Deciphering inflammation and immunomodulation in host-parasite interface: Caco-2 cells and human intestinal organoids response to Anisakis' *messengers of pathogenicity.*

Department of Public Health and Infectious Diseases

PhD course in Infectious Diseases, Microbiology and Public Health

PhD Candidate*:*

Dr. Ilaria Bellini

Tutors*:*

Prof. Stefano D'Amelio

Dr. Serena Cavallero

A.A. 2021- 2022

Index

Introduction

Helminthiasis have a considerable socioeconomic impact, affecting more than 1.5 billion people worldwide and imposing additional burdens on livestock systems, animal health, food safety, and sustainable agriculture [WHO, 2023]. These pathogens can survive for years inside their natural hosts, mainly due to their ability to modulate both the host's immune system and physiological state. Unlike other infectious diseases, helminthiasis are rarely lethal, but their chronicity can lead to fatal consequences such as the development of tumors. Moreover, despite their importance for public health, they are still mostly neglected.

Anisakiasis is a zoonosis caused by the ingestion of the marine parasitic nematode *Anisakis* spp. and even if humans represent accidental hosts, the infection can elicit several illness forms, provoking mild to sometimes severe symptomatology [Baptista-Fernandes et al., 2017]. The disease may also lead to chronic forms, and based on reported cases, it may represent a risk factor for gastric and intestinal cancer. Despite this, the scientific literature about the inflammatory mechanisms, the *Anisakis* messengers of pathogenicity and their targets and the potential consequences of the infection are still very scarce. This PhD thesis has the aim to expand the knowledge in this topic, focusing the attention on human inflammatory response to different *Anisakis* products, using appropriate and innovative cellular models able to recount the human background as faithfully as possible to reality, and investigating, for the first time, a new discovered vehicle of pathogenicity: *Anisakis* extracellular vesicles (EVs).

Chapter 1: *Anisakis* and anisakiasis: features of an accidental zoonosis

1.1 The zoonotic agent: Anisakis *spp. and its biology*

Nematodes of the genus *Anisakis* are cosmopolitan parasites that infect numerous marine hosts to successfully complete their life cycle [Aibinu et al., 2019]. Marine mammals such as whales and dolphins represent the definitive hosts and, once infected, they excrete *Anisakis* eggs through their faeces into the aquatic environment. Individual first (L1) and then second-stage (L2) *Anisakis* larvae develop inside these eggs then hatching to release motile, free-living L2, which are ingested by the intermediate hosts such as crustaceans [Adrhoer et al., 2020]. Here, *Anisakis* larvae reach the third-stage (L3) and intermediate hosts are then predated by fishes or squid (paratenic hosts), in which L3 penetrate the intestine and encapsulate in tissues, particularly in the mesentery and liver. In this way, the paratenic hosts, acting as accumulators of L3, support a trophic network able to reach the definitive hosts. Cetaceans, consuming L3-infected fishes, allow the parasite to reach the fourth-stage larvae (L4s) and subsequently the adult stages (males and females) in the stomach, in which they will reproduce and will lay eggs, beginning the life cycle again [Baird et al., 2014]. In the infected fishes, as a result of different factors such as the increase of temperature, pH or the fish death, *Anisakis* L3 are able to migrate from the body cavities to the muscles, increasing the risk of human infection [Bilska-Zając et al., 2015]. Humans may become infected by *Anisakis* through the ingestion of raw or undercooked seafood infected by L3 [Sakanari et al., 1989], and even if the parasites are unable to develop into reproductively active adults, the interaction with humans can determine a panel of heterogeneous gastrointestinal and/or hypersensitivities symptoms leading to a fish-borne zoonosis called anisakiasis (or anisakidosis).
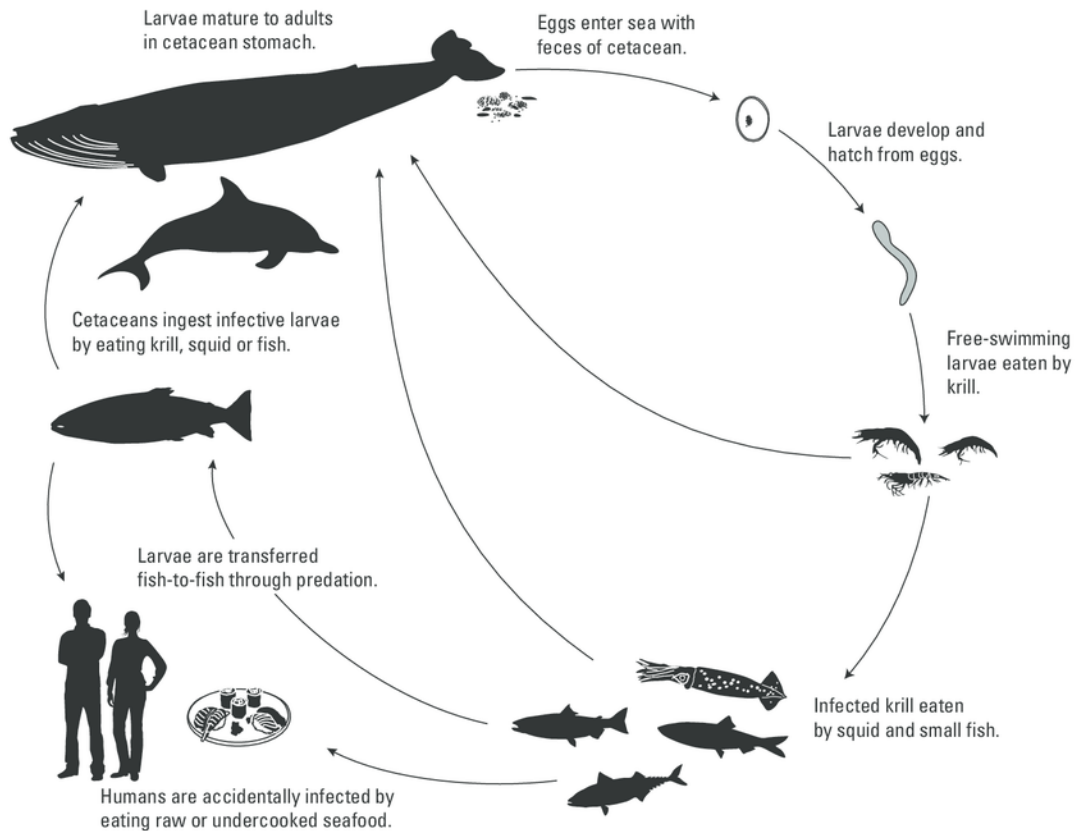
Figure 1: *Graphical representation of the marine nematode* Anisakis spp. *life cycle [Measures et al., 2014].*

Anisakis spp. belong to the subfamily Anisakinae, family Anisakidae, superfamily Ascaridoidea, suborder Ascaridina, order Ascarida [Smith et al., 1978] and there are many species of *Anisakis* which can differ in biology, host range and geographical distributions [Aibinu et al., 2019, Klimpel et al. 2011]. Classification of anisakids at larval stages was originally based on morphological features, such as body width, esophagus length, ventriculus length, extent of the cecum. This led to a classification based on two larval types: *Anisakis* type I and II, according to Berland et al. [1961]. However, the morphological approach is not highly accurate as the genetic analysis. In fact, with the advent of molecular approaches such as sequencing of the internal transcribed spacer (ITS1 and ITS2) region of ribosomal DNA, mitochondrial gene cytochrome oxidase subunit 2 (Cox2), polymerase chain reaction associated to restriction fragment length polymorphism have assisted species-specific

identification representing the best tools to identify and classify taxonomic units of *Anisakis*. Through the molecular analyses, ten species and four distinct clades have been described [Mattiucci et al., 2014; Mattiucci et al., 2018]: Clade 1, also referred as *Anisakis simplex* (s.l.) complex, includes *Anisakis berlandi, Anisakis pegreffii,* and *Anisakis simplex* (s.s.); Clade 2 includes *Anisakis ziphidarum* and *Anisakis nascettii* [Mattiucci 2013]; Clade 3: *Anisakis physeteris, Anisakis paggiae* and *Anisakis brevispiculata* and Clade 4 that include *Anisakis typica*. Additionally, putative hybrids between *A. pegreffii* and *A. simplex* s.s. have been reported in sympatric areas and in the Mediterranean Sea [Abollo et al., 2003; Cavallero et al., 2012] using ITS marker, that allow to detect a recombinant genotype between fixed diagnostic polymorphisms of the two species.
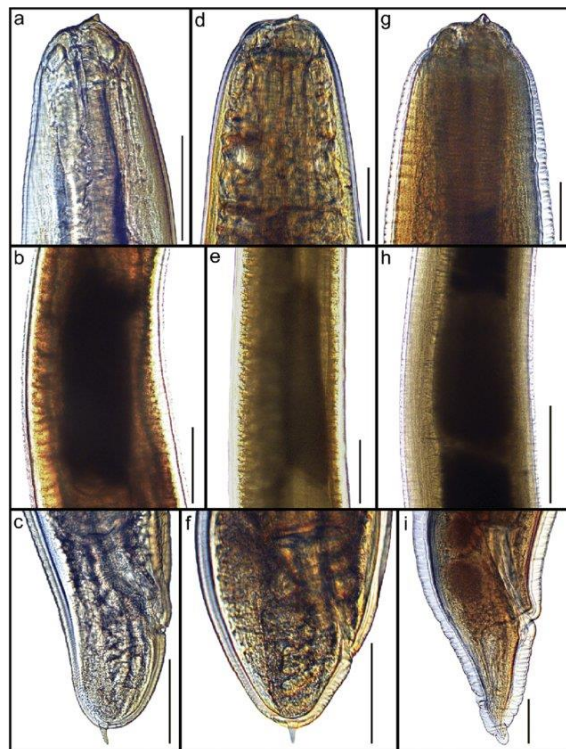
Figure 2: *Optical microscopic images of* Anisakis *L3.* ***(a-c)*** *A. pegreffii.* ***(d-f)*** *A. typica.* ***(g-i)*** *A. physeteris.* ***(a, d, g)*** *Anterior part of the body shows boring tooth. (b, e, h) the Cephalic end. (c, f, i) Caudal end. Scale bars:* ***(a, c, d, f, g, i)*** *50 μm.* ***(b, e, h)*** *20 μm [Sonko et al., 2019].*

However, two species have been correlated with zoonotic infections to humans so far, namely *A. simplex* (s.s.) and *A. pegreffii* [Nieuwenhuizen et al., 2013], and, this doctoral thesis will focus on them exclusively. In particular, *A. simplex s.s*, the most widespread species of the genus *Anisakis*, parasitized oceanic cetaceans of the families Delphinidae, Monodontidae, Phocoenidae, and Balaenopteridae mainly in the Northern waters of Atlantic and Pacific Ocean [Klimpel et al., 2011]. *A. pegreffii* occurs mainly in the Mediterranean Sea and the waters of the Southern Atlantic and Pacific Oceans, and utilizes the family Delphinidae as the preferential final hosts, however, additionally infecting the Ziphiidae, Physeteridae, and Neobalaenidae [Klimpel et al., 2011].

1.2 *Anisakiasis*: *transmission and epidemiology*

Each year, one in ten people feel ill due to the ingestion of contaminated food. According to the World Health Organization (WHO), only in 2010, food-borne diseases resulted in 420.000 deaths [WHO, 2015]. In this scenario, food-borne zoonoses derived from fish and shellfish products acquire even greater public health impact, with a high risk worldwide in both developed and developing countries [EFSA, 2022]. Despite this, the potential pathogens associated with seafood consumption are often neglected, making these zoonoses more likely to occur, as in the case of anisakiasis. The term anisakiasis was first coined around the 1960, following the first record of infection in Netherlands [Van Thiel., 1962], with the intention to define the human disease caused by the third stage larvae of members of Anisakidae family [Van Thiel et al., 1966]. Between 1968 and 1989 almost 12,586 cases were estimated only in Japan [Ishikura, 1990] and in 1988 a standardized nomenclature suggested three different terms: (1) anisakiasis, caused by members of the genus *Anisakis*, (2) anisakidosis, determined by any members of the family Anisakidae and (3) pseudoterranovosis, by members of the genus *Pseudoterranova* [Audicana et al., 2002; Audicana et al., 2008]. Humans could

accidentally be exposed to *Anisakis* L3, through the ingestion of infected raw or undercooked fish (i.e. mackerels, anchovies, cods). Although L3 cannot develop to the adult stage in humans, it is able to reach the gastrointestinal tract causing severe clinical consequences and giving arise to potentially serious pathological conditions [Audicana et al., 2008]. In recent years, the increasing consumption of raw preparations and the trend of exotic dishes worldwide, the lack of awareness about food pathogens have leaded to a consequent increase in fish-borne parasitic zoonosis, considering them as a major food safety problem. The incidence of anisakiasis is strictly dependent by the local dietary habits and Japan alone accounts for around 90% of the total reported cases [Suzuki et al., 2021, Sugiyama et al., 2022]. The remaining cases have been recorded in countries such as Korea, China, Peru, the Netherlands, Germany, France, Spain, Croatia, and Italy [Mattiucci et al., 2022, Jerončić et al., 2020, Year et al., 2018, Herrador et al., 2019]. In particular, in Europe estimates range from 20 to 500 cases/year [Mladineo et al., 2019] according to hospital discharge records and published case reports. In Italy, around 400 symptomatic cases have been estimated based on hospital discharge records in a decade [Cavallero et al., 2018]. However, given several factors such as the large occurrence of L3 in a large variety of fishes, the growing popularity of eating raw or undercooked preparations (sushi, sashimi, carpaccio), the limitations of currently available diagnostic tools and the presence of asymptomatic cases, the global prevalence of anisakiasis is likely to be severely underestimated. About that, WHO estimated approximately 56 million cases deriving from fish parasites due to consumption of infected seafood [WHO, 2012] and a quantitative risk assessment indicated a risk of anisakiasis in between 7700 and 8320 cases annually only in Spain [Bao et al., 2017].

1.3 *Anisakiasis pathogenesis*

Based on larval localization through the gastrointestinal tract, anisakiasis can be classified mostly in three different types: gastric anisakiasis (GA), intestinal anisakiasis (IA) or extra-gastrointestinal anisakiasis (ectopic) [Adroher et al., 2020]. Furthermore, other forms of anisakiasis are identified, such as the gastro-allergic anisakiasis (GAA) caused by an IgE-mediated allergic reaction and an asymptomatic form observed in seropositive cases [Baird et al., 2014]. From 1 h to 6 h after ingestion (Figure 3), *Anisakis* L3 reached the stomach and through a mechanical damage caused by larval migratory behavior associated with the release of secreted proteolytic enzymes (among several: serine, metallo proteases, enolase), adhesion to the gastric mucosa occurred, potentially determining hemorrhagic lesions, worm burrowing and tunnel formation [Audicana et al., 2008].

Time after ingestion	Infection event(s)	Factors released or immune response	Tissue events
<1 h	Mucous adhesion	Proteolytic enzymes	Hemorrhagic lesions; worm burrowing and tunnel formation
4 h to 6 days	Mucosa and submucosa penetration	Chemotactic factors	Eosinophilic phlegmon; erosive lesions
7-14 days	Granuloma formation	Hypersensitivity response induction	Ulcerous lesions
>14 days	Larval death	Persistent inflammation or granuloma	Loss of parasite or chronic ulceration around remains

Figure 3: *Summary of the time-dependent* Anisakis*-humans interactions [Audicana et al., 2008].*

The GA, representing about 95% of the disease burden, is characterized by an early onset of symptoms and, in acute forms, patients experienced abdominal pain, diarrhea, vomiting and allergic reactions such as urticaria or, anaphylactic shock [Shimamura et al., 2016]. Instead, moderate forms of GA are usually characterized by appetite loss, epigastralgia and the occurrence of gastric pseudotumor. On the contrary, acute signs

appear later in IA (around 7 days after eating infected seafood), during this time line *Anisakis* L3 can penetrate intestinal mucosa and submucosa, inducing the release of excrete/secrete and chemotactic factors, potentially eliciting eosinophilic phlegmon and erosive lesions [Audicana et al., 2008]. From a clinical point of view, IA is defined by abdominal pain, nausea, vomiting, mild fever, diarrhea, blood or mucous in stool, leukocytosis and, rarely, eosinophilia. Rare extra-gastrointestinal localizations were documented in the abdominal cavity [Nogami et al., 2016], mesenteries, omentum [Pampiglione et al., 2002], liver [Yamamoto et al., 2015], esophagus. GAA is the most common clinical form, showing the abovementioned gastric symptoms accompanied by an allergic reactions (rush, itching, urticaria, asthma, cough, angioedema, anaphylaxis) [Daschner et al., 2005]. Interestingly, allergic anisakiasis or 'allergy to *Anisakis*' can occur also in the presence of larval allergens derived from the dead parasite [Audícana et al. 2008]. Sensitized patients can also exhibit an allergic response to dead larvae through contact or inhalation of its antigens, following a first exposure to a live L3 [Mazzucco et al., 2018]. This concept is common in occupational settings of fishery and aquaculture workers, cooks, fishmongers and anglers. In such cases, patients exhibit symptoms of rhino-conjunctivitis, asthma, anaphylaxis and dermatitis, which frequently lead to increased incapacity and absenteeism from work [Jerončić et al., 2020]. However, in some cases patient appears asymptomatic but seropositive to *Anisakis* [Mazzucco et al., 2018], thus the broad range of symptoms in association to asymptomatic cases generate misdiagnosis, potentially leading to chronic forms [Park et al., 2018]. Chronic gastrointestinal forms are often associated with appetite loss and epigastralgia and they are difficult to diagnosis solely based on pathological exams or immunological tests. In fact, antibody titers may decrease during chronic periods, and the body of L3 is difficult to identify once it starts to degenerate. In this cases, the most common and incorrect diagnosis is abdominal tumors and intestinal obstruction caused by the granuloma [Nieuwenhuizen., 2016]. About that, even if larvae usually decay within few days in humans, they degenerated in about eight weeks and if host-parasite interaction lasts

over time (> 14 days after ingestion), persistent inflammation, hypersensitivity response, ulcerous lesions and granuloma formation can occur [Audicana et al., 2008]. The main role of granulomas is to protect the host by walling off pathogens or persistent irritants, contributing to a chronic inflammatory microenvironment formation, able to induce an immune response finalized to parasite expulsion or, in some cases, to the disease progression with potentially serious consequences [Nieuwenhuizen., 2016].

1.4 *Diagnosis*

A good medical anamnesis is fundamental to link symptoms to fish consumption, to avoid invasive clinical investigation. A compatible history could be further confirmed by a positive skin prick test or the detection of specific anti-*Anisakis* spp in patients' blood [Chung et al., 2014]. The skin prick test procedure is based on the use of whole body antigen extracts from *Anisakis* spp. but it can produce false positive results due to homology between *Anisakis* spp. allergens and antigens of other nematodes, shellfish or insects (among several: dust mites, crustaceans, mollusks, midges and cockroaches). In addition, one of the criticalities of making a suitable history is that symptoms can occur by exposure to parasite allergens through different routes than the digestive tract. Patients can develop symptoms also being exposed to *Anisakis* spp. proteins through the skin or respiratory tract as it happens in food allergies (Moneo et al., 2017). Given the variety of clinical signs, the similarity to other symptoms of viral, bacterial and parasitic diseases, the presence of asymptomatic cases and the absence of suitable commercial kit, the diagnosis of anisakiasis remain complicated. However, three diagnostic approaches are commonly used: 1) endoscopic and colonoscopy examinations, which may show the presence of the worm, edemas, gastric or duodenal ulcers, lesions and granulomas; 2) X-ray examinations with contrast media, which can show mucosal inflammation and a narrowing of the intestinal lumen; and 3) hematochemical assay, with particular attention to specific and total IgE antibodies [Valle

et al., 2012]. As mentioned before, although there are reports on the usefulness of serology, it is not definitive and easy to interprete because of cross-reactivity. The use of excretory/secretory (ES) products will be useful to improve these aspects since they contain proteins with higher immunogenic potential potentially improving the sensitivity of the diagnostic test used [Moneo et al., 2017]. Moreover, the ES does not contain several somatic allergens such as Ani s 2 (paramyosin) and Ani s 3 (tropomyosin) that would represent a putative source of cross-reactions with the invertebrates. Additional imaging techniques such as computed tomography scan or magnetic resonance may be of diagnostic support. For example, in IA, the computed tomography scan is useful to observe segmental edema of the intestinal wall, proximal dilatation without complete intraluminal occlusion, ascites, and increased attenuation of adjacent fat [Shibata et al., 2014].

