



Cytokine Profile in Patients Infected with *Anisakis simplex* in Endemic Areas: Dietary Intervention with Polyphenols: A Working Hypothesis



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Abstract: Anisakiasis is a zoonosis due to infection with the nematode *Anisakis* (*A.*) *simplex*. Humans represent an accidental host after ingesting raw fish infected with *A. simplex*.

A. simplex larvae lead to a robust host immune response mostly at gastro-intestinal level and, usually, T helper (h)2 lymphocytes play a protective against this nematode. However, larvae can elude immunity triggering activation of T regulatory (Treg) cells, which, in turn, suppress Th2 responses via production of interleukin (IL)-10. According to our own experience in the area of Bari (south Italy) where anisakiasis is endemic, chronic urticaria (CU) is very often associated to *A. simplex* infection. The cytokine profile in CU patients with *Anisakis* infection is characterized by an elevated release of interferon- γ and IL-17, while IL-10 production is very low. This pattern of pro-inflammatory cytokine response was consistently detectable in patients who underwent a raw fish free diet regimen over a period of six months. In the light of the above considerations, we hypothesize that treating these patients with polyphenols it may be beneficial in terms of activation of Treg cell function. In fact, polyphenols have the capacity to expand Treg cell response *in vitro*.

In this regard, activation of human Treg cells, by analogy to other helminthiasis, may lead to a reduction of allergic/inflammatory reaction in the course of human anisakiasis.



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INTRODUCTION

Anisakis spp. are nematodes which belong to the subfamily *Anisakinae*, family *Anisakidae*, superfamily *Ascaroidea*, suborder *Ascaridina*, order *Ascarida* [1]. In humans, *Anisakis* represents an accidental host since marine mammals host adult parasites in their stomach and, then, release unembryonated eggs with faeces [2]. Free living *A. simplex* L2s derived from hatched eggs are ingested by krill and copepods which represent intermediate hosts. Marine fish and cephalopods, which have ingested free living L2 larvae form infected fish, cephalopods and crustaceans, in turn, are ingested by marine mammals that, ultimately, represent the final host. In the context of this life cycle, humans may represent accidental hosts after ingesting raw fish and cephalopods infected with L2s. Of note, humans are not infected by *Anisakis* spp when eating marine mammals. For further details, readers are referred to Nieuwenhuizen and Lopata [3].

The life cycle of *Anisakis* is illustrated in Fig. 1.

Anisakiasis is a zoonosis mostly caused by *A. simplex* and characterized by gastrointestinal symptoms, which may be associated to allergic-type events [4]. The gastrointestinal involvement can be acute or chronic, while IgE-mediated allergic reactions encompass a broad range of clinical manifestations from asthma to urticaria, angioedema and anaphylactic shock [4]. In this framework, in endemic areas *A. simplex* reactivity seems to be associated with chronic urticaria (CU) [5, 6], even if other authors did not report allergic hypersensitivity reactions in acute case of *Anisakis* infection [7, 8].

It is well known that penetration of helminths into a given host usually results in the elicitation of a protective T helper (h)-2 response [9]. However, helminths, tend to escape from the host immune response suppressing Th2 and innate lymphoid cell (ILC)2 functions [9, 10-12]. In fact, expansion and activation of FoxP3+ T regulatory (Treg) cells in response to helminth challenge lead to the release of IL-10 which, in turn, suppresses Th2-related cytokine production [10, 11, 13]. Noteworthy, in *Strongyloides stercoralis* infection,

