Ascaris* vaccine development, termed ‘reverse vaccinology’ (Rappuoli, 2000) was first utilized within the meningococcal B vaccine pipeline (Sette and Rappuoli, 2010). The reverse vaccinology principle is based on expressed transcriptome sequences or the entire protein repertoire to find new potential vaccine candidates, instead of using immunoassays to identify strongly immunogenic proteins against immune sera. This approach allows for streamlined exclusion of potential homologous immunogenic proteins to common allergens or human proteins. Protein microarray profiling also provides a high-throughput screening mechanism compared to traditional immune-screening techniques. Protein microarrays can evaluate multiple antigens to identify and quantify immunodominant antigens of vaccine interest (Peacock, 2014).

New vaccine platforms such as nucleic acid based vaccines (DNA and RNA) have recently gained substantial stride in



vaccinology and could play an important role in *Ascaris* vaccine development. DNA vaccines have been investigated in other helminths such as schistosomiasis but have had minimal advancement to date in *Ascaris* vaccine development (Da'dara and Harn, 2005). The use of mRNA technology has more recently emerged as a high-throughput technology with success in other pathogens of global importance. mRNA platforms allow for standardized production and purification of vaccine targets and can be multi-valent, induce strong, directed immune responses without adjuvant and are non-infectious. However, these technologies may be limited by high-cost and need for specialized production, manufacturing and storage which may not be conducive to parasite endemic regions particularly in LMICs (Versteeg *et al.*, 2019).

Moving forward, a vaccine remains the most likely tool to aid in global eradication of ascariasis. Advances in *Ascaris* genomics, transcriptomics and proteomics may provide the needed link to identify immunogenic products that are developmental-stage specific. This information would be essential to build vaccines that directly target the migrating larval stages, which would prevent both larvae-induced morbidity and the development of adult intestinal worms. These technologies may also provide the path forward for the development of a multi-valent pan-helminthic vaccine to support global STH elimination programmes. A pan-helminthic vaccine would be a major step in eradication of several parasites that share significant geographic overlap and help lead to reduction in over-all morbidity within parasite-endemic regions (Zhan *et al.*, 2014). New technologies and investment in vaccine development on a global scale may overcome vaccine development roadblocks and bring us closer to an effective, safe and affordable *Ascaris* vaccine.

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#### Author contributions.

ACGG, PHGG and JW conceived and designed the study. ACGG, PHGG and JW wrote the article.

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