1.5 *Treatment and prevention*

The gastric or colonic endoscopic approach can be also curative for gastric and intestinal anisakiasis, allowing the L3 removal using conventional forceps (Figure 4). These methods are mostly used in severe cases, such as IA presenting as bowel obstruction, or granulomas that can resemble other acute abdominal problems.


Figure 4: *Image of* Anisakis *L3 remotion from the gastric mucosa of a 60-year-old Caucasian female, using the standard biopsy forceps [Shimamura et al., 2016].*

To date, the anthelmintic drugs used are albendazole and ivermectin, although several doubts emerged about their administration [Pacios et al., 2013]. The possibility to use alternative drugs, such as gastric mucosal protectants or gastric acid secretion inhibitors, are under evaluation in in-vivo models [Gomez-Mateos et al., 2021]. Regarding this, EFSA expressed its opinion in 2010 [EFSA, 2010], stating that, having not identified specific and targeted pharmacological treatments for the effective killing of the parasite in-vivo, the most efficient treatment remains prevention. The first step for anisakiasis prevention is to educate about the risks of eating raw fish. Public health agencies [FDA, 2022] and experts committees [EFSA-BIOHAZ, 2010] have established a series of measures regarding the methods of fishing and disposal of fish viscera, fish inspection, fish handling before sale, that from 2012 are included in current legislation of UE and other countries such as Japan [Adrhoer et al., 2020]. It's well known as Anisakids are able to survive and be resistant to different treatment conditions such as freezing, microwaving, salting [Tejada et al., 2006]. Studies carried out to investigate the survival of *A. simplex* in fresh arrowtooth flounder (*Atheresthes stomias*) showed that L3 were killed by 96, 60, 12, and 9 h at temperatures of −15, −20, −30, and −40 °C, respectively [Adams et al., 1999; Adams et al., 2005]. Hence, FDA guidelines recommended to cook seafood above 60°C for at least 1 min at the core or to freeze it for at least 24 hours at −20°C or 15 hours at −35°C. [FDA, 2022].

1.6 *Anisakiasis and tumorigenic potential*

Helminths (e.g. flukes) are capable of significant tumor-promoting activity [Scholte et al., 2018]. In particular, *Schistosoma haematobium* (urine bladder), *Clonorchis sinensis* (bile duct carcinoma), and *Opisthorchis viverrini* (liver carcinoma) are classified as carcinogenic agents for humans (IARC group 1) [IARC, 2012]. *Schistosoma japonicum*

is classified in group 2B as possibly carcinogenic, and *Schistosoma mansoni* is classified in group 3 as not classifiable yet to its carcinogenicity. The key factor in the initiation and development of tumor microenvironment is chronic inflammation associated to suppressor-gene inactivation, oncogene activation and somatic mutations [Hibino et al., 2021]. The persistent stimulation occuring during chronic inflammation triggered inflamed cells to generate free radicals (such as reactive oxygen species: ROS) and nitrogen species, which can oxidize and damage DNA leading to genetic instabilities and malignant transformation [Hibino et al., 2021]. Even if to date there are no scientific evidences that officially proves an involvement of the nematode *Anisakis* in tumorigenesis processes, reports of tumor co-localization with *Anisakis* L3 and L3 mimicking metastatic lesions are increasing [Petithory et al., 1990; Eskesen et al., 2001; Mineta et al., 2006; Kang et al., 2014; Sonoda et al., 2015; Gonzalez et al., 2023] (Figure 5) and, interestingly, cases have been reported mainly in countries with a higher prevalence of anisakiasis [Rawla et al., 2019; Aibinu et al., 2019]. A peculiar feature of *Anisakis* L3 is their tendency to adhere to ulcerous, lesioned and cancerous mucosa maybe for local defect in acid secretion, change in mucin and other structural alterations. However, despite it is still unclear if anisakiasis and carcinoma are causatively related or accidental incidences [Petithory et al., 1990], neoplasia and embedded larvae share a common site in all of the reported cases [Mineta et al., 2006].
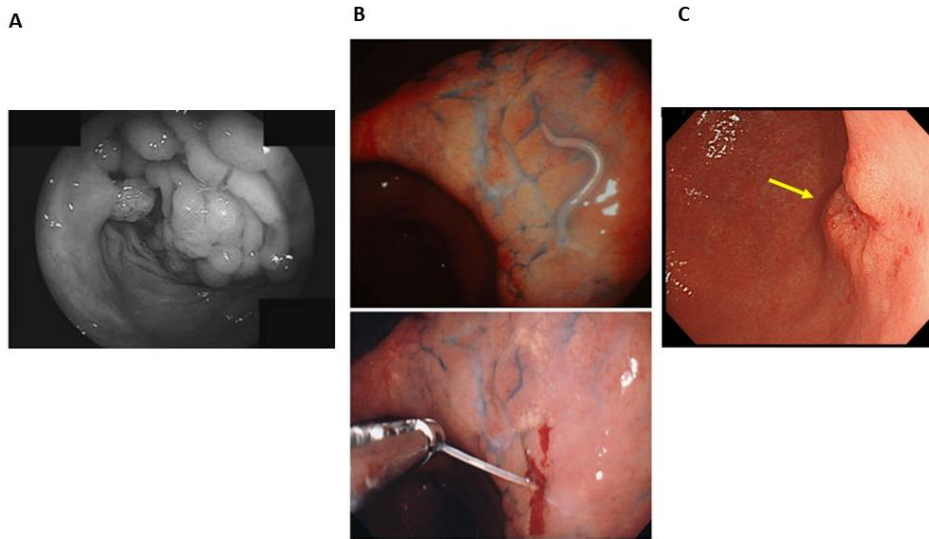
Figure 5: *Representative images of reported cases of* Anisakis *L3 on tumors sites. **A.** Colonoscopic view of a lumen with multiple polypoid lesions, diffuse granular redness, and edematous mucosa associated to chronic anisakiasis [Mineta et al 2006] in a 69 years old female. **B.** Anisakis L3 attached to an early gastric cancer of a 63 years old Japanese male [Sonoda et al. 2015]. **C.** Gastric endoscopy of a 40 years old man with chronic anisakiasis revealed superficial adenocarcinoma (arrow) on the posterior wall in the corpus of the stomach [Sakurai et al 2021].*

Nonetheless, exposure to *Anisakis* is suggested as a risk factor for gastric or colon adenocarcinoma [Garcia-Perez et al., 2015]. To date, only two studies have investigated the tumorigenic potential in the framework of anisakiasis. The first study using hamster ovary cells and Sprague–Dawley rats, revealing an increase in cell proliferation, a reduction of apoptosis, and changes in the expression of serum cancer-related miRNAs in rats [Corcuera et al., 2018]. Moreover, an increased level of P53 and Reactive Oxygen Species (ROS) were observed in the fibroblast cell line, HS-68, treated with *Anisakis* ES products and crude extracts (CE) [Messina et al., 2016]. However, data about the pathogenetic mechanisms, consequences and *Anisakis* potentially messengers of pathogenicity are very scarce, and in this scenario, investigations about human-*Anisakis* interactions acquired even greater importance.

Chapter 2*.* Host- parasite Interaction: *Anisakis* VS human hosts

2.1 Anisakis *messengers of pathogenicity*: *The live larvae (L3)*

The two sibling species most commonly causing human infections are *A. simplex* (s.s) and *A. pegreffii.* Even if they sympatrically occur in several countries, *A. simplex* (s.s.) is known to be prevalent in Japan, while clinical cases due to *A. pegreffii* has been reported mostly in southern Europe such as Italy, Spain, and Croatia [Baird et al., 2014]. In order to understand this epidemiological differences between the two species and to deepen mechanisms of infection in humans, studies on their pathogenic potential have been carried out. In human host, *Anisakis* live L3 could carry out its pathogenicity through almost two different ways: the mechanical action due to its migrating behavior and somatic and excrete/secrete (ES) antigens.

2.1.1   The mechanical action due to L3 migrating behavior

The ability to migrate and penetrate tissues of natural and/or accidental hosts, or to survive in gastric and intestinal microenvironment determining tissues damage could be indicative of L3 pathogenicity, and to date the few studies aimed to explore such abilities in humans revealing interesting outcomes in the pathogenic potential among *Anisakis* species. Scientific evidences demonstrated how the migratory behavior of L3 was non-synchronous and L3 larvae showed no preference for penetration site within vertebrate host tissues (Buselic et al., 2018). Thus, *A. simplex* (s.s.) showed higher tolerance for artificial gastric juice and acid [Jeon et al., 2015] and penetrates both agar and fish muscles [Arizono et al., 2012; Cipriani et al., 2015], as well as digestive tract tissues of Wistar rats more efficiently than *A. pegreffii* [Romero et al., 2013]. Histopathological analysis of lesions caused by the migratory behavior of *Anisakis* L3 in infected Sprague-Dawley rats revealed an acute and strong tissue inflammation, mostly dominated

by macrophages and neutrophils [Hrabar et al., 2019]. In particular, stomach and intestine tissues were characterized by extensive submucosal hemorrhages as well as perivascular necrosis, which allowed leukocytosis [Buselic et al., 2018; Hrabar et al., 2019]. Thus, microRNA expression analysis was performed in Sprague-Dawley rats stomach and intestine affected by L3 migration [Hrabar et al., 2019] and rno-miRNA-451-5p and -223-3p were differentially expressed in the stomach while the same miRNA-451-5p and -672-5p were differentially expressed in the intestine. Therefore, their induction may serve to limit neutrophil infiltration and tissue damage, and gradual substitution with other immune cells, being involved in impairing neutrophil chemotaxis and negatively affecting secretion of IL-6 and CCL3, respectively [Hrabar et al., 2019]. To our knowledge, only one study was aimed to test the mechanisms by which human cells respond to *Anisakis* live L3 in relation to cellular oxidative stress and immunological and inflammatory responses during infection [Napoletano et al., 2018]. Mature and immature dendritic cells (DCs) exposed to *A. pegreffii* live L3 showed a significant decreased in IL10, CXCL10, CCL4 and ICAM expression and upregulation of CCL3. Interestingly, the action of live L3 on DCs affected cellular viability and maturation by reducing the expression of pivotal molecules, such as HLA-DR, CD86, CD83 and CCR7, involved in antigen presentation and migration and by increasing ROS level. At the same time, autologous CD4+ T cells, stimulated with DCs previous exposed to *A. pegreffii* live L3 failed to produce IL-4, IL17 and IL10, suggesting a less reactive phenotype of DCs not sufficient to drive a Type 2 (Th2)/Type 17 (Th17) immune response.

2.1.2 *Somatic and excrete/secrete (E/S) antigens*

The somatic antigens and the release of active molecules (ES) able to facilitate the penetration and survival through the human digestive wall are important pathogenetic factors in the human host. Nevertheless, a comparative analysis of

transcriptomes carried out on *Anisakis* pharyngeal tissues and the whole L3 of *A. simplex* (s.s.) and *A. pegreffii* allowed the identification of groups of molecules with putative roles in mechanisms of parasite pathogenicity [Cavallero et al., 2019]. Among these molecules, the cysteine-rich secretory proteins (CRISPs), belonging to the CAP superfamily (cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins), were significantly upregulated in the pharyngeal tissues of both species. Transcripts encoding for peptidases involved in host tissue penetration and digestion were also present, such as metalloproteinases (i.e. aminopeptidases and neprilysins), that were particularly abundant in *A. simplex* (s.s.) and astacins with anticoagulant functions and protease inhibitors, usually involved in cuticle formation able to prevent attack by host proteolytic enzymes. Moreover, proteomic profiling of *A. simplex* (s.s.) L3 secretome [Kochanowski et al., 2022] revealed an high presence of proteases useful in host-parasite interaction (17% of ES *Anisakis* proteins); antioxidant enzymes able to protect against the toxic contents released by host immune effector cells; proteins essential for parasite life and survival (21% of the E/S); pathogenicity-related proteins such as Heat Shock Proteins (HSPs), and allergens like Anis 4 important for its heat- and pepsin-resistant properties and its ability to induce anaphylaxis. Furthermore, fibroblast cell lines HS-68 exposed to ES revealed an increase in ROS, TNF and P53 levels, an induction of inflammation, cell proliferation and apoptosis inhibition [Messina et al., 2017], interesting features associated with DNA damage and tumorigenic potential.

2.2 Anisakis *messengers of pathogenicity: the crude extract (CE)*

Anisakis crude extract (CE) represents a cocktail of somatic, excrete/secrete antigens (allergens, toxins etc..) derived from pestled whole body of L3, useful to study host-pathogen interaction. Even if the precise number of potential *Anisakis* spp. allergens is

still unknown, the characterization of such messengers of pathogenicity is necessary to deepen the pathogenic cycle of *Anisakis* in accidental human host, to develop robust and reliable diagnostic tools for anisakiasis and potentially to improve effective therapeutic strategies. To date, 14 allergens have been described and characterized experimentally [D'Amelio et al., 2020]. Among them, Ani s 1, Ani s 5, Ani s 7, and Ani s 9 have being recognized by serum antibodies in patients affected by allergic anisakiasis. Interestingly, Ani s 1, Ani s 4, Ani s 5, Ani s 8, Ani s 9, Ani s 10, and Ani s 11 are characterized by heat stability [Rodriguez et al., 2008; Carballeda-Sangiao et al., 2014; Carballeda-Sangiao et al., 2016], and this could lead to the onset of an allergic form even though the infected fish is eaten cooked. In particular, Ani s 1 is a major *Anisakis* allergen, it was detected in parasitic excretory glands by Moneo et al. [Moneo et al., 2000] and it belongs to the animal Kunitz serine protease inhibitor family (which includes peptidases and their inhibitors), revealing that the serine protease inhibitor activity inhibited trypsin and elastase but not chymotrypsin. Ani s 2 or paramyosin is a highly conserved protein that shows a high frequency (88%) of IgE binding sites, for this reason it has been suggested as strongly immunogenic as in other helminthic parasitic infections induced by *Taenia*, *Schistostoma* and *Dirofilaria* [Perez-Perez et al., 2000]. Tropomyosin or Ani s 3 is a pan-allergen, with homology to several known allergens including paramyosin [Guarnieri et al., 2007]. Ani s 7 is a major allergen and the only one identified in 100% of infected patients [Rodríguez et al., 2008], its allergenic potential is related to larval status, in fact during the acute infection, the immune system of rats reacts only with live larvae. Additionally, the proteomic analysis of *A. simplex* L3 identified 17 novel putative allergens, including enzymes like enolase and endochitinase [Fæste et al., 2014]. Furthermore, allergenicity of Ani s 1 and Ani s 9 was studied by intranasal administration of the allergens [Cho et al., 2015] demonstrating a high ability to trigger Th2 (IL-4, IL-5, IL-13 and IL-25) and Th17 (IL-6, IL-17, IL-25 and CXCL1) responses in the lung.

Anisakis CE currently represent the most used product for pathogenetic studies aimed to investigate anisakiasis mechanisms. In fact, human epithelial colorectal adenocarcinoma cells (Caco-2), an in-vitro model widely used to recount human intestinal barrier, exposed to CE revealed a decrease in Caspase-3 activation and marked COX-2 expression, suggesting that exposure to *Anisakis* may affect different mechanisms crucial not only in the inflammatory pathways, but also in cells proliferation and death [Speciale et al., 2017]. Recently, the same in-vitro model was used to study the influence of *A. simplex* CE on the intestinal integrity and permeability in relation to the Ani s 4 allergen [Carballeda-Sangiao et al., 2020]. Data showed a decrease in trans-epithelial electrical resistance after CE treatment, associated to an altered integrity and intestinal barrier function with increased ROS production and changes in tight junction protein localization. Similar results were collected also with other in-vitro models such as fibroblasts cell lines (HS-68), in which altered ROS production, P53 expression and HSP70 were observed [Messina et al., 2016], or Chinese hamster ovary cells (CHO) in which CE induces cell proliferation and apoptosis inhibition [Corcuera et al., 2018]. All of these data represent the first hallmarks to support the existence of damage conditions where a potential tumorigenic microenvironment can occur. Similar outcomes emerged also in in-vivo studies. Corcuera et al. [2018] demonstrated that the gastric mucosa CE inoculation in Sprague-Dawley rats determined the differential expression of 6 miRNAs, among which miR-10b-5p, miR-218a-5p and miR-224-5p are highlighted for their involvement in gastric and colorectal cancer. In addition, studies carried out on Interleukin-4 receptor alpha-deficient BALB/c mice exposed to *Anisakis* proteins, the most used in-vivo model to investigate allergy, revealed allergic reactions in sensitized mice by IL-4/IL-13-mediated mechanisms [Nieuwenhuizen et al., 2006]. The oral administration of *A. typica* CE to BALB/c mice [Haryadi et al., 2019] showed the elicitation of patterns not totally related to food antigen allergy, showing Th1/Th2- related cytokines associated to inflammation and in fact, some inflammatory-related markers were triggered (CD11c+IL-6+, CD4+IFN-γ+, CD4+IL-4+, CD4+IL-5+), indicating that *A. typica*

induces DCs maturation to secrete IL-6 as a pro-inflammatory cytokine. At the same time, some other markers were reduced (CD4+CD25+CD62L+), suggesting a depletion of naïve Tregs population after one week.

2.3 Anisakis *messengers of pathogenicity: extracellular vesicles (EVs)*

A peculiarity of helminths, including nematodes, is that, despite being macropathogens, they are able to establish long-term, chronic and rarely lethal infections in the hosts. Even if the immune system is capable of parasite expulsion, in natural infections, an altered immune response in which the host seemed to tolerate the invader is frequently observed [Maizels et al., 2016]. This phenomenon appears such a re-setting of host immunity and it results from both host reparative responses to physical damage from tissue-migrating parasites and/or through active immunomodulation by their molecular products. Excretory/secretory (ES) products represent the focus of investigation for host's immunoregulation, due to their possible involvement in pathogenesis and disease progression, even promoting tumorigenesis [Mehrdana et al., 2017; Maizels et al., 2018]. Among them, the recent discovery of EVs, as an innovative inter-kingdom cross-talk system and their detection in helminths, has revealed a new paradigm in the study of host-parasite relationships [Coakley et al., 2015]. EVs are nano-scale particles of 100-1000 nm of endosomal biogenesis that are produced and released by almost all cell types (Figure 6). They could vary in size, properties, and secretion pathway in based of their source of origin [Gurunathan et al., 2021].
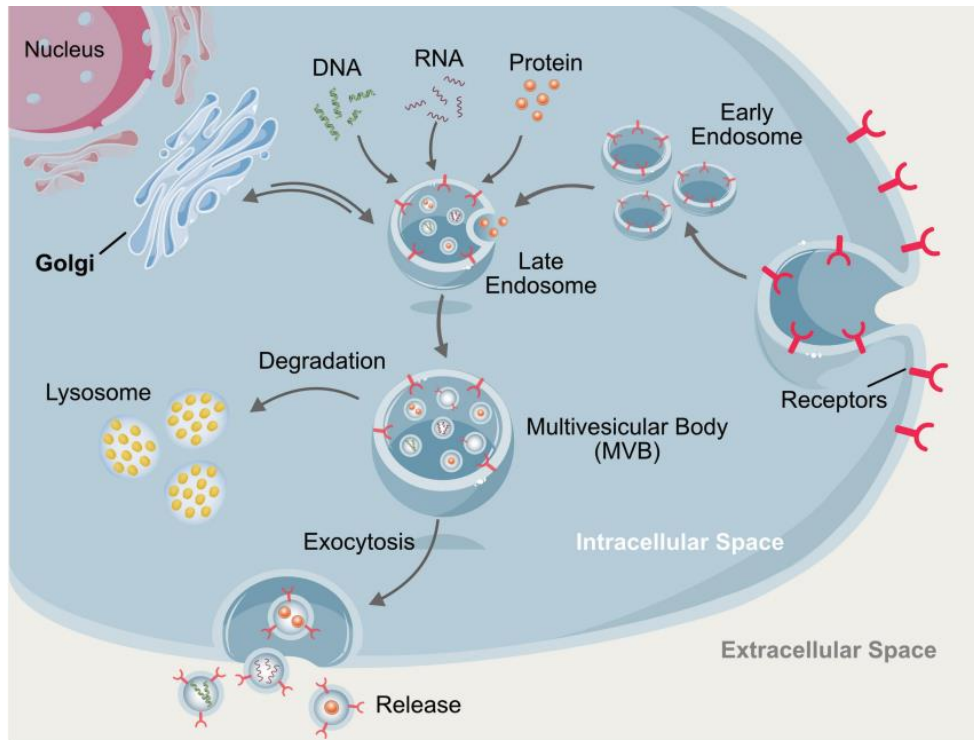
Figure 6*: Extracellular vesicles biogenesis. Internalized cargoes are sorted into early endosomes that then mature into late endosomes or multivesicular bodies. These are specialized endosomal compartments rich in intraluminal vesicles, which sequester proteins, lipids, cytosolic elements and potential EVs cargoes delivered also from trans-Golgi network. Multivesicular bodies containing EVs cargoes get transported to the plasma membrane, fuse with the cell surface and then get secreted as extracellular vesiscles [Gurunathan et al., 2021].*