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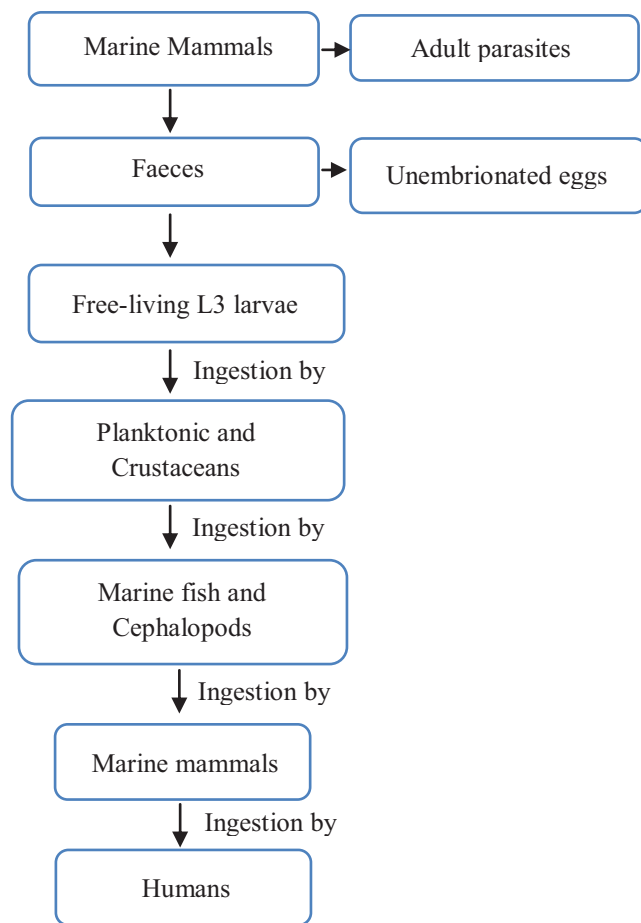


Fig. (1). Life cycle of *Anisakis*. Humans are accidental hosts after ingesting raw fish infected with larvae. Marine mammals are the final hosts, and release unembrionated eggs with faeces, thus initiating the life cycle.

the role of Treg cells is quite controversial since they can aggravate disease abrogating Th2-mediated protection, on the one hand [13]. On the other hand, Treg cells are also able to attenuate Th2-mediated immunopathology, in *e.g.*, filariasis [14].

The immunoregulatory network in the course of helminth infections is depicted in Fig. 2.

On these grounds, here we will try to clarify the complex spectrum of human immune responses in the course of anisakiasis, mostly placing special emphasis on CU associated to *A. simplex* infection. Finally, we will hypothesize the use of natural products, such as polyphenols, to treat anisakiasis in endemic areas.

SPECTRUM OF IMMUNE RESPONSES TO *A. simplex*

A robust local immune response can be observed in gastrointestinal cases of *Anisakis* infection. In histological samples, an eosinophilic phlegmonous inflammation, observed in human anisakiasis, was reproduced by intraserosal injection of eosinophil cationic protein (ECP) into the ileum of rabbits [15]. It is well known that eosinophils are the major cells involved in parasitocidal

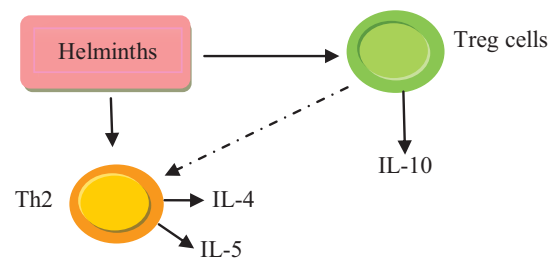


Fig. (2). Putative role of Treg cells in anisakiasis. Activated Treg cells in response to *Anisakis* larvae release IL-10 in the gastrointestinal milieu. IL-10, in turn, may suppress protective Th2 response, on the one hand. On the other hand, IL-10 may be able to attenuate immunopathology caused by *Anisakis* in the chronic phase of disease.

activity *via* release of major basic protein (MBP) and IgE- or IgG-mediated cytotoxicity [9]. For instance, in guinea pigs and rabbits, injection of *Anisakis* extracts leads to a massive recruitment of eosinophils and their infiltration within a few hours from administration [16]. In human anisakiasis detection of ECP in serum and MBP in biopsic samples, respectively, supports the primary involvement of eosinophils in this disease [17]. Besides ECP and MBP release, also production of nitric oxide (NO) by eosinophils seems to contribute to worm eradication [17]. However, prolonged release of these mediators may lead to local damage [18]. Larvae death is followed by a granuloma formation prevalently composed by macrophages (M0) and lymphocytes, which may rapidly disappear or persist, thus, leading, in this last case, to chronic symptoms [19].