In addition to their physiological roles, EVs may represent a new potential vehicle of pathogenicity exploited by pathogens to attack their hosts or as co-factors in cancer development [Mashouri et al., 2019]. EVs content is characterized by key elements such as lipids, nucleic acids, proteins and miRNAs, that are involved in a complex regulatory network and modulated genes expression [Saliminejad et al., 2019]. In helminths-derived EVs, this packaged cargo suggests a strong immunomodulatory potential able to suppress the host's type 2 innate immune response [Buck et al., 2014], to ameliorate host inflammatory pathways [Zakeri et al., 2018] and to modulate the transcription of related target genes, affecting the human immune system. These features depicted EVs as interesting new potential candidates for immunotherapies, diagnosis and prevention of different autoimmune or chronic inflammation diseases such as IBD [Eichenberger et

al., 2018, Roig et al., 2018, Gao et al., 2021]. Nonetheless, EVs are involved in the creation of a tumor environment in diseases such as opisthorchiasis or schistosomiasis [Chaiyadet et al., 2015; Yuan et al., 2021]. So far, studies on EVs and their content have been carried out in few nematodes such as *Heligmosomoides polygirus* [Coakley et al., 2017] *, Brugia malayi* [Ricciardi et al., 2021]*, Ascaris suum* [Hansen et al., 2019], and *Trichuris muris* [Eichenberger et al., 2018] showing immunomodulatory effects able to suppress key pathways related to immunity. According to Coakley et al. [2017], EVs secreted by *H. polygirus* are internalized by macrophages and modulate their activation and functions downregulating type 1 and type 2 immune-response-associated molecules (IL-6 and TNF-α, and Ym1 and RELMa), and inhibits expression of the IL-33 receptor subunit (ST2) usually involved in parasite expulsion mechanisms. On the other hand, Ricciardi et al. [2021] showed as *B. malayi* downregulates the phosphorylation of mTOR in human monocytes (THP-1 cells), a highly conserved serine/threonine protein kinase, critical in regulating cellular growth, proliferation, and metabolism, to the same degree that rapamycin (mTOR inhibitor) does. Furthermore, data deriving from the analysis of *A. suum* EVs content showed the presence of miRNAs such as asu-miR-5361-5p, asu-let-7-5p, asu-lin-4-5p, able to target i) CD80, involved in T-cell proliferation and cytokine production, ii) CD86, expressed by antigen-presenting cells and involved in T-cell activation, and iii) SLA-DOB, that encodes MHC class II, respectively [Hansen et al., 2019]. Even if anisakiasis is an accidental zoonosis, it shows interesting and similar features, such as the induction of chronic inflammation and the association with tumor lesions that need to be investigated and in which EVs could play a crucial role. To date, three studies aimed to investigate *Anisakis* EVs demonstrating, using a fluorescent labelling, that also this nematode is able to produce these vesicles [Boysen et al., 2020] (Figure 7), deepen investigating their cargo, focusing on miRNAs [Cavallero et al., 2022], and trying to evaluate their effects on the human host [Bellini et al., 2022]. Results from the latter research represent the majority of the data showed in this PhD thesis.
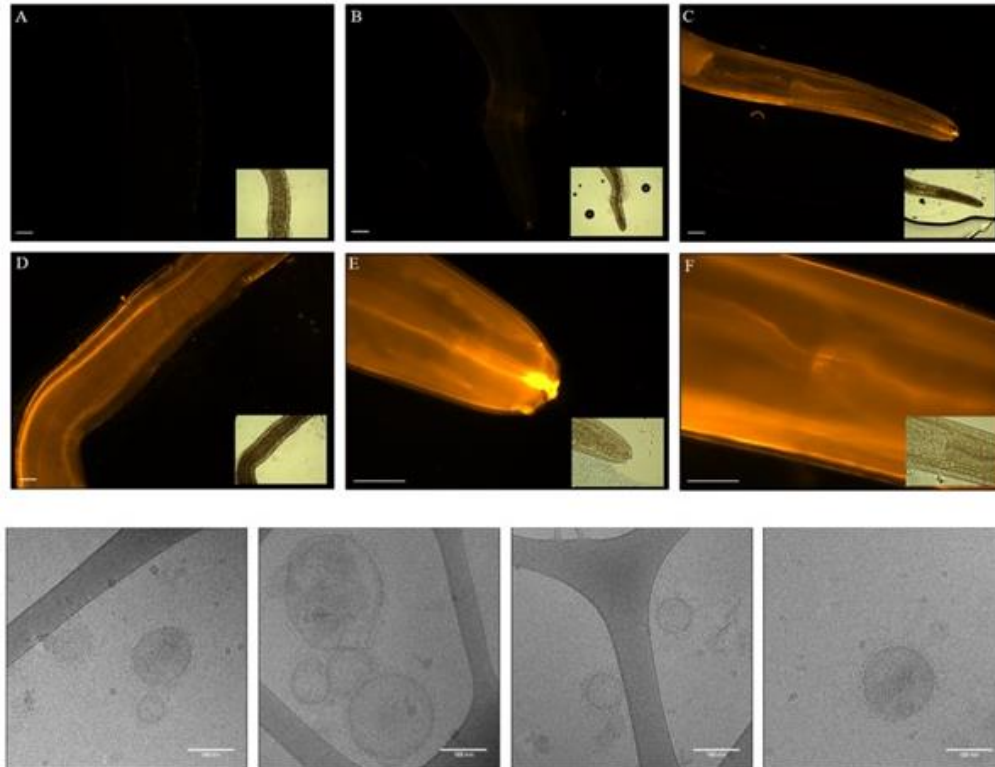
*Figure 7: The first panel showed by Boysen et al. [2020] represented Anisakis spp. L3 cultured in 0 μM (**A**), 1 μM (**B**) or 4 μM (**C–F**) of DOPE-Rho, a fluorescent lipid analogue efficiently incorporated by EVs in their structures. Scale bar: 100 μm. In the second panel, using a Cryo-TEM, Boysen et al. visualized, for the first time, Anisakis EVs, at high resolution, in their native state [Boysen et al., 2020].*

In particular, Cavallero et al. [2022] listed twenty most abundant miRNAs in infective third-stage of *A. pegreffii* larvae (L3) and EVs and interestingly, among several, ape-miR-100a-5p and ape-lin-4 were abundantly expressed also in EVs. Previous studies suggested that a dysregulation of miR-100-5p may be related with several types of human cancers [Chen et al., 2017; Takebayashi et al., 2020; Wang et al., 2021] and similarly, the potential targets of ape-lin-4 are genes involved in cellular proliferation, tumor suppression and induction of apoptosis [Cavallero et al., 2022].

## 2.4 *Human host defense: immune response to helminths*

The tendency to establish stable chronic infections that can endure for surprisingly long (up to 20 years) in an individual host is a result of adaptation processes or dynamic co-evolution between helminths and their hosts. Nevertheless, infection with helminth parasites and their immunomodulators (ES) determine a damage to the host tissues and a release of danger signals able to trigger the innate and adaptive human immune responses [Inclan-Rico et al., 2018]. The large size of helminths represent a condition that prevents them from being quickly captured by phagocytic cells. Therefore, immunomodulating messengers released by immune cells and the parasite itself play a crucial role in host–parasite interactions, contributing to the onset of an inflammatory microenvironment able to induce a Th2 response [Gause et al., 2020]. The first cells involved are the epithelial cells, macrophages and dendritic cells. The epithelial cells represent a physical barrier also equipped with microbial-detection mechanisms, signaling circuits and inflammatory mediators. Their activation results in the release of "alarmin" chemokines as CXCL1, CXCL2, CXCL8, and eotaxins, able to attract neutrophils and eosinophils from the periphery to sites of damage, and cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, IL-4, IL-5, IL-13, IL-9 and IL-33, also involved in parasite expulsion mechanisms, proliferation and activation of terminally differentiated innate cell populations and type 2 response initiation [Inclan-Rico et al., 2018]. Being unable to exert phagocytosis action against the parasite, the inflammatory microenvironment exerts selective pressure on macrophages, promoting their polarization of alternatively activated or M2 macrophages, responsible of tissue reparation [Inclan-Rico et al., 2018]. On the other hand, DCs as mediators between innate and adaptive immunity, play a pivotal role in the recognition, capture, processing, and presentation of helminth molecules to T cells [Everts et al., 2010]. In fact, DCs are able to detect multiple ES by expressing different innate immune receptors such as the molecular patterns highly conserved in pathogens or PAMPs, Toll-like receptors (TLRs),

C-type lectin receptors (CLRs), and nucleotide binding domain leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs) [Motran et al., 2018]. At this point, mature DCs migrate to lymph nodes, priming T naïve cells and giving arise to a Th2 clonal proliferation. This leads to a stereotyped signal cascade of effector mechanisms, including immunoglobulin E isotype-switched B-cell responses, increased permeability, epithelial cell turnover, smooth muscle contractility, mucus production, eosinophilia and mastocytosis, with potential consequent parasite killing and expulsion [Motran et al., 2018]. Interestingly, in order to survive for a long time inside the host, helminths have developed a series of strategies able to target many of the mechanisms described above. Some example include: the alarmin release inhibitor HpARi produced by *H. polygyrus,* that suppresses IL-33 [McSorley et al., 2012; Osbourn et al., 2017], cytokines involved in parasite expulsion mechanisms [Maizels et al., 2018]; the chemokine binding protein SmCKBP released by *S. mansoni,* able to neutralize chemokine activity (CXCL8, CCL3, CX3CL1,CCL2, CCL5); inhibiting neutrophil migration, *B. malayi* TGF-β homolog-2, that ligates mammalian TGF-β receptor and suppresses T-cell responses; Ace-MTP-2*,* a tissue inhibitor of metalloproteases, released by *Ancylostoma ceylanicum* able to reduce MHC-I and MHC-II molecules on DCs, which consequently induce CD4 and CD8 Treg cells [Maizels et al., 2018]. In this regards, also EVs represent an important immunomodulant instrument for helminths. *Nippostrongylus brasiliensis* EVs suppressed inflammatory cytokines IL-6 and IFNγ, as well as upregulated the anti-inflammatory cytokine IL-10 in in-vivo models of colitis [Eichenberger et al., 2018]. DCs exposed to *Fasciola hepatica* EVs inhibit antigen-specific production of the T-cell growth factor IL-2 by T-cells, and did not activate the Th2 responses normally seen during *F. hepatica* infection [Murphy et al., 2020]. In this setting almost every facet of the immune system seems to be modified or even recalibrated, displaying a state of immune hyporesponsiveness in the host that can be considered a form of immunologic tolerance, which may lead to a chronicity with severe consequences for the host. However, the extraordinary helminths prevalence bears witness and their success at defeating host

defenses suggest that we have much to learn from how these parasites modulate our own immune system.

2.5 *Human host defense: immune response in anisakiasis*

A co-evolutive adaptation concept is not likely to be extended to anisakiasis, as humans represent accidental hosts; however, some interesting features emerged from the few studies aimed to explore *Anisakis* ability in modulating humans' inflammatory and immune response [Napoletano et al., 2018; Rodero et al., 2021; Zamora et al., 2021]. Despite this, the fine molecular mechanisms related to anisakiasis pathogenesis and related clinical outcomes are still mostly obscure. Once *Anisakis* reached the gastro-intestinal tract, several sources as ES molecules from living larvae, somatic antigens from living or dead L3 are detected by the first responder epithelial cells through immune receptors such as TLRs (Figure 8) [Audicana et al., 2008]. Interestingly, Zamora and colleagues [Zamora et al. 2021] demonstrated as *A. simplex* ES and CE seemed to affect the response unleashed by TLR2, 4, and 9 agonists, which are the main TLRs involved in helminths detection, mainly acting over cytokine's levels (IL-10, IL-12, TNF-α) in BALB/c and C57BL/6J mice.
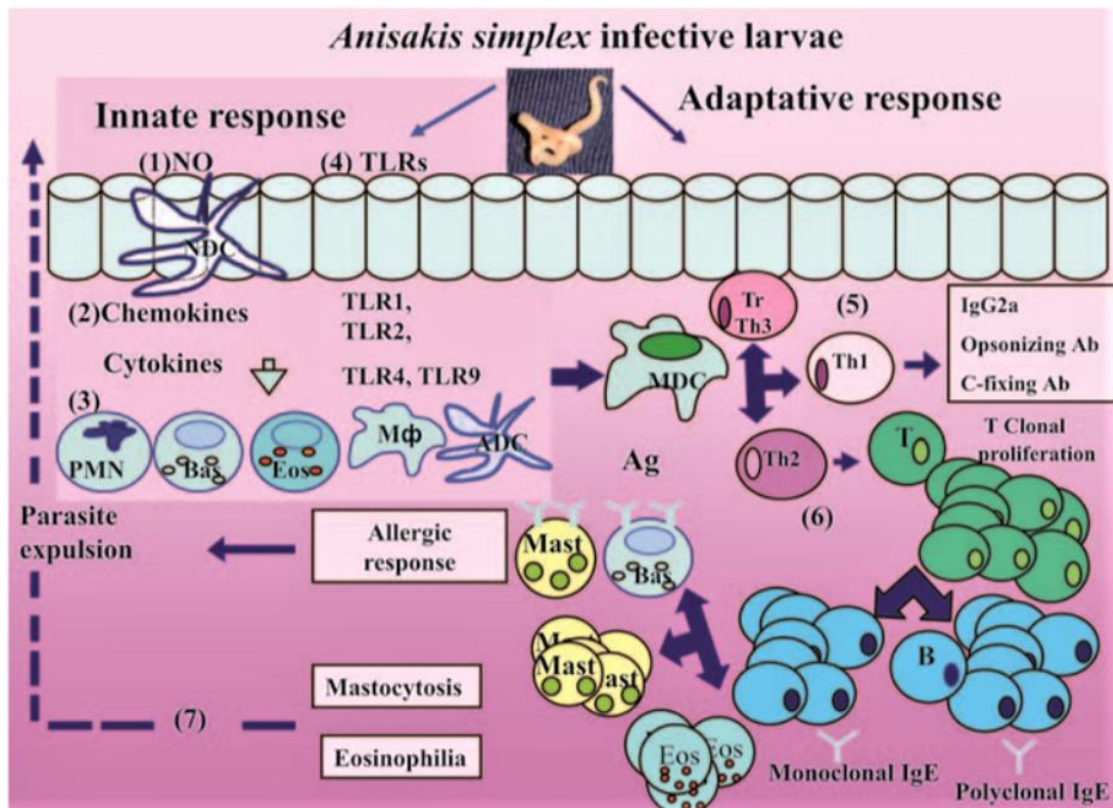
Figure 8: *schematic representation of the innate and adaptive human immune response to* Anisakis *spp.* *[Audicana et al., 2008].*

However, this recognition induced the release of cytotoxic molecules such as nitric oxide (NO), ROS [Messina et al., 2016], giving arise to an inflammatory microenvironment (TNF-α, COX-2, p53, Caspase-3) able to trigger and attract innate immune cells such as DCs, neutrophils, basophils, macrophages and polymorphonuclear leukocytes. Antigen presentation by activated DCs stimulates a dual response of Th1 and Th2, and other T cells can be recruited as T-regulatory cells [Audicana et al., 2008]. Th1 cytokines such as IFN-γ, TNF-α, IL-2, and IL-13 lead the production of IgG2a antibodies, macrophage activation, and delayed-type hypersensitivity. Th2 cytokines (IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13) promote IgG1 and IgA production and by T- clonal proliferation and B cells maturation, antigen-specific IgE and total/polyclonal IgE are produced. Mastocytosis and eosinophilia are induced by a Th2 response and chemoattractive cytokines and may be responsible for parasite expulsion and for a great deal of tissue damage observed

surrounding the parasite in both acute and chronic infections, in fact, eosinophil infiltration is one of the features of anisakiasis tissue lesions [Hrabar et al., 2019]. In-vitro experiments carried out on DCs exposed to *Anisakis* L3 and CE showed that the parasite tend to modulate their activity through the induction of a less responsive phenotype not able to trigger a strong T cells activation [Napoletano et al., 2018]. In some cases, parasite expulsion can fail and *Anisakis* L3 usually die within about 14 days in humans, during which larval debris is surrounded by edema, necrosis and cellular inflammation characterized mostly by eosinophils but also neutrophils, lymphocytes and monocytes, fibrotic tissues that lead to granuloma formation [Nieuwenhuizen et al., 2016].

2.6 *Limits in scientific literature*

Despite Anisakids global distribution, their medical and socio-economic impact, and the hypothesis of tumors correlation, most of the research efforts found in the scientific literature focused on food safety, taxonomic, ecological or phylogenetic aspects, while specific disease pathways leading to host inflammatory response and chronic reactions as well as the parasitic tumorigenic potential are still obscure. The study of host-pathogen interactions in parasitology and in particular in nematology is characterized by several limitations such as the lack of suitable model systems that are able to recapitulate the microenvironment in which the parasite acts and reliable and robust genomics data. Additionally, working with parasitic helminths could be challenging, as most species cannot be maintained in culture for their whole life cycle, or in conditions that closely mimic the host environment and natural infection. In the case of anisakiasis, the scientific literature is extremely scarce and difficult to compare. Furthermore, the few in-vitro studies aimed to investigate human-*Anisakis* mechanisms of infection and the consequences of this zoonosis on human host showed several limitations:

- A lack of standardization in terms of experimental settings (quantity/dose of starting materials such as CE, amount of L3 used, timing of incubations, kind of materials used, such as live L3, CE, E/S, coadjuvants)

- In vitro models applied rarely resembled the real niche of infection.

- Problems in data comparison.

Furthermore, the discovery of EVs has revealed a new way forward in the study of host–parasite relationships [Coakley et al., 2015], adding complexity and curiosity. Decoding this interaction will be a fundamental key to control parasites in the future and in this framework, the importance of ES products in governing these interplays is indisputable. Recommendations proposed by the International Society for Extracellular Vesicles for the mammalian and other eukaryotic EVs have been recently implemented also for studies dealing with helminths parasites [White et al., 2023]. In this scenario, the investigation of *Anisakis* EVs, their role in pathogenic mechanisms, their potential effects on the host or their involvement in anisakiasis are still in their infancy. For these reasons, the need to deepen these lines of research, the search for and use of models that could better recount the natural environment in which the infection occurs, and the standardization of experimental settings could prove to be important points to acquire more data regarding anisakiasis and its potential consequences in humans.

Chapter 3: suitable cellular models

3.1 *Human colorectal adenocarcinoma cells: Caco-2 in vitro model*

Caco-2 (*Ca*ncer *co*li-2) cells were established from a human colorectal adenocarcinoma by Jorgen Fogh at the Sloan-Kettering Cancer Research Institute [Fogh et al., 1977], with the objective to investigate mechanisms in tumors development. However, Caco-2 cells expressed several morphological and functional features typical of small bowel intestine (Figure 9).

Table 10.1 Properties of Caco-2 cells

Growth	Grows in culture as an adherent monolayer of epithelial cells
Differentiation	Takes 14–21 days after confluence under standard culture conditions
Cell morphology	Polarized cells with tight junctions and brush border at the apical side
Electrical parameters	High electrical resistance
Digestive enzymes	Expresses typical digestive enzymes, membrane peptidases and disaccharidases of the small intestine (lactase, aminopeptidase N, sucrase-isomaltase and dipeptidylpeptidase IV)
Active transport	Amino acids, sugars, vitamins, hormones
Membrane ionic transport	Na^+/K^+ ATPase, H^+/K^+ ATPase, Na^+/H^+ exchange, $Na^+/K^+/Cl^-$ cotransport, apical Cl^- channels
Membrane non-ionic transporters	Permeability glycoprotein (P-gp, multidrug resistance protein), multidrug resistance-associated protein, lung cancer-associated resistance protein
Receptors	Vitamin B12, vitamin D3, EGFR (epidermal growth factor receptor), sugar transporters (GLUT1, GLUT2, GLUT3, GLUT5, SGLT1)
Cytokine production	IL-6, IL-8, TNFα, TGF-β1, thymic stromal lymphopoietin (TSLP), IL-15

Figure 9: Table showing the main properties characterizing Caco-2 cells [Lea et al. 2015].

Towards confluence, they polarized acquiring an apical brush border with microvilli, cells-cells tight junctions, expressed enzyme activities peculiar of enterocytes (among several: aminopeptidase N, lactase, dipeptidylpeptidase IV etc..) [Lea et al. 2015] and showed 4 times higher trans-epithelial electrical resistance (TEER) values compared to HT29 cell lines (human adenocarcinoma cell line), more similar to the in-vivo condition. Additionally, Caco-2 cells express receptors, transporters and drug metabolizing

enzymes usually present in normal intestinal epithelium and they are excellent tools for high-throughput screening, genetic manipulation and biobanking [Lea et al., 2015]. Today, these properties allowed them to be widely used as a model of the intestinal epithelial barrier, also suitable for investigation of host-pathogens interactions both for viral [Bautista et al., 2015], bacterial [Ambrosi et al., 2015] and parasitic diseases [Ma'ayeh et al., 2018]. In scientific literature, intestinal helminths and their interactions with hosts have been modelled mainly with common cells lines that allow to recapitulate small intestine and colon epithelial function such as HT-29, Caco-2 and T84. Although, different cell lines have been used to investigate the epithelial cells response to different nematodes such as *H. polygirus* [Coakley et al. 2017]*,* and despite the gut resemble the microenvironment in which also *Anisakis* spp. accidental infection occurs, only two studies used Caco-2 cells to investigate human anisakiasis [Speciale et al., 2017; Carballeda-Sangiao et al., 2020]. If compared with normal intestinal epithelium, the Caco-2 cells model present several limitations. First of all, the normal epithelium is characterized by an heterogenicity in cells population (enterocytes, paneth cells, goblet cells etc..). Secondly, no mucus layer is present. Finally, Caco-2 cells derived from cancerous cells, limiting their use in understanding physiological conditions in healthy tissues [Lea et al., 2015]. Nevertheless, to date, Caco-2 cells provide a powerful and not so expensive tool for studying properties of the intestinal epithelium and deepen host-pathogen interface, for these reasons mostly of this PhD thesis involved studies using this in-vitro model.

3.2 *Human Intestinal Organoids (HIO): a new tool to decipher host-parasite interaction*

The modern term "organoid" refers to cells growing in an in-vitro bi or three-dimensional (3D) environment to create mini-clusters of various cell types capable of self-organization, self-renewal, and exhibiting the architecture and functionality of the organ

of origin, for these reasons they are also called "mini-organs" [Corrò et al., 2020]. Organoids can be derived from induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs) and neonatal or adult stem cells (ASCs), when cultured in precise conditions that resemble those of the stem cell niche. These conditions involve the use of an extracellular matrix supported by the use of growth factors and morphogens that direct the differentiation and division of cells into the heterogeneous population of the targeted organ. The first attempt of in-vitro organism regeneration was exploited by Henry Van Peters Wilson in 1907 [Wilson et al., 1907], describing that dissociated sponge cells can self-organize to regenerate a whole organism. Later, research groups start to perform studies leading to increasingly innovative discoveries concerning the generation of different types of organs from dissociated amphibian pronephros [Holtfreter et al., 1944] and chick embryos [Weiss et al., 1960], the thermodynamics of cell sorting and rearrangement [Steinberg et al., 1964]. Stem cell first isolation (1981), culture (1998) and reprogramming, the improvement of cell culture conditions through the simulation of the in-vivo microenvironment (Figure 10) [Corrò et al., 2020].
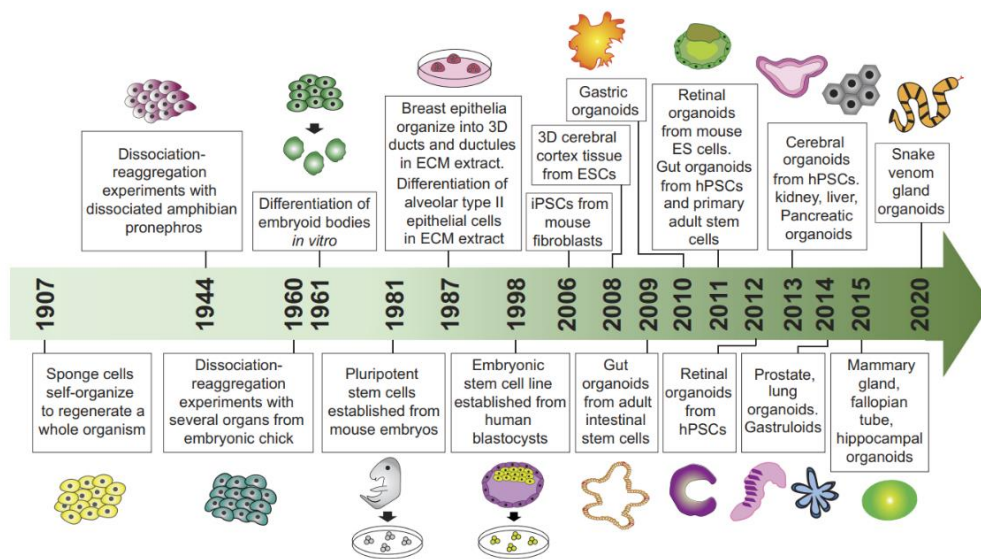
Figure 10: history of organoids. In particular, human intestinal organoids were the first 3D cultures that have been created in laboratory, in 2009 [Corrò et al., 2020].

In 2009, Sato et al. [2009] demonstrated that adult intestinal stem cells expressing the single leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5)- expressed in adult intestinal stem cells were able to generate 3D- intestinal organoids if cultured in Matrigel presenting the crypt-villus structures. To date, organoids have been developed for a variety of organs and tissue such as liver, lung, stomach, esophagus, brain, kidney, etc.. opening new lines of investigation in biomedical research [Clevers et al., 2016]. Organoids are used also as suitable models for different infectious diseases, including bacterial, viral and parasitic. While they are gaining popularity as model for studies on protozoan infections, such as *Toxoplasma gondii* infection in bovine and porcine intestinal organoids [Derricott et al., 2019], the investigation of *Cryptosporidium parvum* life cycle in murine and human small intestinal and lung organoids [Wilke et al., 2019; Heo et al., 2018], their use for helminths investigations is on its infancy. Recently, organoids have been exploited as a model for indirectly investigate the effects on the intestinal epithelia induced by intestinal helminths infections using ES products of different nematodes. EVs derived from *T. muris, A. suum and N. brasiliensis* have been successful microinjected into 3D organoids, recapitulating parasite interaction with the host epithelium [Duque-Correa et al., 2020; Chandra et al., 2019; Eichenberger et al., 2018], demonstrating their uptake in host cells and immunomodulatory properties such as protection against chemically induced colitis and a downregulation of specific targets such as interferon stimulated genes (ISGs). However, since the microinjection requires specialized training and instruments, 2D organoids, growing in semi-permeable membranes, represent a suitable option to overcome such limitations, allowing the apical delivery of parasite and their products, and being amenable to high-throughput applications [Duque-Correa et al., 2020]. In general, organoids are a valid alternative bridging in-vitro, in-vivo and ex-vivo models, they can be generated from biopsies deriving from hosts of interest and they can be expanded and cryopreserved as individual lines, allowing to perform several experiments. Differently from classic cell lines, their

multicellularity allows for the investigations of the role and the effects of different cells populations in helminth invasion and pathogenesis, they retain organ architecture and genome stability that permit to better evaluate the cellular and molecular interactions of helminths with their host [Duque-Correa et al., 2020]. On the other hand, in-vivo tissue is provided with vascularization, innervation, microbiota and immune cells, which are totally missing in the model. At the same time, their use is expensive and their dimensional conformation and size could affect the delivery and the culture of only precise parasitic stages [Duque-Correa et al., 2020]. To our knowledge, no studies aimed to investigate the effect of *Anisakis* and its products on human intestinal organoids are available. In this scenario, the preliminary experiments and data described in this PhD thesis represent the first attempt of 2D human intestinal organoids stimulated with *Anisakis* EVs.

Chapter 4: Objectives of the thesis

The general aim of this PhD thesis is to provide new knowledge about pathogenetic mechanisms depicting anisakiasis, focusing on the inflammatory response of human host.

Is Anisakis spp. able to induce an inflammatory response in its pathogenic cycle inside the accidental host? Can we identify *the effects of* Anisakis *EVs on human host?*

To achieve this goal, the project has been structured in two sections:

1- *Caco-2 cells exposed to three* Anisakis *derived messengers of pathogenicity above described;*
2- *HIO exposed to* Anisakis *EVs.*

4.1 *Caco-2 cells exposed to three* Anisakis *derived messengers of pathogenicity*

In this first section, the host-pathogen interplay is investigated using Caco-2 cells exposed to *Anisakis* derived products of pathogenicity carried by three challenges. These mimic two pivotal phases of *Anisakis* cycle in humans: i) L3, as a model for the initial contact with the intestinal epithelium; ii) the crude extract CE, representing the whole body of senescent larvae, mirroring the moment in which the parasite, failing to complete its life-cycle, and eventually dying, and iii) the exosomal enriched fraction of EVs , as a potential vehicle of pathogenicity and immunomodulation. In particular, the gene expression and the protein product abundance of two crucial pro-inflammatory cytokines (IL-8 and IL-6), mostly expressed by Caco-2 cells, have been assessed by a quantitative real-time PCR (qRT-PCR) and a sandwiched enzyme-linked immunosorbent assay (ELISA), respectively. IL-8 is a strong chemokine secreted by different cell types including blood monocytes, fibroblasts, and epithelial and endothelial cells, and it has a crucial role in the recruitment of neutrophils and granulocytes to the site of infection [Ha

et al., 2017]. IL-6 is a pleiotropic cytokine with a pivotal role in inflammation and hematopoiesis, it represents a link between the innate and acquired immune response, and it is often involved in autoimmune disease and tumorigenesis [Jones et al., 2005].

4.2 *HIO exposed to* Anisakis *EVs*

To expand the knowledge on *Anisakis* EVs and their effects on human host, the second section has the aim to investigate, for the first time, the impact of *Anisakis* EVs on HIO cultures, highlighting the potential effects, risks and consequences of these intricate host-pathogen dynamics. To achieve this goal, the specific objectives are:

o To develop from human biopsy and assess an innovative tool for anisakiasis based on human intestinal organoids.

o To perform gene expression analyses (qRT-PCR) on transcripts of interest (*Il33, Il1β*, *Il8*) involved in inflammatory response.

The secretion of the alarmin cytokine IL-33 represent a potent signal for type 2 response initiation, as it is involved mainly in parasite expulsion mechanisms. As shown in two previous studies carried out on *H.polygirus*, IL-33 pathway seems to be an ideal target for parasite immunomodulation to determine the persistence of infection [Coakley et al. 2017; Osburn et al., 2017]. For this reason, it will be interesting to investigate *Il33* gene expression also in anisakiasis. IL-1β is a pro-inflammatory cytokine usually upregulated in pathological conditions such as rheumathoid arthitis and type 2 diabetes [Zaiss et al 2013], and also in the intestine of IBD patients. Moreover, according to Helmby et al. [2004], IL-1β plays a key protective role in chronic gastrointestinal nematode infections, being a crucial component for the development of Th2 responses useful for parasite expulsion. Thus, the investigation of IL-1β production could be attractive in investigating the potential chronicity pathways of intestinal helminth infection. Unfortunately, due to very low basal *Il6* gene expression in this model, qRT-PCR efficiency was not

comparable with the other genes analyzed, and for this reason, the results about *Il6* gene expression in human intestinal organoids are not showed in this thesis.

Chapter 5: Materials and methods

5.1 Parasite sampling

Anisakis live third-stage infective larvae (L3) were collected from the visceral cavity of 21 European hakes *Merluccius merluccius* purchased at markets between 2018 and 2022 and captured from area FAO 37 (Mediterranean Sea). L3 were washed three times in a 0.22 μm filtered phosphate buffer saline (PBS). After the experiments, all the L3 used for the study were stored at −20°C for subsequent identification of species through a molecular diagnostic key based on the PCR-RFLP of the internal transcribed spacers of the nuclear ribosomal DNA, according to D'Amelio et al. [2000].

5.2 Crude extract

A pool of L3 (n = three) was used to obtain crude extract samples. CE was prepared as follows: PBS 10X was added to each sample and then homogenated with pestles. The mixture (0.13 mg/mL) was centrifuged at 13000 rpm for 30 min. at 4 °C, according to Mattiucci et al. [2017]. Lastly, the supernatant was used for the challenge with Caco-2 cells, whereas the pellets were stored in 70% ethanol for the subsequent identification of species. The protein concentration of samples were evaluated using a Qubit4 (Thermo Fisher Scientific, Waltham, MA, USA).

5.3 Isolation and EVs-enriched fraction characterization

Pools of L3 (n = 20 for Caco-2 experiments and n = 50 for HIO experiments) were incubated in RPMI with 1X Pen/Strep for 24 h at 37 °C with 5% CO2. After incubation, the culture media were collected to isolate the exosomal-enriched fraction of the EVs using an ExoQuick kit (System Biosciences, Palo Alto, CA, USA) in accordance with the

manufacturer's protocol. The samples obtained were diluted in a 0.22 µm filtered PBS and immediately used for incubation with Caco-2 cells and human intestinal organoids, while the larvae were stored for the subsequent identification of species. The size distribution and concentration of particles in the recovered fractions after the exosome enrichment were measured using nanoparticle tracking analysis (NTA), using a Nanosight NS300 (Malvern Panalytical, Malvern, UK). Five measurements were performed with a 60 s duration for each measurement and the data were analyzed using NTA software version 3.4. To further characterize the presence of extracellular vesicles in our samples, western blotting analysis of the isolated EVs against the cytosolic EVs' marker ALG-2-interacting protein X (Alix) antibodies were undertaken. The protein concentration of the EV samples was evaluated using a Qubit4 (Thermo Fisher Scientific, Waltham, MA, USA). The CE sample (used as the larval control), Caco-2 cells (used as a positive control for the anti-body), and the EV samples were lysed in a 5X (CE and EVs) and 1X (Caco-2 cells) sample buffer (50 mMTris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol). About 8 µg of proteins were resolved on a 12% Tris–glycine SDS-PAGE and transferred onto nitrocellulose membranes (Hybond-P GE-Healthcare, Sigma-Aldrich St. Louis, MO, USA). Western blot analyses were performed using polyclonal, rabbit anti-Alix (1:500, TBS-T, 5% NFDM) from Thermo Fisher Scientific, USA (Catalog # PA5-52873). Anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad) was used as a secondary antibody (1:2000). Blots were visualized by an enhanced chemiluminescence system (GE-Healthcare Bio-Sciences, Milan, Italy).

5.4 Caco-2 cells culture and challenging experiments

Caco-2 cells (ATCC HTB-37) were grown in a Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and grown at 37 °C in the presence of 5% CO2. Cells were seeded at a density of 1×10^5 cell/mL in 35 mm cell culture dishes

and grown for 48 h prior to being challenged with CE and EVs. Otherwise, cells were grown for 14 days (full-confluent and polarized monolayers) before being incubated with live L3 larvae. To ensure that the Caco-2 monolayer was not affected and disrupted by the larval mechanical action, we performed two-time points for ELISA incubation (6 h and 24 h) while for the CE and EVs challenge, a single time point at 24 h was assessed. To evaluate a potential temporal dynamic early host–pathogen interaction on the cytokines' gene expression [Ebner et al., 2018], three-time points at 1 h, 6 h, and 24 h for the qRT-PCR were assessed. After the indicated time points, cell supernatants were collected and stored at −80°C for ELISA analyses, while cells were lysed and whole cell extracts were used for qRT-PCR analyses.

5.5 *Human intestinal organoids culture and challenge with* Anisakis *EVs*

Three biological human colonic samples were used for the experiments. Two samples were obtained by the collaboration with HUB-Hubrecht Organoid Technology and they were cultured following manufacturer's protocol, while one biological sample were established from colonic biopsies from a healthy human donor by the collaboration with Prof. Anna Maria Pronio, from the Chirurgic Department of Policlinico Umberto I. Both samples from HUB and Policlinico were obtained after the acceptance of the ethical committees. Colonic biopsy sample was obtained from an individual (Female, 62 ages) that had been admitted to the hospital, for suspected inflammation. The individual was classified as healthy based on standard histological examination of biopsy. Crypts were isolated from colon biopsy washing them with cold DMEM/F12 and incubated with 10 mM EDTA for 30 min. After harvesting the crypts containing supernatant, EDTA was washed away and crypts were seeded in 50% Matrigel (Corning®, Kaiserslautern, Germany) in 24-well plates (Corning®, Kaiserslautern, Germany). Growth medium (STEMCELL technologies, Vancouver, Canada) was further supplemented with 1X

Pen/Strep and gentamicin (1:1000). Organoids were incubated in a humidified chamber with 5% CO_2 at 37°C. Medium was refreshed every 2–3 days, and organoids were passaged every 7 days. After two passages, HIO were mechanical destroyed and generation of an organoid-based 2D monolayer culture was performed. Cells were counted and $1 * 10^5$ cells seeded on precoated (Matrigel 1:50 in PBS) Transwells® (Corning®, Kaiserslautern, Germany; diameter: 12 mm; pore size: 0.4 µm). Basolateral chamber was filled with 600 µl of growth medium, apical chamber with 0.1 ml growth medium, respectively. After 14 days of cultivation, growing medium was changed to differentiation medium for another 7 days until experiments were carried out. 2D HIO were exposed two times with 20 µl of *Anisakis* EVs during 48 h. After incubation, total RNA extraction from 2D human intestinal monolayer was performed.

5.6 *2D Human intestinal monolayer immunostaining*

2D organoids cultures were washed with PBS and fixed in 4% formaldehyde for 20 min at room temperature (RT), then washed once with PBS and stored at 4°C in PBS until stained. Cell monolayers were permeabilized with PBS 0.5 % TRITON-X (Sigma) and blocked with a solution containing 1% BSA (Sigma) and 3% normal goat (Gibco) for 2 h at RT. Cells were washed once with PBS and incubated overnight at 4°C with diluted primary antibodies, mouse anti-zonulin-1 (1:500) and mouse anti-villin (1:250) (both from Invitrogen), in PBS containing 1% BSA. After washing three times with PBS with 0.1% TRITON-X, cells were incubated with anti-IgG secondary antibodies conjugated with TRITC and FITC (Jackson Immuno Research), diluted 1: 250 for 1h at RT. Cells were washed three times with PBS with 0.1% TRITON-X, then stained with 50 µl of PBS with 2 µg/ml DAPI (Invitrogen) for 10 min at RT and then wash three times with PBS with 0.1% TRITON-X. Membranes were excised from the transwells, mounted on a glass slides and then stored at 4°C. Images were recorded with a Leica DM5000B microscope

equipped with DFX340/DFX300 camera and processed using the Leica Application Suite 2.7.0.R1 software (Leica).

5.7 Cytokines' measurements

Supernatants from Caco-2 cells challenged with live *Anisakis* L3, EVs, and CE were analyzed with ELISA assays (Thermo Fisher Scientific, Milan, Italy) to determine IL-6 and IL-8 amounts, according to the manufacturer's protocol.

5.8 Relative quantification of gene expression by Real-Time PCR analyses

Total RNA was obtained from Caco-2 cells and HIO using a TRIsure™ reagent (Bioline, London, UK), and contaminating genomic DNA was removed using a Turbo DNA-free kit (Life Technologies, Carlsbad, CA, USA). The amount of RNA was evaluated by spectrophotometric measurements using the Take3 module of the plate reader, BioTek SynergyHT and GEN5™, and the RNA integrity was evaluated through a run in an agarose gel 1.5%, stained with Syber safe (Invitrogen Waltham, Massachusetts, USA). A total amount of 1µg RNA was reverse-transcribed for each sample, using SuperScript II RT (Invitrogen Waltham, Massachusetts, USA) and OligodT (Invitrogen Waltham, Massachusetts, USA) according to the manufacturer's protocol. Target genes for the relative quantification by real-time PCR were *Il6* and *Il8* for Caco-2 cells and *Il8*, *Il33* and *Il1β* for HIO experiments, using *GAPDH* as the endogenous control. cDNA templates were mixed with a 2× PowerUp™ SYBR™ Green Master Mix (Applied Biosystem, Foster City, CA, USA) and specific primers [Borkowski et al., 2014; Tiwari et al., 2011;Perez et al., 2020]. Reactions included an initial holding stage of 2 min at 50 °C and of 2 min at 95°C, followed by 40 cycles of PCR (95 °C, 15 s; 60 °C, 1 min); a final stage for melting

curves was included to verify the specificity of the amplifications. A fold change in the expression level was calculated using the ΔΔCt (Delta–Delta Ct) method.