In human anisakiasis, analysis of T cell infiltrates within biopsic samples demonstrate that Th2-related cytokines can be detected with a prevalence of IL-4 and IL-5 in the absence of interferon (IFN)- γ and IL-2 [17]. It is well known that IL-4 induces switch of B cell response toward IgE, while IL-5 contributes to eosinophil proliferation, thus leading to eradication of larvae [20].

As demonstrated by Perteguer and Cuellar [21] the genetic background of the host in the elaboration of the immune response to *Anisakis* plays an important role. In this framework, in IL-4R $\alpha^{-/-}$ mice (upon reinfection after resolution of the primary *Anisakis* infection), IgG and IgE levels were lower than those observed in the reinfected wild counterpart [22]. On the other hand, in the latter mice, allergic symptoms were more marked than in the former counterpart and characterized by itching, diarrhoea, respiratory mucus and cell infiltration. On these bases, one can explain the broad spectrum of clinical manifestations in humans, ranging from asymptomatic to allergic-inflammatory events. In this regard, in humans, various cytokine profiles have been demonstrated according to the clinical manifestations of *Anisakis* infection. In patients with urticaria-angioedema signs and/or anaphylaxis, serum increase in total and specific IgE, IL-4 and IL-5 and serum decrease in IFN- γ have been reported [23]. *Viceversa*, elevated levels of serum IFN- γ and mild allergic symptoms have been detected in patients with gastrointestinal *Anisakis* [23]. In another study, Th1 and Th2

serum cytokines were assayed in patients with chronic urticaria (CU) associated (CU⁺) or not associated (CU⁻) to *A. simplex* sensitization [24]. Of note, gastro-allergic anisakiasis (GAA) patients and healthy donors were selected as controls. In CU⁺ and CU⁻ patients, serum levels of IL-10 and IFN- γ were lower than those detected in healthy donors. Conversely, in GAA individuals levels of IL-17 were higher than those observed in both CU⁺ and CU⁻ patients.

Respiratory manifestations, such as airway hyper-responsiveness, are also present during infection with *Anisakis* larvae, as observed in wild type and IL-4R α ⁻ mice, where IL-4/IL-13 on the one hand, and, IFN- γ , on the other hand, seem to be involved in clinical manifestations [25]. In this framework, evidence has been provided that a 24 kDa protein of *A. simplex* (As22U) was able to elicit airway inflammation in mice, triggering both Th2 and Th17 responses [26].

According to the type of individual responses to *Anisakis* larvae, Th1 responders tend to develop a chronic inflammation [23] with prevalent gastrointestinal manifestations [27]. By contrast, Th2 responders are able to expel ingested larvae with vomiting and diarrhoea as a result of a robust allergic reaction [28]. Also worm burden seems to determine the type of immune response since there is evidence that in rats and humans high numbers of *Anisakis* larvae may lead to IgE suppression [29].