5.9 *Statistical Analysis*

ELISA protocols for IL-6 and IL-8 accounted for the calibrated reference, according to the manufacturer. The relative quantifications of *Il6*, *Il8*, *Il33* and *Il1β* gene expression in RT-PCR were obtained using *GADPH* as a reference gene. The statistical significance of data was determined using a Student's paired t-test to analyze statistical differences between two groups and a One-way analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison test to compare three or more groups. F-values, as variable used to describing the variation between sample means / variation within the samples for multiple comparisons, are reported in brackets as follows: (F(degree of freedom considering groups, degree of freedom considering individuals) = value). T-values used in the case of pairwise comparisons, are reported as follows (t(degree of freedom considering individuals) = value, p = value). Statistical significance was considered with $p \leq 0.05$.

Chapter 6: Results

6.1 Parasite samples and identification

Caco-2 and HIO incubations requested a total of 855 L3, that were collected and identified following a restriction analysis of the nuclear ribosomal ITS with *Hinf*I. The enzymatic cuts produced different banding patterns: three fragments of ~370, 300, and 250 bp in *A. pegreffii*; two fragments of ~620 and 250 bp, plus one additional band at 80 bp in *A. simplex* sensu stricto, and all of the four fragments in the hybrid genotype of the two mentioned species (Figure 11). Once identified, the L3 were used for further experiments: 33 (46% *A. pegreffii*, 50% *A. simplex* s.s., and 4% putative hybrids) for the challenge with the live parasites, 72 (70% *A. pegreffii*, 17% *A. simplex* s.s, and 13% putative hybrids) for the crude extracts isolation, and 750 (61% *A. pegreffii*, 23% *A. simplex* s.s, and 16% putative hybrids) for the EVs-enriched fraction isolation.
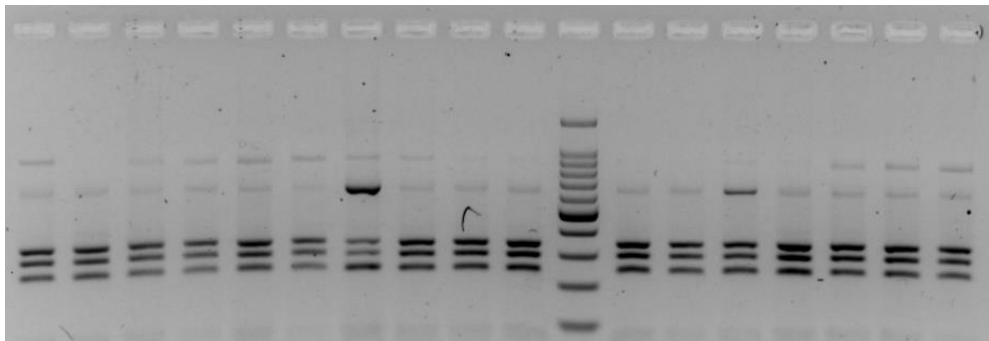
Figure 11: Representative 1.5% agarose gel image of L3 identification after enzymatic digestion of ITS using Hinf*I. Banding patterns are attributable mainly to* A. pegreffii*, and two samples are hybrids.*

6.2 EVs-enriched fraction characterization

Nanoparticle tracking analysis (NTA) using Nanosight technology estimated the size distribution and concentration of the particles in the exosomal-enriched fraction. Two representative measures of a sample used for Caco-2 experiments (B: pool of 20 L3)

and a sample used for HIO exposition are reported (A: pool of 50 L3). The mean size of the particles in the *Anisakis* EVs samples used for Caco-2 incubation was 141.7 nm (mode, 107.4). Three main numbers of peaks were obtained (main peak, 115 nm), and a concentration of 1.32×10^{10} particles/mL was reported (Figure 12 B). The mean size of particles in *Anisakis* EVs samples used for HIO incubation was 138,1 nm (mode, 100,8 nm). A main peak were obtained at 99 nm, and a concentration of $9,78 \times 10^{09}$ particles/mL was reported (Figure 12 A).
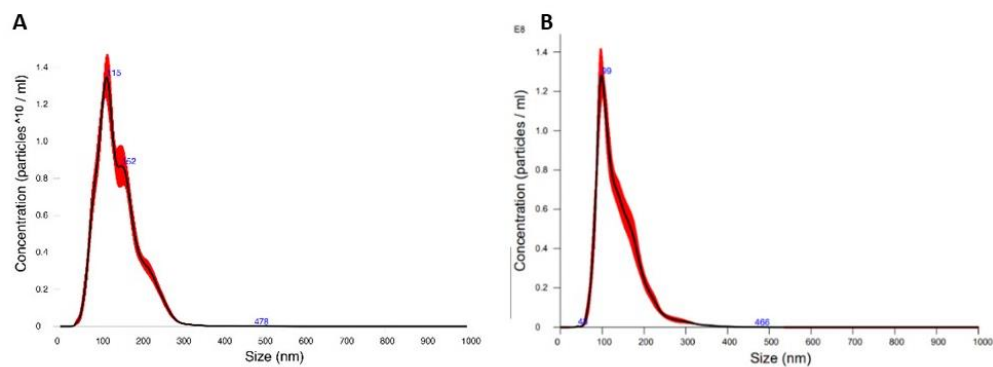
Figure 12: *Finite track length adjustment (FTLA) concentration/size image for nanoparticle tracking analysis (NTA) of extracellular vesicles secreted by third-stage larvae of* Anisakis spp. *(A. simplex sensu stricto, A. pegreffii, and the hybrid form). The number of particles is intended as ^10.*

A Western blot with anti-Alix antibody, performed on the *Anisakis* EV-enriched fraction, the *Anisakis* CE, and on the Caco-2 cells as the controls, showed a band at 110 kDa in the positive controls (Caco-2), while in the CE and EVs, the bands were specific but at a higher molecular weight of around 130 kDa (Figure 13), probably due to post-transcriptional modifications of the protein.
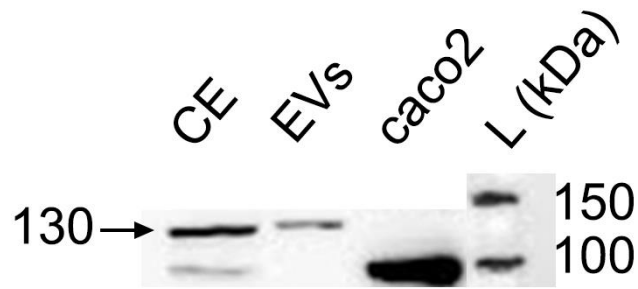
Figure 13: *Western Blot of the extracellular vesicles marker ALIX.*

(CE: crude extract; EVs: extracellular vesicles; Caco-2 cells; L: ladder) [Bellini et al., 2022].

6.3 Caco-2 and HIO RNA quality check

Total RNA isolated from Caco-2 cells (Figure 14A) and HIO (Figure 14B) ,running in 1.5% agarose gel, showed three specific intact bands representing eukaryotic ribosomal subunit at 5.8s, 18s and 28s (Figure 14).
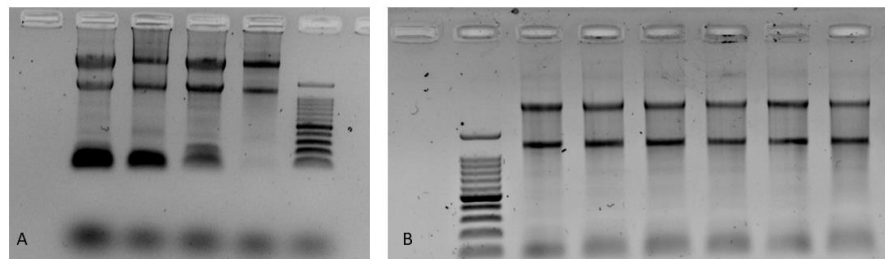
Figure 14: A. Representative image of 1.5 % agarose gel, in which RNA from Caco-2 control, Caco-2 exposed to L3, Caco-2 exposed to EVs and Caco-2 exposed to CE along with molecular ladder (Promega 100 bp) are loaded, respectively. B. Representative image of 1.5% agarose gel in which RNA from HIO controls from the three biological samples and the triplicates of HIO exposed to Anisakis EVs with molecular ladder (Promega 100 bp) are loaded, respectively.

6.4 Intestinal epithelial cells' response to live L3 actions

An additional time point at 6h was included in the incubation with the L3, in order to monitor its potential mechanical activity on the cells' monolayer integrity. This time point has been analyzed by ELISA, too. No morphological changes, such as cytoplasmic vacuolization, shrinkage, plasma membrane blebbing, and chromatin condensation, were observed in the Caco-2 cells after 1 h, 6 h, and 24 h post-contact with the *Anisakis* products or in the unexposed Caco-2 cells. The challenge with the live L3 showed a progressive decrease of IL-6 in the Caco-2 cells incubated at 6 h (not significant) and 24 h ($F_{(2,37)} = 8.143$, $p = 0.0001$) if compared with the non-treated cells (Figure 15A). In order to evaluate the gene expression and cellular response over time, qRT-PCR analyses were performed (GAPDH Eff: 99.97%, *Il8* Eff: 98.71%, and *Il6* Eff: 90.59%) at three different time points (1 h, 6 h, 24 h). However, the results obtained for the *Il6* gene expression showed no significant changes. On the other hand, levels of the neutrophil chemotactic factor IL-8 (Figure 15B) were consistently reduced in the Caco-2 cells incubated with the live L3 for 6 h ($F_{(2,37)} = 17.85$, $p < 0.0001$), if compared to the controls, followed by a significant increase at 24h (6 h vs 24 h: $p < 0.01$), with the IL-8 levels at 24 h not significantly differing from the controls. Similarly, the gene expression of *Il8* showed a slight decrease at 1h compared to the non-treated cells (not significant), followed by an increase at 6 h ($F_{(2,33)} = 3.060$, $p = 0.0422$, 1 h vs 6h: $p < 0.05$) that tended to reach the level of expression as in the controls (controls vs 6 h = ns, and controls vs 24 h = ns) (Figure 15B).
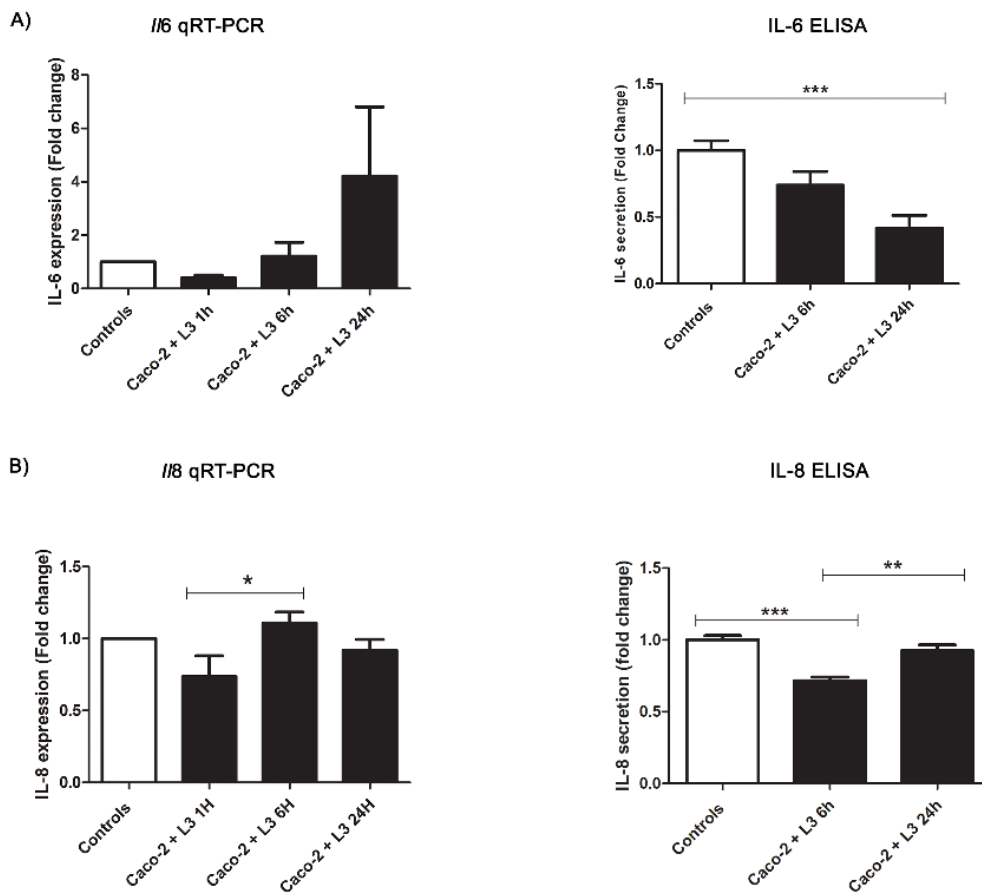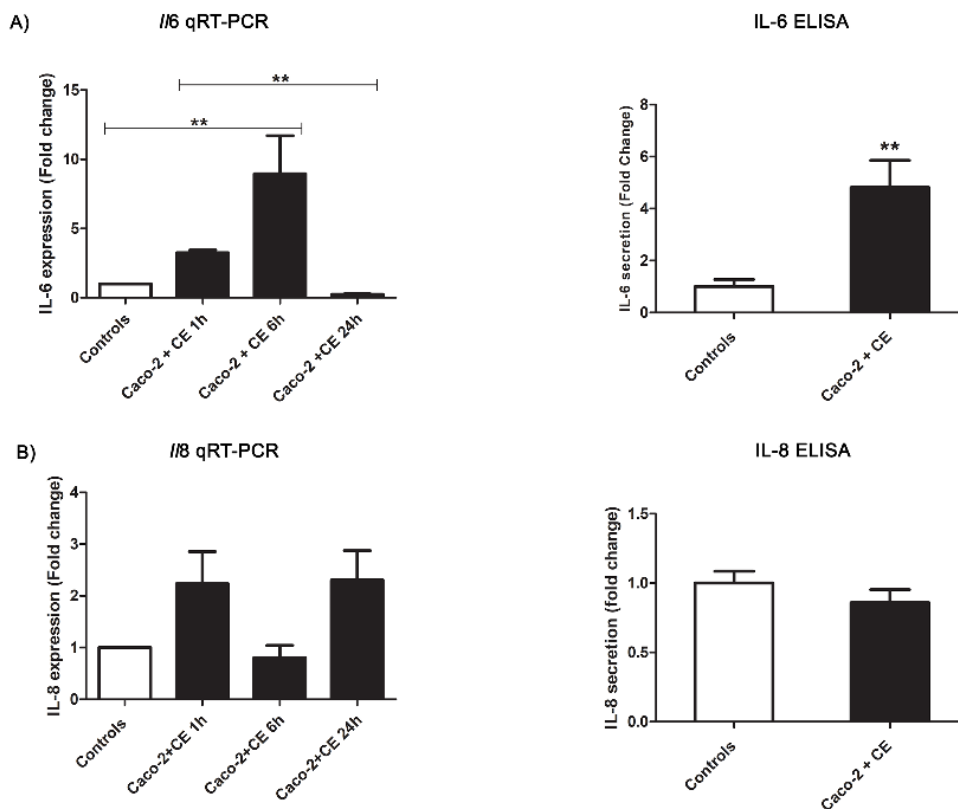
Figure 15: Anisakis *live larvae (L3) modulation of cytokines' secretion in Caco-2 cell monolayers after different times of incubation (1 h, 6 h, and 24 h). (A) Il6 gene expression and IL-6 levels in Caco-2 cell monolayers. (B) Il8 gene expression and IL-8 levels in Caco-2 cell monolayers. Data are expressed as a fold change compared to the control samples and as means ± SEM (standard error mean). Significance was evaluated using an ANOVA test and Bonferroni multiple comparison test. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 [Bellini et al., 2022].*

6.5 Intestinal epithelial cells' response to Anisakis crude extract

The number of proteins observed in the CE samples used for the Caco-2 cells challenge was about 900 ng/µL. The Caco-2 cells exposed to the *Anisakis* CE for 24h showed a strong, relevant increase in IL-6 secretion ($t(22) = 3.64$, $p = 0.0054$) compared to the control group (Figure 16A). This evidence was also supported by the qRT-PCR analyses showing an increasing trend in the *Il6* gene expression at 1 h (not significant) and 6 h ($F(3,20) = 8.143$, $p = 0.0010$). Then, the *Il6* expression decreased significantly (6 h vs 24

h: $p < 0.05$), reaching the expression level reported in the controls (Figure 16A). Interestingly, the CE slightly affected the IL-8 secretion, showing a mild but not significant decrease in the ELISA test compared to the controls ($t(24) = 1.49$, $p = 0.1612$) (Figure 16B). According to that, a slight decrease in the *Il8* gene expression was observed at 6h, with respect to the other check points analyzed (1 h and 24 h), however, without statistical support ($F(3,20) = 2.805$, $p = 0.0661$) (Figure 16B).

Figure 16: Anisakis *crude extract (CE) modulation of cytokines' secretion in Caco-2 cell monolayers after different times of incubation (1 h, 6 h, and 24 h). (A) Il6 gene expression and IL-6 levels in Caco2 cell monolayers. (B) Il8 gene expression and IL-8 levels in Caco-2 cell monolayers. Data are expressed as a fold change compared to the control samples and as means ± SEM (standard error mean). Significance was evaluated using an ANOVA test and Bonferroni multiple comparison test and a Student's t-test pairing for the Caco-2 cells' controls vs the Caco-2 cells exposed. **$p \leq 0.01$ [Bellini et al., 2022].*

6.6 Intestinal epithelial response to Anisakis *EVs*

The challenges of the Caco-2 cells with *Anisakis* EVs revealed a decreasing trend for the two cytokines of interest. In particular, IL-6 was not detected in the ELISA assay compared to the controls ($t(29) = 3.42$, $p = 0.0065$) (Figure 17A). The relative quantification of the *Il6* gene expression showed a statistically relevant ($F(3,18) = 9,205$, $p = 0.0007$) upregulation at 1 h ($p < 0.001$), followed by a significant decrease in gene expression at 6 h and 24 h (1 h vs 6h: $p < 0.05$; 1 h vs 24 h: $p < 0.01$, respectively), reaching the expression level reported in the controls (Figure 17A). A similar pattern was also observed for the IL-8 assays. IL-8 in the Caco-2 cells treated with the EVs after 24 h appeared decreased ($t(31) = 2.92$, $p = 0.0127$) compared to the controls, and the *Il8* gene expression analysis showed a statistically relevant ($F(3,20) = 5.762$, $p = 0.0052$) upregulation at 1 h ($p < 0.05$) that decreased at the other checkpoints (1 h vs 6 h: $p < 0.05$; 1 h vs 24 h: $p < 0.05$), reaching the control levels of the gene expression (Figure 17B).
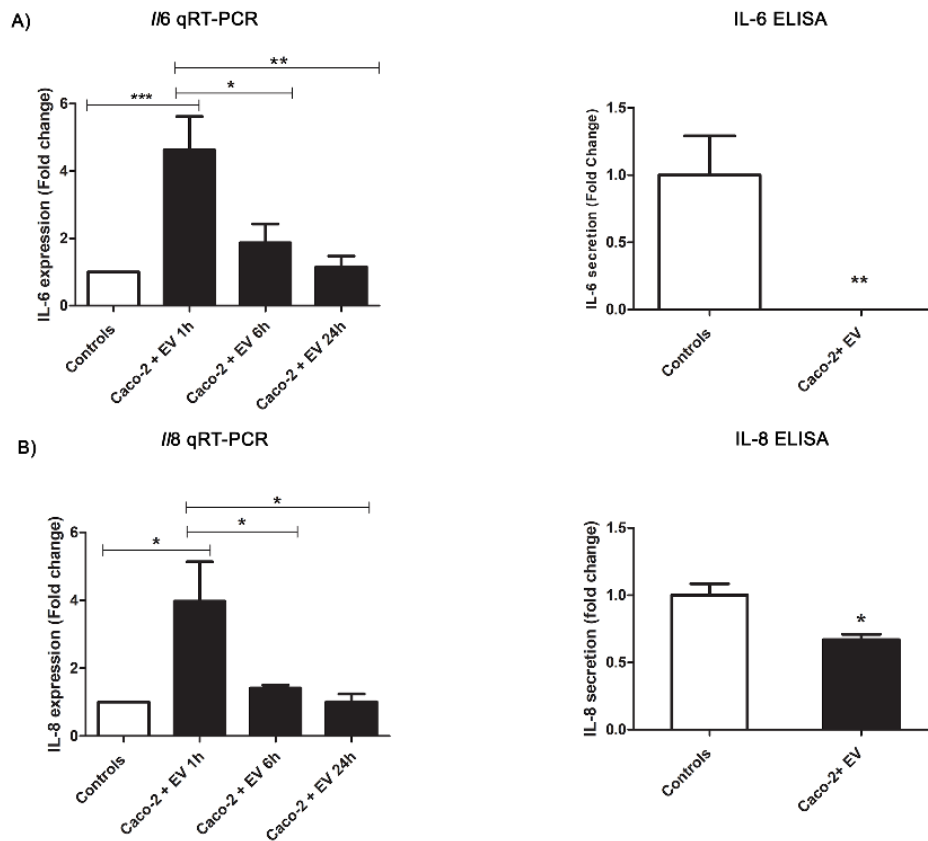
Figure 17: Anisakis *extracellular vesicles (EVs) modulation of cytokines' secretion in Caco-2 cell monolayers after different times of incubation (1 h, 6 h, and 24 h). (A) Il6 gene expression and IL-6 levels in Caco-2 cell monolayers. (B) Il8 gene expression and IL-8 levels in the Caco-2 cell monolayer. Data are expressed as a fold change compared to the control samples and as means ± SEM (standard error mean). Significance was evaluated using an ANOVA test and Bonferroni multiple comparison test and a Student's t-test pairing for the Caco-2 cells' controls vs the Caco-2 cells exposed. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 [Bellini et al., 2022].*

6.7 Human intestinal organoids 2D- cultures and immunostaining

2D- cultures of HIO starting by colon biopsies from a healthy donor were efficiently established in collaboration with Dr. Daniela Scribano from the Organoid laboratory of Microbiology Section of the Department of Public health and infectious diseases of Sapienza University of Rome, as shown in Figure 18. Before exposure to *Anisakis* EVs, 2D HIO were induced to differentiate and immunostaining for Zonulin and Villin was

performed, in order to confirm such differentiation. As reported in Figure 19, 2D HIO cultures showed positivity for the presence of both markers used.
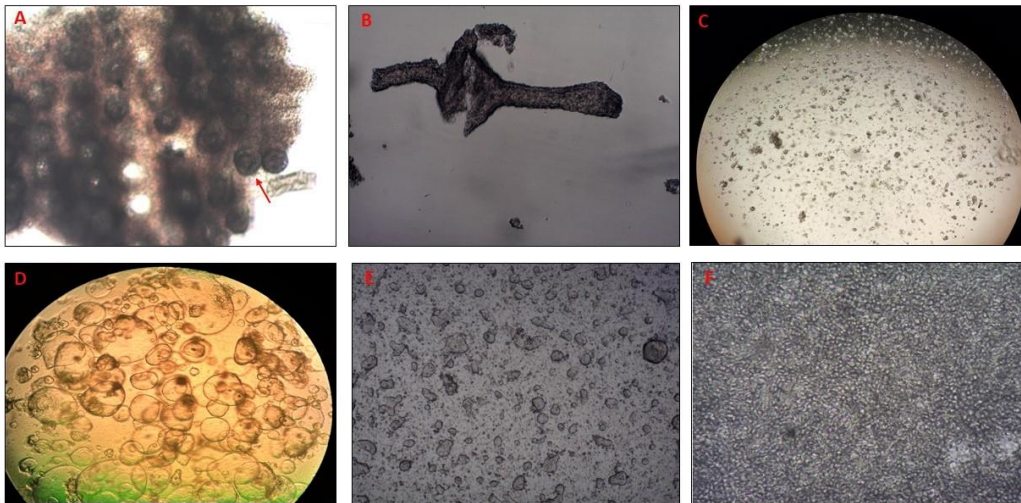
Figure 18: 2D- cultures of HIO, starting by the isolation of intestinal crypts (B) from colon biopsy of a healthy donor (A). After the mechanical disruption of crypts, the material is seeded in Matrigel drops (C), intestinal organoids started to grown in 3D-, cystic, conformation (D), and after a mechanical disruption they were seeded in transwell (E), where they start to grow in 2D- conformation.
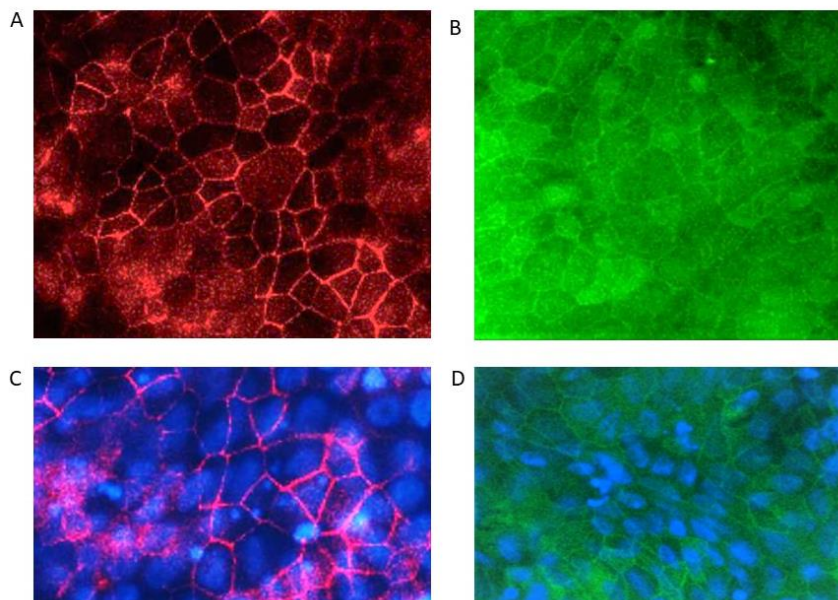
Figure 19: immunostaining of whole 2D- organoids after differentiation of A. Zonulin (red) the tight junction protein as marker of enterocytes. B. Villin (green) a protein forming the structure of microvilli as a marker of the brush border C. D. merged pictures.

6.8 Human intestinal organoids response to Anisakis EVs

In order to evaluate the gene expression and human intestinal organoids response to *Anisakis* EVs, qRT-PCR analyses were performed at 48 h post exposure (*GAPDH* Eff: 100%, *Il8* Eff: 111%; *Il33* Eff: 102%; *Il1β* Eff:112%). The challenge of HIO with *Anisakis* EVs revealed a decreasing trend for *Il33* and *Il1β* gene expression at 48 h, even if without a statistical significance if compared to controls (*Il33:* t(12) = 1.019; *p* = 0,3550; *Il1β:* t(12) = 0.7915; p = 0.4645) (Figure 20B-C). As confirmed in Caco-2 experiments, no significant relevant alterations of *Il8* gene expression were detected at 48 h after exposure (t(12)= 0.04571; *p* = 0,9653) (Figure 20A).
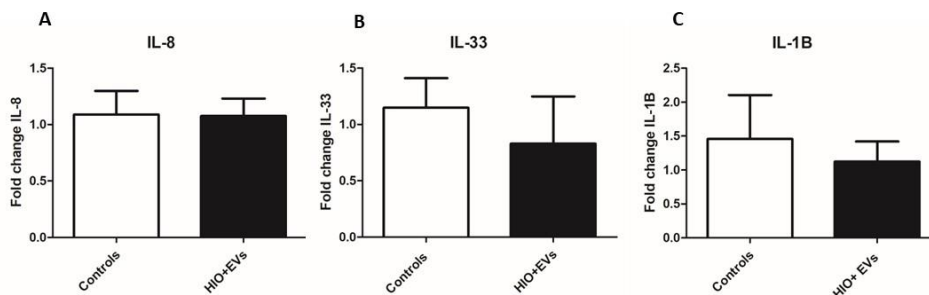
Figure 20: Anisakis *extracellular vesicles (EVs) modulation of cytokines' secretion in HIO 2D-cultures after 48 h of exposure. (A) Il8 gene expression in HIO. (B) Il33 gene expression in HIO and (C) Il1β gene expression in HIO. Data are expressed as a fold change compared to the control samples and as means ± SEM (standard error mean). Significance was evaluated using a Student's t-test pairing for the HIO controls vs the HIO exposed.*

Chapter 7: Discussion

Helminths parasites are widely distributed in marine and terrestrial ecosystems and afflict humans with usually chronic and rarely lethal diseases [Stoltzfus et al., 2017], however inflicting disability and suffering [Pullan et al., 2014]. Despite often overlooked due to low rates of direct mortalities, helminthiasis could be controlled or eradicated, but stronger scientific research efforts on several biological and clinical aspects appear needed. Such a concept could be applicable also for anisakiasis, where knowledge about *Anisakis* ability in modulating humans' inflammatory and immune response, molecular mechanisms related to its pathogenesis and clinical outcomes, is still scarce, although evidence about carcinogenic potential are emerging. To date, the two studies that used Caco-2 cells to investigate human anisakiasis were not focused on cytokines production or modulation [Speciale et al., 2017; Carballeda-Sangiao et al., 2020]; hence, the present investigation was performed using Caco-2 cells and HIO as suitable models able to recapitulate the microenvironment for *Anisakis* infection. For Caco-2 cells experiments, we tested three challenges mimicking two pivotal phases of *Anisakis* pathogenic cycle in humans:

1. active penetration of the tissue (L3) and communication with the intestinal epitheliums (EVs);
2. the larval dying (CE).

Additionally, given the paucity of studies and the scientific interest on parasitic EVs topic, in this PhD thesis, we decided to expand the knowledge on *Anisakis* EVs and their effect on the human host using a cutting-edge model: HIO. In particular, the present study is the first attempt to explore the cellular response after exposure to the EVs derived from the infective third-stage larvae of the zoonotic nematode *Anisakis* spp. (*A. pegreffii, A. simplex s.s.,* and their hybrid form) for both the models used. Data about inflammatory pathways triggered by the challenges with *Anisakis*-derived products, revealed

interesting and variable outcomes on cytokines' production and gene expression. As mentioned before, once ingested, the L3 released proteolytic enzymes and chemotactic factors that adhere to and penetrate the mucosa and submucosa [Kim et al., 2018; Kochanowski et al., 2022], inducing hemorrhagic and erosive lesions [Audicana et al., 2008], accompanied by a production of parasitic ES factors able to interact and elicit the human first line of defense: the intestinal epithelium [Mehrdana et al., 2017]. Surprisingly, results here obtained showed that Caco-2 cells were not significantly stimulated by the presence of the live L3. Thus, the observed decrease in the IL-6 and IL-8 cytokines' amount could represent a first strategy by which *Anisakis* L3 modulates the epithelial barrier response prompted by the initial contact with the host, quickly returning to homeostasis for successful long-lived infections. This concept was also demonstrated for the phylogenetic-related ascarid *Ascaris suum* [Ebner et al., 2018]. In fact, the transcriptional analysis derived from the incubation of *A. suum* L3 with porcine intestinal epithelial cells showed a low magnitude of inflammatory response driven mainly by IL-8 and NF-kB suppression. Our results, based on ELISA and qRT-PCR analyses, demonstrated that live *Anisakis* L3 activate an early and transient downregulation of host cytokines' gene expression and protein release. Moreover, the effect on the IL-6 quantity was longer than those observed on IL-8 that tend to turn to the basal level at 24 h. This is in agreement with a previous study, in which no IL-8 mRNA alterations in patient sera affected by anisakiasis was observed [Del Pozo et al., 1999]. To our knowledge, only Napoletano et al., [2018] have investigated IL-6 production in *Anisakis* live L3-human interaction using DCs exposed to seven live specimens of L3 for six days and demonstrated an increase in IL-6 production and a less reactive phenotype of DCs not sufficient to trigger a Th2/Th17 response. In the present study, using a different in-vitro model and experimental settings, consistent outcomes about a suppressive action of *Anisakis* live L3 on the first line of human defense were obtained but based on the decrease of the IL-6.

Besides the mechanical action of live L3, their derived EVs are an additional pivotal factor that should be evaluated in the early pathogenicity exerted by *Anisakis*. EVs are a newly discovered messenger of early communication between pathogens and their host, since they determine strong immunomodulatory effects [Drurey et al., 2022], as described for other nematodes [Hansen et al., 2019; Eichenberger et al., 2018; Buck et al., 2014]. According to Boysen et al. [2020] and Cavallero et al. [2022], *Anisakis* live L3 is able to release EVs packed with potential immune modulators able to affect pivotal pathways in human host.

In this PhD project, *Anisakis* EVs have been successful characterized trough NTA and WB analyses [Bellini et al., 2022]. In particular, in WB analyses, Alix from *Anisakis* could be heavier respect to the ones expressed by human cells however, so far, there are no evidences to support this hypothesis. As mentioned in chapter 2, paragraph 2.6, to date there are several troubles in finding reliable EVs markers for organisms other than eukaryotic model organisms and with the present data we add some knowledge about EVs in parasitic nematodes. Additionally, available data on eukaryotic Alix show that it exists in monomeric (low molecular weight) and dimeric/multimeric (high molecular weight) configurations, and the dimeric form seems to be mainly associated to cell cytoskeleton and to exosomes [Qiu et al., 2022]. Moreover, this protein undergoes to several different post-translational modifications such as ubiquitination, phosphorylation, and palmitoylation increasing its size, allowing the interaction with its binding partners but also its membrane association. In fact ALIX/ALG2 orthologue mol weight of the peptides gives around 100kDa but this is without glycosylations and there are 33 N residues, so it could be larger due to this and it will be possible that in the Crude extract all the forms of the protein have been identified. The EVs-enriched fraction used in this study is the result of the incubation of a pool of 20 to 50 larvae, while usually a single or few L3 infect humans. Consequently, in the experiments carried out using Caco-2 cells with comparable EVs and L3 data, a stronger activity of such EVs-enriched fraction

compared to a single larva is expected. According to this hypothesis and to published data [Coakley et al., 2017; Buck et al., 2014], IL-6 was not detected and a reduction in IL-8 in the ELISA at 24 h was observed. However, the gene expression of the *Il6* and *Il8* showed different trends. The exposure to EVs elicits an early upregulation of both genes, followed by a progressive downregulation over time, even if not statistically supported, compared to the basal level of gene expression in the controls (at 6 h and 24 h). Based on these results, the attenuation of IL-6 and IL-8 may have occurred via post-transcriptional mechanisms, potentially due to proteases, as demonstrated for other intestinal parasites [Cotton et al., 2014] and usually released also by *Anisakis* [Kim et al., 2018], or to other potential regulatory factors such as miRNAs packed in EVs. Alternative explanations may be related to the selected time points that may not be representative of the precise time of the cytokines' mRNA downregulation. A crucial factor to consider is the timing of the EVs' uptake by the cells. *H. polygyrus* EVs are efficiently internalized by epithelial cells (MODE-K) at 24 h (55%) in comparison to 1h (10%) [Coakley et al., 2017]; *T. muris* EVs are internalized by murine colonic organoids within 3 h at 37 °C [Eichenberger et al., 2018], and *Anisakis*-derived EVs are internalized by human macrophage-Like THP-1 cells, but a precise indication of the uptake timing are lacking [Boysen et al., 2020]. In this framework, the early upregulation of the *Il6* and *Il8* gene expressions observed in Caco-2 cells exposed to EVs, could be mainly due to the interaction between the host cellular receptors and the *Anisakis* EVs' surface proteins, rather than an effect associated to the EVs' cargo. Consequently, the observed immunomodulatory strategy exerted by live L3-releasing EVs on intestinal epithelial cells may be defined by several not-exclusive mechanisms of action. The probability that IL-8 is not fundamental in anisakiasis pathogenesis emerged also in preliminary data achieved from HIO exposed to *Anisakis* EVs. RT-PCR analyses showed that *Il8* gene expression seems to be not affected after 48 h of exposure, respect to controls. On the other hand, the tendency of *Il33* and *Il1β* gene expression revealed interesting decreasing outcomes. Previous studies carried out using *H. polygirus* [Coakley et al.,

2017] and *N. brasiliensis* EVs [Eichenberger et al., 2018] demonstrated as these pathways could be targeted by parasites EVs. In particular, *H.polygirus EVs* suppress ST2/IL-33R expression in RAW246.7 macrophages cell line during type 2 responses and murine intestinal organoids treated with *N. brasiliensis* EVs showed a statistically relevant suppression of pro-inflammatory cytokines IFN-γ, IL-6, IL-17a, and IL-1β resulting in suboptimal type 2 immunity and potentially leading to chronicity. About that, to validate this hypothesis and to add new tiles in depicting anisakiasis framework, data derived from a comparative transcriptomic analyses still ongoing in the laboratory, associated to further analyses on cytokines measurement in HIO medium during the incubation with EVs will be helpful.

A different scenario is observed in the CE exposure regarding IL-6 release. CE represents a cocktail of L3 elements that may be clinically representative of the moment in which L3 decay (around 14 days after ingestion) occurs, allowing parasite's expulsion or granuloma formation and chronic inflammation [Audicana et al., 2008]. The in-vitro studies available in scientific literature, demonstrated a pro-inflammatory activity induced by *Anisakis* CE products, including the upregulation of oxidative stress, inhibition of apoptosis-related biomarkers, the barrier's integrity alteration, and inflammation [Messina et al., 2016; Speciale et al., 2017; Carballeda-Sangiao t al., 2020]. In agreement to that, our data about Caco-2 cells exposed to CE showed a strong upregulation of IL-6 release and in the *Il6* gene expression. This pro-inflammatory enhancement could be a trigger for the host immune response, capable to activate and recruit immune cells (DCs, macrophages, neutrophils and eosinophils) to the site of damage, leading to the arrest or the disease progression. Moreover, such results are in agreement with those obtained by previous in vivo and in vitro studies on *Anisakis* CE [Messina et al., 2016; Napoletano et al., 2018; Corcuera et al., 2018]. These studies highlighted a pathological condition associated to chronic inflammation and, in turn, to a potential progressive increased risk of the host's DNA damage and cancer, as showed

for other helminths [Brindley et al., 2017]. To date, only two studies have investigated these neglected features in the framework of anisakiasis. The tumorigenic potential of *Anisakis* was explored with hamster ovary cells and Sprague–Dawley rats, revealing an increase in cell proliferation, a decrease of apoptosis, and alterations in the expression of serum cancer-related miRNAs [Corcuera et al., 2018]. Additionally, an increased level of P53 and ROS in the fibroblast cell line (HS-68) incubated with *Anisakis* ES and CE was observed [Messina et al., 2016]. Interestingly, also in this case, from our data emerged that CE did not particularly affect the *Il8* gene expression and secretion by Caco-2 cells, suggesting that this cytokine is not a crucial factor involved in anisakiasis.

Chapter 8: Conclusions

In conclusion, the results obtained indicate an intricate interplay between *Anisakis* and its accidental host. In this role play, the pathogenic cycle could be divided in two phases: an early stage in which the live, active larva and its released EVs seem to silence the host's immune response at the intestinal epithelium level, to find a long-lasting niche to remain alive. In a later phase, the dying larva could induce the activation of the immune strategy of the host, potentially leading to parasite expulsion, eosinophilia, and/or granuloma formation. Furthermore, the three challenges showed how the effects produced by *Anisakis* on the human host could be manifold, as also suggested in previous studies [Messina et al., 2016] (Figure 21).
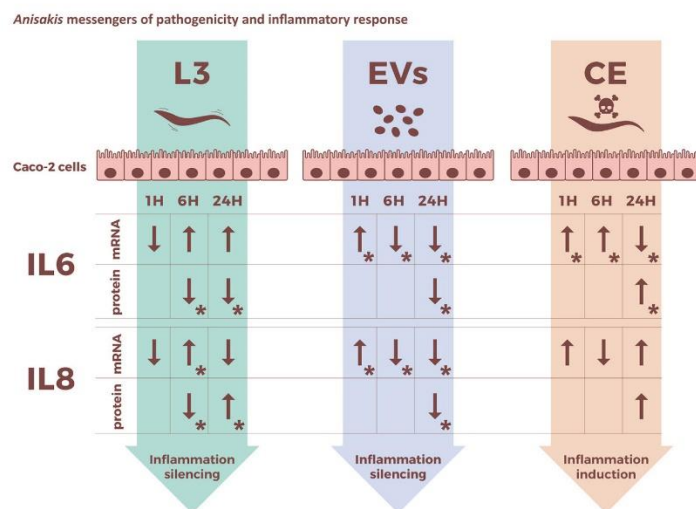
Figure 21: Graphical summary of Caco-2 experiments and outputs from the different exposure to Anisakis *messengers of pathogenicity [Bellini et al.2022].*

In this scenario, highlighted all the limitations that characterized this topic to date and its countless factors that need to be explored, it would be interesting to deeply investigate the molecular mechanisms at the base of this *Anisakis*-humans interface, expanding the research to other factors that could add pivotal information about anisakiasis, as well as about the tumorigenic potential of these parasites.

References:

Abollo E, Paggi L, Pascual S, D'Amelio S. Occurrence of recombinant genotypes of *Anisakis simplex* s.s. and *Anisakis pegreffii* (Nematoda: Anisakidae) in an area of sympatry. *Infect Genet Evol.* **2003**; 3(3):175-81.

Adams AM, Miller KS, Wekell MM, Dong FM. Survival of *Anisakis simplex* in microwave-processed arrowtooth flounder (*Atheresthes stomias*). *J Food Prot.* **1999**; 62(4):403-9.

Adams AM, Ton MN, Wekell MM, MacKenzie AP, Dong FM. Survival of *Anisakis simplex* in arrowtooth flounder (*Atheresthes stomia*) during frozen storage. *J Food Prot.* **2005**; 68(7):1441-6.

Adroher-Auroux FJ, Benítez-Rodríguez R. Anisakiasis and *Anisakis*: An underdiagnosed emerging disease and its main etiological agents. *Res Vet Sci.* **2020**;132:535-545.

Aibinu IE, Smooker PM, Lopata AL. *Anisakis* Nematodes in Fish and Shellfish- from infection to allergies. *Int J Parasitol Parasites Wildl.* **2019**; 9:384-393.

Ambrosi C, Pompili M, Scribano D, Limongi D, Petrucca A, Cannavacciuolo S. The *Shigella flexneri* OspB effector: An early immunomodulator. *Int. J. Med. Microbiol.* **2015**; 305, 75–84.

Arizono N, Yamada M, Tegoshi T, Yoshikawa M. *Anisakis simplex* sensu stricto and *Anisakis pegreffii*: Biological characteristics and pathogenetic potential in human anisakiasis. *Foodborne Pathog. Dis.* **2012;** 9, 517–521.

Audicana M.T., Ansotegui I.J., de Corres L.F., Kennedy M.W. *Anisakis simplex*: Dangerous-dead and alive?. *Trends Parasitol.* **2002**; 18, 20–25.

Audicana M.T., Kennedy M.W. *Anisakis simplex*: From obscure infectious worm to inducer of immune hypersensitivity. *Clin. Microb. Rev.* **2008**; 21, 360–379.

Baird FJ, Gasser RB, Jabbar A, Lopata AL. Foodborne anisakiasis and allergy. *Mol and Cell Probes.* **2014**; 28, 4,167-174, ISSN 0890-8508.

Bao M., Pierce G.J., Pascual S., González-Muñoz M., Mattiucci S., Mladineo I., Cipriani P., Bušelić I., Strachan N.J. Assessing the risk of an emerging zoonosis of worldwide concern: Anisakiasis. *Sci. Rep.* **2017**; 7, 43699.

Baptista-Fernandes T, Rodrigues M, Castro I, Paixão P, Pinto-Marques P, Roque L, Belo S, Ferreira PM, Mansinho K, Toscano C. Human gastric hyperinfection by *Anisakis simplex*: A severe and unusual presentation and a brief review. *Int J Infect Dis.* **2017**; 64:38-41.

Bautista D, Rodríguez LS, Franco MA, Angel J, Barreto A. Caco-2 cells infected with rotavirus release extracellular vesicles that express markers of apoptotic bodies and exosomes. *Cell Stress Chaperones.* **2015**; 20, 697–708. 39.

Bellini I, Scribano D, Sarshar M, Ambrosi C, Pizzarelli A, Palamara AT, D'Amelio S, Cavallero S. Inflammatory Response in Caco-2 Cells Stimulated with *Anisakis* Messengers of Pathogenicity. Pathogens. **2022**; 20;11(10):1214.

Berland B. Nematodes from some Norwegian marine fishes. **1961;** Sarsia 2, 1–50.

Bilska-Zając E, Różycki M, Chmurzyńska E, Karamon J, Sroka J, Kochanowski M, Kusyk P, Cencek T. Parasites of Anisakidae Family—Geographical Distribution and Threat to Human Health. Journal of Agricultural Science and Technology. **2015**; 5, 146-152.

Borkowski J, Li L, Steinmann U, Quednau N, Stump-Guthier C, Weiss C, Findeisen P, Gretz N, Ishikawa H, Tenenbaum T, Schroten H, Schwerk C. *Neisseria meningitidis* elicits a pro-inflammatory response involving IκBζ in a human blood-cerebrospinal fluid barrier model. *J Neuroinflammation*. **2014**; 13, 11:163.

Boysen AT, Whitehead B, Stensballe A, Carnerup A, Nylander T, Nejsum P. Fluorescent Labeling of Helminth Extracellular Vesicles Using an In Vivo Whole Organism. *Approach Biomed*. **2020**; 8, 213.

Brindley PJ, Loukas A. Helminth infection–induced malignancy. *PLoS Pathog*. **2017**; 13, e1006393.

Buck A, Coakley G, Simbari F, McSorley HJ, Quintana JF, Le Bihan T, Kumar S, Abreu-Goodger C, Lear M, Harcus Y, Ceroni A, Babayan SA, Blaxter M, Ivens A, Maizels RM. Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. *Nat. Commun.* **2014**; 5, 5488.

Bušelic I, Trumbi ´c Ž, Hrabar J, ´Vrbatovic A, Bo ´cina I and Mladineo I. Molecular and Cellular Response to Experimental *Anisakis pegreffii* (Nematoda, Anisakidae) Third-Stage Larval Infection in Rats. *Front. Immunol.* 2008; 9:2055.

Carballeda-Sangiao N, Olivares F, Rodriguez-Mahillo AI, Careche M, Tejada M, Moneo I, González-Muñoz M. Identification of autoclave-resistant *Anisakis simplex* allergens. *J. Food Prot.* **2014**; 77, 605–609.

Carballeda-Sangiao N, Rodríguez-Mahillo AI, Careche M, Navas A, Caballero T, Dominguez-Ortega J, Jurado-Palomo J, González-Muñoz M. Ani s 11-Like Protein Is a Pepsin- and Heat-Resistant Major Allergen of *Anisakis* spp. and a Valuable Tool for *Anisakis* Allergy Component-Resolved Diagnosis. *Int. Arch. Allergy Immunol.* **2016**; 169, 108–112.

Carballeda-Sangiao N, Sánchez-Alonso I, Navas A, Arcos SC, de Palencia PF, Careche M, González-Muñoz M. *Anisakis simplex* products impair intestinal epithelial barrier function and occludin and zonula occludens-1 localisation in differentiated Caco-2 cells. *PLoS Negl. Trop. Dis.* **2020**; 14, e0008462.

Cavallero S, Bellini I, Pizzarelli A, Arcà B, D'Amelio S. A miRNAs catalogue from third-stage larvae and extracellular vesicles of *Anisakis pegreffii* provides new clues for host-parasite interplay. *Sci. Rep.* **2022**; 12, 9667.

Cavallero S, Costa A, Caracappa A, Gambetta B, D'Amelio S. Putative hybrids between two *Anisakis* cryptic species: Molecular genotyping using High Resolution Melting. *Exp Parasit.* 2014; 146, 87-93, 0014-4894.

Cavallero S, Lombardo F, Salvemini M, Pizzarelli A, Cantacessi C, D'Amelio S. Comparative Transcriptomics Reveals Clues for Differences in Pathogenicity between *Hysterothylacium aduncum*, *Anisakissimplex* sensu stricto and *Anisakis pegreffii*. Genes (Basel). **2020**; 18;11(3):321.

Cavallero S., Martini A., Migliara G., De Vito C., Iavicoli S., D'Amelio S. Anisakiasis in Italy: Analysis of hospital discharge records in the years 2005–2015. *PLoS ONE*. **2018**; 13, e0208772.

Chaiyadet S, Sotillo J, Smout M, Cantacessi C, Jones MK, Johnson MS, Turnbull L, Whitchurch CB, Potriquet J, Laohaviroj M, Mulvenna J, Brindley PJ, Bethony JM, Laha T, Sripa B, Loukas A. Carcinogenic Liver Fluke Secretes Extracellular Vesicles That Promote Cholangiocytes to Adopt a Tumorigenic Phenotype. *J Infect Dis*. **2015**; 15, 212(10):1636-45.

Chandra L, Borcherding DC, Kingsbury D, Atherly T, Ambrosini YM, Bourgois-Mochel A, Yuan W, Kimber M, Qi Y, Wang Q, Wannemuehler M, Ellinwood NM, Snella E, Martin M, Skala M, Meyerholz D, Estes M, Fernandez-Zapico ME, Jergens AE, Mochel JP, Allenspach K. Derivation of adult canine intestinal organoids for translational research in gastroenterology. *BMC Biol*. **2019**;17(1):33.

Chen P, Lin C, Quan J, Lai Y, He T, Zhou L, Pan X, Wu X, Wang Y, Ni L, Yang S, Wang T, Lai Y. Oncogenic miR-100-5p is associated with cellular viability, migration and apoptosis in renal cell carcinoma. *Mol Med Rep*. **2017**;16(4):5023-5030.

Cho MK, Park MK, Kang SA, Park SK, Lyu JH, Kim DH, Park HK, Yu HS. TLR 2-dependent amelioration of allergic airway inflammation by parasitic nematode type II MIF in mice. *Parasite Immunol*. **2015**; 37, 180–191

Chung YB, Lee J. Clinical characteristics of gastroallergic anisakiasis and diagnostic implications of immunologic tests. *Allergy Asthma Immunol Res*. **2014;** 6(3):228-33.

Cipriani P, Smaldone G, Anastasio, A, Acerra V, D'Angelo L, Bellisario B, Palma G, Nascetti G, Mattiucci S. Genetic identification and distribution of the larval parasites *Anisakis pegreffii* and *A. simplex* (s. s.) in fish tissues of *Merluccius merluccius* from Tyrrhenian Sea and Spanish Atlantic coast: Implications for food safety. *Int. J. Food Microbiol*. **2015**; 198, 1–8. 52.

Clevers H. Modeling Development and Disease with Organoids. *Cell*. **2016**; 16,165(7),1586-1597.

Coakley G, Maizels RM, Buck AH. Exosomes and Other Extracellular Vesicles: The New Communicators in Parasite Infections. *Trends Parasitol*. **2015**;31(10):477-489.

Coakley G, McCaskill JL, Borger JG, McSorley HJ, Maizels RM, Buck AH. Extracellular Vesicles from a Helminth Parasite Suppress Macrophage Activation and Constitute an Effective Vaccine for Protective Immunity. *Cell Rep*. **2017**; 19, 1545– 1557.

Corcuera MT, Rodríguez-Bobada C, Zuloaga J, Gómez-Aguado F, Rodríguez-Perez R, Mendizabal Á, González P, Arias-Díaz J, Caballero ML. Exploring tumourigenic potential of the parasite *Anisakis*: A pilot study. *Parasitol. Res*. **2018**; 117, 3127–3136.

Corrò C, Novellasdemunt L, Li VSW. A brief history of organoids. *Am J Physiol Cell*. **2020**; 1, 319(1), C151-C165.

Cotton JA, Bhargava A, Ferraz JG, Yates RM, Beck PL, Buret AG. *Giardia duodenalis* cathepsin B proteases degrade intestinal epithelial interleukin-8 and attenuate interleukin-8-induced neutrophil chemotaxis. *Infect Immun*. **2014**; 82, 2772–2787.

D'Amelio S, Lombardo F, Pizzarelli A, Bellini I, Cavallero S. Advances in Omic Studies Drive Discoveries in the Biology of Anisakid Nematodes. *Genes (Basel)*. **2020**;11(7):801.

D'Amelio S, Mathiopoulos KD, Santos CP, Pugachev ON, Webb SC, Picanço M, Paggi, L. Genetic markers in ribosomal DNA for the identification of members of the genus

Anisakis (Nematoda: Ascaridoidea) defined by polymerase-chain-reaction- based restriction fragment length polymorphism. *Int. J. Parasitol.* **2000**; 30, 223–226.

Daschner A, Pascual CY. *Anisakis simplex*: sensitization and clinical allergy. *Curr Opin Allergy Clin Immunol.* **2005**;5(3):281-5.

del Carmen Romero M, Valero A, Concepción Navarro-Moll M, Martín-Sánchez J. Experimental comparison of pathogenic potential of two sibling species *Anisakis simplex* s.s. and *Anisakis pegreffii* in Wistar rat. *Trop Med and Int Health.* **2013**; 18, 8, 979-984.

Del Pozo V, Arrieta I, Tuñon T, Cortegano I, Gomez B, Cárdaba B. Immunopathogenesis of human gastrointestinal infection by *Anisakis simplex. J. Allergy Clin. Immunol.* **1999**; 104, 637–643.

Derricott H, Luu L, Fong WY, Hartley CS, Johnston LJ, Armstrong SD, Randle N, Duckworth CA, Campbell BJ, Wastling JM, Coombes JL. Developing a 3D intestinal epithelium model for livestock species. *Cell Tissue Res.* **2019**;375(2):409-424.

Drurey C, Lindholm HT, Coakley G, Poveda MC, Löser S, Doolan R, Gerbe F, Jay P, Harris N, Oudhoff MJ et al. Intestinal epithelial tuft cell induction is negated by a murine helminth and its secreted products. *J. Exp. Med.* **2022;** 219, e20211140.

Duque-Correa MA, Maizels RM, Grencis RK, Berriman M. Organoids - New Models for Host-Helminth Interactions. *Trends Parasitol.* **2020**; 36(2), 170-181.

Duque-Correa MA, Schreiber F, Rodgers FH, Goulding D, Forrest S, White R, Buck A, Grencis RK, Berriman M. Development of caecaloids to study host-pathogen interactions: new insights into immunoregulatory functions of *Trichuris muris* extracellular vesicles in the caecum. *Int J Parasitol.* **2020**; 50(9):707-718.

Ebner F, Kuhring M, Radonić A, Midha A, Renard BY, Hartmann S. Silent Witness: Dual-Species Transcriptomics Reveals Epithelial Immunological Quiescence to Helminth Larval Encounter and Fostered Larval Development. *Front Immunol.* **2018**; 15, 9, 1868.

EFSA Journal **2022**; 20():e200409. Foodborne and zoonotic diseases.

Eichenberger RM, Talukder MH, Field MA, Wangchuk P, Giacomin P, Loukas A, Sotillo J. Characterization of *Trichuris muris* secreted proteins and extracellular vesicles provides new insights into host-parasite communication. *J Extracell Vesicles.* **2018**; 21;7(1):1428004.

Eichenberger RM, Ryan S, Jones L, Buitrago G, Polster R, Montes de Oca M, Zuvelek J, Giacomin PR, Dent LA, Engwerda CR, Field MA, Sotillo J, Loukas A. Hookworm Secreted Extracellular Vesicles Interact With Host Cells and Prevent Inducible Colitis in Mice. *Front. Immunol.* **2018**; 9:850.

Eskesen A, Strand EA, Andersen SN, Rosseland A, Hellum KB, Strand OA. Anisakiasis presenting as an obstructive duodenal tumor. A Scandinavian case. *Scand. J. Infect. Dis.* **2001**; 33, 75–76. 15.

Everts B, Smits HH, Hokke CH, Yazdanbakhsh M. Helminths and dendritic cells: sensing and regulating via pattern recognition receptors, Th2 and Treg responses. *Eur J Immunol.* **2010**; 40(6):1525-37.

Fæste CK, Jonscher KR, Dooper MM, Egge-Jacobsen W, Moen A, Daschner A, Egaas E, Christians U. Characterisation of potential novel allergens in the fish parasite *Anisakis simplex*. *EuPA Open Proteom*. **2014**; 4, 140–155.

FDA, Fish and Fishery Products Hazards and Controls Guidance. **2022.**

Fogh J, Fogh JM, Orfeo T. One Hundred and Twenty-Seven Cultured Human Tumor Cell Lines Producing Tumors in Nude Mice, *JNCI: Journal of the National Cancer Institute*. **1977**; 59, 1, 221–226.

Gao X, Yang Y, Liu X, Wang Y, Yang Y, Boireau P, Liu M, Bai X. Extracellular vesicles derived from *Trichinella spiralis* prevent colitis by inhibiting M1 macrophage polarization. *Acta Tropica*. **2021**; 213, 105761,ISSN 0001-706X,

Garcia-Perez JC, Rodríguez-Perez R, Ballestero A, Zuloaga J, Fernandez-Puntero B, Arias-Díaz J, Caballero ML. Previous Exposure to the Fish Parasite *Anisakis* as a Potential Risk Factor for Gastric or Colon Adenocarcinoma. *Medicine*. **2015**; 94, e1699.

Gause WC, Rothlin C, Loke P. Heterogeneity in the initiation, development and function of type 2 immunity. *Nat Rev Immunol*. **2020**;20(10):603-614.

Gómez-Mateos M, Arrebola F, Navarro MC, Romero MC, González JM, Valero A. Acute Anisakiasis: Pharmacological Evaluation of Various Drugs in an Animal Model. *Dig. Dis. Sci*. **2021**; 66, 105–113.

Gonzalez RS, Pastrián LG, Pyatibrat S, Arias HDQ, Gil YR, Booth AL, de la Peña Navarro I, Garmendia-Irizar M, Lapointe JR, Mobarki M, Nova-Camacho LM, Parini G, Romio E, Alayza AR, Pritt BS, Ruz-Caracuel I. Submucosal Necrotic Nodule of the Colon. *Arch Pathol Lab Med*. **2023**.

Guarnieri F, Guarnieri C, Benvenga S. Cross-reactivity of *Anisakis simplex*: Possible role of Ani s 2 and Ani s 3. *Int. J. Dermatol*. **2007**; 46, 146–150.

Gurunathan S, Kang MH, Kim JH. A Comprehensive Review on Factors Influences Biogenesis, Functions, Therapeutic and Clinical Implications of Exosomes. *Int J Nanomedicine*. **2021**;17,16:1281-1312.

Ha H, Debnath B, Neamati N. Role of the CXCL8-CXCR1/2 Axis in Cancer and Inflammatory Diseases. *Theranostics*. **2017**; 7, 1543. 44.

Hansen EP, Fromm B, Andersen SD, Marcilla A, Andersen KL, Borup A, Williams AR, Jex AR, Gasser RB, Young ND. Exploration of extracellular vesicles from *Ascaris suum* provides evidence of parasite–host cross talk. *J. Extracell. Vesicles*. **2019**; 8, 1578116.

Haryadi L, Suprayitno E, Aulanni'am A, Hariati AM. Immune response evaluation in Balb/c mice after crude extract of *Anisakis typica* sensitization. *Vet. World*. **2019**; 12, 1529–1534.

Helmby H, Grencis RK. Interleukin 1 plays a major role in the development of Th2-mediated immunity. *Eur J Immunol*. **2004**;34(12):3674-81.

Heo I, Dutta D, Schaefer DA, Iakobachvili N, Artegiani B, Sachs N, Boonekamp KE, Bowden G, Hendrickx APA, Willems RJL, Peters PJ, Riggs MW, O'Connor R, Clevers H. Modelling *Cryptosporidium* infection in human small intestinal and lung organoids. *Nat Microbiol*. **2018**;3(7):814-823.

Herrador Z., Daschner Á ., Perteguer M.J., Benito A. Epidemiological Scenario of Anisakidosis in Spain Based on Associated Hospitalizations: The Tip of the Iceberg. *Clin. Infect. Dis.* **2019**; 69, 69–76.

Hibino S, Kawazoe T, Kasahara H, Itoh S, Ishimoto T, Sakata-Yanagimoto M, Taniguchi K. Inflammation-Induced Tumorigenesis and Metastasis. *Int J Mol Sci.* **2021**; 21, 22(11):5421.

Holtfreter J. Experimental studies on the development of the pronephros. *Rev Can Biol.* **1944***;* 3, 220 –250.

Hrabar J, Trumbić Ž, Bočina I, Bušelić I, Vrbatović A, Mladineo I. Interplay between proinflammatory cytokines, miRNA, and tissue lesions in *Anisakis*-infected Sprague-Dawley rats. *PLoS Negl Trop Dis.* **2019;** 15;13(5):e0007397.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Biological agents. *IARC Monogr Eval Carcinog Risks Hum.* **2012**; 100(Pt B):1-441.

Inclan-Rico JM, Siracusa MC. First Responders: Innate Immunity to Helminths. *Trends Parasitol.* **2018**; 34(10):861-880.

Ishikura H. Anisakidae and Anisakidosis. *Progress in Clinical Parasitology*. Eds, Springer-Verlag. **1990**; 3, 3.

Jeon CH, Kim JH. Pathogenic potential of two sibling species, *Anisakis simplex* (s.s.) and *Anisakis pegreffii* (Nematoda: Anisakidae): In vitro and in vivo studies. *Biomed. Res. Int.* **2015**; 2015, 983656.

Jerončić A., Nonković D., Vrbatović A., Hrabar, J., Bušelić I., Martínez-Sernández V., Rocamonde S.A.L., Ubeira F.M., Jaman S., Jeličić E.Č., et al. *Anisakis* Sensitization in the Croatian fish processing workers: Behavioral instead of occupational risk factors? *PLoS Negl. Trop Dis.* **2020**; 14, e0008038.

Jones SA. Directing transition from innate to acquired immunity: Defining a role for IL-6. *J. Immunol.* **2005**; 175, 3463–3468.

Kang DB, Park WC, Lee JK. Chronic gastric anisakiasis provoking a bleeding gastric ulcer. *Ann. Surg. Treat. Res.* **2014**; 86, 270–273. 17.

Kim JH, Kim JO, Jeon CH, Nam UH, Subramaniyam S, Yoo SI, Park JH. Comparative transcriptome analyses of the third and fourth stage larvae of *Anisakis simplex* (Nematoda: Anisakidae). *Mol. Biochem. Parasitol.* **2018**; 226, 24–33.

Klimpel, S.; Palm, H.W. Anisakid Nematode (Ascaridoidea) Life Cycles and Distribution: Increasing Zoonotic Potential in the Time of Climate Change? In Progress in Parasitology; Parasitology Research Monographs 2; Mehlhorn H. Eds., Springer: Berlin/Heidelberg, Germany, **2011**.

Kochanowski M, Dąbrowska J, Różycki M, Sroka J, Karamon J, Bełcik A, Korpysa-Dzirba W, Cencek T. Proteomic Profiling and In Silico Characterization of the Secretome of *Anisakis simplex* Sensu Stricto L3 Larvae. *Pathogens.* **2022**; 14;11(2):246.

Lea T. Caco-2 Cell Line. In The Impact of Food Bioactives on Health: In Vitro and Ex Vivo Models. Verhoeckx K, Cotter P, López- Expósito I, Kleiveland C, Lea T, Mackie A, Requena T, Swiatecka D, Wichers H, Eds.; Springer: Cham, Switzerland, **2015**.

Ma'ayeh SY, Knörr L, Sköld K, Garnham A, Ansell BRE, Jex AR, Svard SG. Responses of the Differentiated Intestinal Epithelial Cell Line Caco-2 to Infection with the *Giardia intestinalis* GS Isolate. Front. *Cell Infect. Microbiol.* **2018**; 8, 244.

Maizels RM, McSorley HJ. Regulation of the host immune system by helminth parasites. *J Allergy Clin Immunol.* **2016**;138(3):666-675.

Maizels RM, Smits HH, McSorley HJ. Modulation of Host Immunity by Helminths: The Expanding Repertoire of Parasite Effector Molecules. *Immunity.* **2018**; 20;49(5):801-818.

Mashouri L, Yousefi H, Aref AR, Ahadi AM, Molaei F, Alahari SK. Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance. Mol Cancer. **2019**; 2;18(1):75.

Mattiucci S, Cipriani P, Levsen A, Paoletti M, Nascetti G. Molecular Epidemiology of *Anisakis* and Anisakiasis: An Ecological and Evolutionary Road Map. *Adv Parasitol.* **2018**; 99:93-263.

Mattiucci S, Cipriani P, Webb SC, Paoletti M, Marcer F, Bellisario B, Gibson DI, Nascetti G. Genetic and morphological approaches distinguish the three sibling species of the *Anisakis simplex* species complex, with a species designation as *Anisakis berlandi* n. sp. for *A. simplex* sp. C (Nematoda: Anisakidae). *J Parasitol.* **2014**;100(2),199-214.

Mattiucci S, Colantoni A, Crisafi B, Mori-Ubaldini F, Caponi L, Fazii P, Nascetti G, Bruschi F. IgE sensitization to *Anisakis pegreffii* in Italy: Comparison of two methods for the diagnosis of allergic anisakiasis. *Parasite Immunol.* **2017**; 39, e12440.

Mattiucci S., Palomba M., Cavallero S., D'Amelio S. Anisakiasis. In Helminth Infections and their Impact on Global Public Health, 2nd ed; Bruschi, F., Ed.; Springer: Berlin/Heidelberg, Germany, **2022;** pp. 451–495.

Mazzucco W, Raia DD, Marotta C, Costa A, Ferrantelli V, Vitale F, Casuccio A. *Anisakis* sensitization in different population groups and public health impact: A systematic review. *PLoS One.* **2018**; 20;13(9):e0203671.

McSorley HJ, O'Gorman MT, Blair N, Sutherland TE, Filbey KJ, Maizels RM. Suppression of type 2 immunity and allergic airway inflammation by secreted products of the helminth *Heligmosomoides polygyrus*. *Eur J Immunol.* **2012**; 42(10):2667-82.

Measures LN. Anisakiosis and pseudoterranovosis: Reston, Va., U.S. Geological Survey Circular **2014**; 34, 2, 1393.

Mehrdana F, Buchmann K. Excretory/secretory products of anisakid nematodes: biological and pathological roles. *Acta Vet Scand.* **2017**; 59, 42.

Messina CM, Pizzo F, Santulli A, Bušelić I, Boban M, Orhanović S, Mladineo I. *Anisakis pegreffii* (Nematoda: Anisakidae) products modulate oxidative stress and apoptosis-related biomarkers in human cell lines. *Parasit Vectors.* **2016**, 9, 607.

Mineta S, Shimanuki K, Sugiura A, Tsuchiya Y, Kaneko M, Sugiyama Y, Akimaru K, Tajiri T. Chronic anisakiasis of the ascending colon associated with carcinoma. *J. Nippon. Med. Sch.* **2006**; 73, 169–174. 16.

Mladineo I. Anisakiasis in Europe: emerging, neglected, misdiagnosed, or all of the above? Veterinaska Stanika. **2019**; 50, (5).

Moneo I, Caballero ML, Gómez F, Ortega E, Alonso M.J. Isolation and characterization of a major allergen from the fish parasite *Anisakis simplex*. *J. Allergy Clin. Immunol.* **2000**; 106, 177–182.

Moneo I, Carballeda-Sangiao N, González-Muñoz M. New Perspectives on the Diagnosis of Allergy to *Anisakis* spp. *Curr Allergy Asthma Rep.* **2017**; 17, 27.

Motran CC, Silvane L, Chiapello LS, Theumer MG, Ambrosio LF, Volpini X, Celias DP and Cervi L. Helminth Infections: Recognition and Modulation of the Immune Response by Innate Immune Cells. *Front. Immunol.* 2018; 9:664.

Murphy A, Cwiklinski K, Lalor R, O'Connell B, Robinson MW. *Fasciola hepatica* Extracellular Vesicles isolated from excretory-secretory products using a gravity flow method modulate dendritic cell phenotype and activity. *PLOS Negl Trop Dis.* **2020***;* 14(9): e0008626.

Napoletano C., Mattiucci S., Colantoni A., Battisti F., Zizzari I.G., Rahimi H., Nuti M., Rughetti A. *Anisakis pegreffii* impacts differentiation and function of human dendritic cells. *Parasite Immunol.* **2018**; 40, e12527.

Nieuwenhuizen N, Lopata AL, Jeebhay MF, Herbert DR, Robins TG, Brombacher F. Exposure to the fish parasite *Anisakis* causes allergic airway hyperreactivity and dermatitis. *J. Allergy Clin. Immunol.* **2006**; 117, 1098–1105.

Nieuwenhuizen, N.E.; Lopata, A.L. *Anisakis*—A food-borne parasite that triggers allergic host defences. *Int. J. Parasitol.* **2013**; 43, 1047–1057.

Nogami Y, Fujii-Nishimura Y, Bann K, Suzuki A, Susumu N, Hibi T, Murakami K, Yamada T, Sugiyama H, Morishima Y. et al. Anisakiasis mimics cancer recurrence: Two cases of extragastrointestinal anisakiasis suspected to be recurrence of gynecological cancer on PET-CT and molecular biological investigation. *BCM Med. Imaging.* **2016**; 26, 16–31. 29.

Osbourn M, Soares DC, Vacca F, Cohen ES, Scott IC, Gregory WF, Smyth DJ, Toivakka M, Kemter AM, le Bihan T, Wear M, Hoving D, Filbey KJ, Hewitson JP, Henderson H, Gonzàlez-Cìscar A, Errington C, Vermeren S, Astier AL, Wallace WA, Schwarze J, Ivens AC, Maizels RM, McSorley HJ. HpARI Protein Secreted by a Helminth Parasite Suppresses Interleukin-33. *Immunity.* **2017**; 17;47(4):739-751.e5.

Pacios E, Arias-Diaz J, Zuloaga J, Gonzalez-Armengol J, Villarroel P, Balibrea JL. Albendazole for the Treatment of Anisakiasis Ileus. *Clin. Infect. Dis.* **2013**; 41, 1825–1826.

Pampiglione S, Rivasi F, Criscuolo M, De Benedittis A, Gentile A, Russo S, Testini M., Villan M. Human anisakiasis in Italy: A report of eleven new cases. *Pathol. Res. Pract.* **2002**; 198, 429–434. 30.

Panel EFSA. On biological hazards (BIOHAZ) scientific opinion on risk assessment of parasites in fishery products. *EFSA J.* **2010**; 8, 1543.

Park EY, Baek DH, Kim GH, Lee BE, Lee SJ, Park DY. Endosonographic Findings and the Natural Course of Chronic Gastric Anisakiasis: A Single-Center Experience. *Gastroenterol Res Pract.* **2018**; 20;2018:8562792.

Perez F, Ruera CN, Miculan E, Carasi P, Dubois-Camacho K, Garbi L, Guzman L, Hermoso MA, Chirdo FG. IL-33 Alarmin and Its Active Proinflammatory Fragments Are Released in Small Intestine in Celiac Disease. *Front Immunol.* **2020**; 8;11:581445.

Pérez-Pérez J, Fernández-Caldas E, Marañón F, Sastre J, Bernal ML, Rodríguez J, Bedate CA. Molecular Cloning of Paramyosin, a New Allergen of *Anisakis simplex. Int. Arch. Allergy Immunol.* **2000**; 123, 120–129.

Petithory JC, Paugam B, Buyet-Rousset P, Paugam A. *Anisakis simplex*, a co-factor of gastric cancer? *Lancet.* **1990**; 336, 1002. 14.

Pullan RL, Smith JL, Jasrasaria R, Brooker SJ. Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. *Parasit Vectors.* **2014**;7:37.

Qiu X, Campos Y, van de Vlekkert D, Gomero E, Tanwar AC, Kalathur R, Weesner JA, Bongiovanni A, Demmers J, d'Azzo A. Distinct functions of dimeric and monomeric scaffold protein Alix in regulating F-actin assembly and loading of exosomal cargo. *J Biol Chem.* **2022**; 298(10):102425.

Rawla P, Sunkara T, Barsouk A. Epidemiology of colorectal cancer: Incidence, mortality, survival, and risk factors. *Prz. Gas- troenterol.* **2019**; 14, 89–103. 24.

Ricciardi A, Bennuru S, Tariq S, Kaur S, Wu W, Elkahloun AG, Arakelyan A, Shaik J, Dorward DW, Nutman TB. Extracellular vesicles released from the filarial parasite *Brugia malayi* downregulate the host mTOR pathway. *PLoS Negl. Trop Dis.* **2021**;15, e0008884.

Rodero M, Cuéllar C. Modulation by *Anisakis simplex* antigen of inflammatory response generated in experimental autoimmune encephalomyelitis. *Int. Immunopharmacol.* **2021**; 90, 107241. 46.

Rodríguez E, Anadón AM, García-Bodas E, Romarís F, Iglesias R, Gárate T, Ubeira, F.M. Novel sequences and epitopes of diagnostic value derived from the *Anisakis simplex* Ani s 7 major allergen. *Allergy.* **2008**, 63, 219–225.

Rodríguez-Perez R, Moneo I, Rodríguez-Mahillo AI, Caballero M.L. Cloning and expression of Ani s 9, a new *Anisakis simplex* allergen. *Mol. Biochem. Parasitol.* **2008**; 159, 92–97.

Roig J, Saiz Maria L, Galiano A, Trelis M, Cantalapiedra F, Monteagudo C, Giner E, Giner RM, Recio MC, Bernal D, Sánchez-Madrid F, Marcilla A. Extracellular Vesicles From the Helminth *Fasciola hepatica* Prevent DSS-Induced Acute Ulcerative Colitis in a T-Lymphocyte Independent Mode. *Frontiers in Microbiology.* **2018**; 9.

Sakanari JA, McKerrow JH. Anisakiasis. *Clin Microbiol Rev.* **1989**; 2(3):278-84.

Saliminejad K, Khorram Khorshid HR, Soleymani Fard S, Ghaffari SH. An overview of microRNAs: Biology, functions, therapeutics, and analysis methods. *J. Cell Physiol.* **2019**; 234, 5451–5465. 32.

Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nat.* **2009***;* 459, 262–265.

Scholte LLS, Pascoal-Xavier MA and Nahum LA. Helminths and Cancers From the Evolutionary Perspective. *Front. Med.* **2018**; 5:90.

Shibata E, Ueda T, Akaike G, Saida Y. CT findings of gastric and intestinal anisakiasis. *Abdom Imaging*. **2014**;39(2):257-61.

Shimamura Y, Muwanwella N, Chandran S, Kandel G, Marcon N. Common Symptoms from an Uncommon Infection: Gastrointestinal Anisakiasis. *Can J Gastroenterol Hepatol.* **2016**; 5176502.

Smith JW, Wootten R. *Anisakis* and anisakiasis. *Adv Parasitol*. **1978**;16:93-163.

Sonko P, Chih-Cheng Chen S, Chou CM, Huang YC, Hsu SL, Barčák D, Oros M, Fan CK. Multidisciplinary approach in study of the zoonotic *Anisakis* larval infection in the blue mackerel (*Scomber australasicus*) and the largehead hairtail (*Trichiurus lepturus*) in Northern Taiwan. *J Microbiol Immunol Infect*. **2020**; 53(6):1021-1029.

Sonoda H, Yamamoto K, Ozeki K, Inoye H, Toda S, Maehara Y. An *Anisakis* larva attached to early gastric cancer: Report of a case. *Surg. Today*. **2015**; 45, 1321–1325.

Speciale A, Trombetta D, Saija A, Panebianco A, Giarratana F, Ziino G, Minciullo PL, Cimino F, Gangemi S. Exposure to *Anisakis* extracts can induce inflammation on in vitro cultured human colonic cells. *Parasitol. Res*. **2017**; 116, 2471–2477.

Steinberg MS. The problem of adhesive selectivity in cellular interactions. In: Cellular Membranes in Development. Ed: Locke M. *New York and London: Academic Press*. **1964**.

Stoltzfus JD, Pilgrim AA, Herbert DR. Perusal of parasitic nematode 'omics in the post-genomic era. *Mol Biochem Parasitol*. **2017**; 215:11-22.

Sugiyama H, Shiroyama M, Yamamoto I, Ishikawa T, Morishima Y. Anisakiasis Annual Incidence and Causative Species, Japan, 2018-2019. *Emerg Infect Dis.* **2022;** 28(10):2105-2108.

Suzuki J., Murata R., Kodo Y. Current Status of Anisakiasis and *Anisakis* Larvae in Tokyo, Japan. *Food Saf*. **2021**; 9, 89–100.

Takebayashi K, Nasu K, Okamoto M, Aoyagi Y, Hirakawa T, Narahara H. hsa-miR-100-5p, an overexpressed miRNA in human ovarian endometriotic stromal cells, promotes invasion through attenuation of SMARCD1 expression. *Reprod Biol Endocrinol*. **2020**; 16;18(1):31.

Tejada M, Solas MT, Navas A, Mendizábal A. Scanning electron microscopy of Anisakis larvae following different treatments. *J Food Prot*. **2006;** 69(6):1379-87

Tiwari RL, Singh V, Singh A, Barthwal MK. IL-1R-associated kinase-1 mediates protein kinase Cδ-induced IL-1β production in monocytes. *J Immunol*. **2011**; 1,187(5):2632-45.

Valle J, Lopera E, Sánchez ME, Lerma R, Ruiz JL. Spontaneous splenic rupture and *Anisakis* appendicitis presenting as abdominal pain: A case report. *J. Med. Case Rep*. **2012**; 6, 114.

Van Thiel PH. Anisakiasis. [Abstract]. *Parasitology*,**1962**; 52, 16P–17P

Van Thiel PH., Van Houten H. The Herring Worm Anisakis marina as a Human Parasite outside the Wall of the Gastro-Intestinal Tract. [Abstract]. Nederlands Tijdschrift Voor Geneeskunde, **1966**; 110, 1524–1528.

Wang G, Yang L, Hu M, Hu R, Wang Y, Chen B, Jiang X, Cui R. Comprehensive Analysis of the Prognostic Significance of Hsa-miR-100-5p and Its Related Gene Signature in Stomach Adenocarcinoma. *Front Cell Dev Biol*. **2021**; 17, 9:736274.

Weiss P, Taylor AC. Reconstitution of complete organs from single-cell suspensions of chick embryos in advanced stages of differentiation. *Proc Natl Acad Sci*. **1960**; USA 46, 1177–1185.

White R, Sotillo J, Ancarola ME, Borup A, Boysen AT, Brindley PJ, Buzás EI, Cavallero S, Chaiyadet S, Chalmers IW, Cucher MA, Dagenais M, Davis CN, Devaney E, Duque-Correa MA, Eichenberger RM, Fontenla S, Gasan TA, Hokke CH, Kosanovic M, Kuipers ME, Laha T, Loukas A, Maizels RM, Marcilla A, Mazanec H, Morphew RM, Neophytou K, Nguyen LT, Nolte-'t Hoen E, Povelones M, Robinson MW, Rojas A, Schabussova I, Smits HH, Sungpradit S, Tritten L, Whitehead B, Zakeri A, Nejsum P, Buck AH, Hoffmann KF. Special considerations for studies of extracellular vesicles from parasitic helminths: A community-led roadmap to increase rigour and reproducibility. *J Extracell Vesicles*. **2023**; 12(1):e12298.

Wilke G, Funkhouser-Jones LJ, Wang Y, Ravindran S, Wang Q, Beatty WL, Baldridge MT, VanDussen KL, Shen B, Kuhlenschmidt MS, Kuhlenschmidt TB, Witola WH, Stappenbeck TS, Sibley LD. A Stem-Cell-Derived Platform Enables Complete *Cryptosporidium* Development In Vitro and Genetic Tractability. *Cell Host Microbe*. **2019**; 26(1):123-134.e8.

Wilson HV. A new method by which sponges may be artificially reared. *Science*. **1907**; 25, 912–915.

World Health Organization, 2012. World Health Organization. **2012**.

World Health Organization. Soil-transmitted helminth infection. **2023** .

World Health Organization, 2015. World health statistics **2015**.

Yamamoto T, Miyazaki T, Kurashima Y, Ohata K, Okawa M, Tanaka S, Uenishi T, Miyaji K, Fukumoto N. Solitary hepatic eosinophilic granuloma accompanied by eosinophilia without parasitosis: Report of a case. *Int. Surg*. **2015**; 100, 1011– 1017

Year H., Fréalle É., Dutoit E., Dupouy-Camet J. A national retrospective survey of anisakidosis in France (2010–2014): Decreasing incidence, female predominance, and emerging allergic potential. *Parasite*. **2018**; 25, 23.

Yuan Y, Zhao J, Chen M, Liang H, Long X, Zhang B, Chen X, Chen Q. Understanding the Pathophysiology of Exosomes in Schistosomiasis: A New Direction for Disease Control and Prevention. *Front in Immun*. **2021**; 12, 1664-3224

Zaiss MM, Maslowski KM, Mosconi I, Guenat N, Marsland BJ, Harris NL. IL-1β suppresses innate IL-25 and IL-33 production and maintains helminth chronicity. *PLoS Pathog*. **2013**; 9(8), e1003531.

Zakeri A, Hansen EP, Andersen SD, Williams AR, Nejsum P. Immunomodulation by Helminths: Intracellular Pathways and Extracellular Vesicles. *Front. Immunol*. **2018**; 9, 2349.

Zamora V, Andreu-Ballester JC, Rodero M, Cuéllar C. *Anisakis simplex*: Immunomodulatory effects of larval antigens on the activation of Toll like Receptors. *Int. Immunopharmacol*. **2021**; 100, 1567–5769.