According to our own experience, the area of Bari (south Italy) is endemic for *A. simplex* infection because of the habit to eat raw fish products [5]. Among the many cases of acute and chronic anisakiasis observed by us, major symptoms include urticaria, respiratory distress, ocular manifestation, skin rash and anaphylaxis [5]. Clinical manifestations are present either in raw fish consumers and/or in individuals who manipulate fish in domestic and occupational habitats. In a previous report of our group, in our area, we have investigated in 919 adults the association between CU and *A. simplex* sensitization [5]. Of 919 subjects, 213 met the criteria for CU and 106/213 were sensitized to *A. simplex*. In sensitized patients, a raw fish free dietary regimen was recommended for 6 months. Following the dietary regimen, urticaria disappeared in 82/106 cases. Of 75 responders after additional 3 months of follow up, CU relapsed in 88% of patients who had reintroduced raw fish in their diet. Since *A. simplex* infection leads to a robust immune response into the host, in connection with our previous results [5], in a group of 20 patients with CU associated to *A. simplex* infection, also belonging to the same endemic area, we have investigated the profile of peripheral cytokines (unpublished data). In patients under six month raw fish free dietary regimen, peripheral blood was collected at time 0 (T0, before diet), T1 (after one month of diet) and T2 (after six month of diet), respectively. Isolated lymphomonocytes were stimulated with phorbol-myristate acetate (PMA) plus ionomycin for 24 h and supernatants were assayed for cytokine detection by ELISA. In these patients, the cytokine profile, upon stimulation with PMA, was characterized by more elevated levels of IFN- γ and IL-17 and lower levels of IL-10 in comparison to unstimulated cell cultures. This pattern of cytokine release was consistently observed at all time points considered, regardless of disappearance of CU

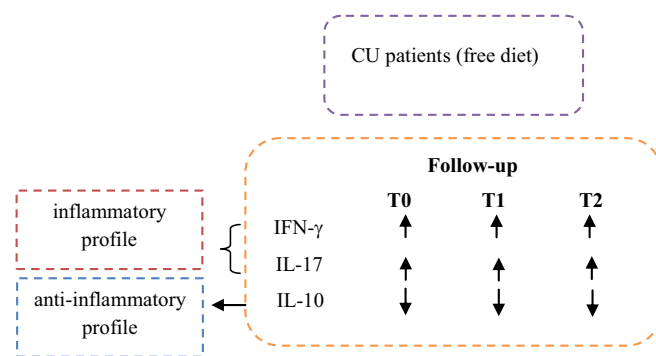


Fig. (3). Cytokine profile in CU patients infected with *A. simplex* in an endemic area (Bari, south Italy). Over a period of six months, 20 patients under a free raw fish diet were followed-up for cytokine profile. IFN- γ and IL-17 exhibited higher values than those observed with IL-10 when lymphomonocytes were stimulated with PMA.

manifestations. These findings indicate that in these habitual consumers of raw fish the profile of cytokines is allergic/inflammatory with a trend to chronicization of disease. In this context, reintroduction of raw fish into the diet may trigger further release of pro-inflammatory cytokines with re-exacerbation of CU clinical symptoms. In fact, the impaired anti-inflammatory pathway, due to low levels of IL-10 does contribute to the maintenance of Th1 and Th17 responses, which both favor allergic/inflammatory clinical conditions. This pattern of cytokine responses is indicated in Fig. 3.

DIETARY APPROACHES

Anisakis larvae do not survive or reproduce in the human gastrointestinal tract but, if not removed, may lead to a chronic inflammation which may simulate irritable bowel syndrome, inflammatory bowel disease or, in some cases, gastric cancer [4]. The major measures of prevention of anisakiasis are to eat fish that has been deep-frozen (-20°C) for at least 24 h [30]. In alternative, cooked fish should reach a temperature of at least 60°C over a period of 10 min or longer [31]. In the United States, uncooked fish or shell fish is blast-frozen to -35°C or below for 15 h or frozen at -23°C or below for 7 days [FDA, <http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsInformation/Seafood/ucm091704.htm>].

As additional therapeutic measures, chronic anisakiasis patients can be treated with anti-helminthics, as suggested by Pacios *et al.* [32]. However, no other evidences are reported in literature concerning their use in Anisakiasis.

In the light of the evidence that a number of natural products can exert anti-helminthic effects, their use in the treatment of *Anisakis* infection should be taken into serious consideration. In particular, dietary intervention with polyphenols will be proposed in the next paragraphs.

Polyphenols are ubiquitously present in fruits and vegetables and are classified as flavonoids and non

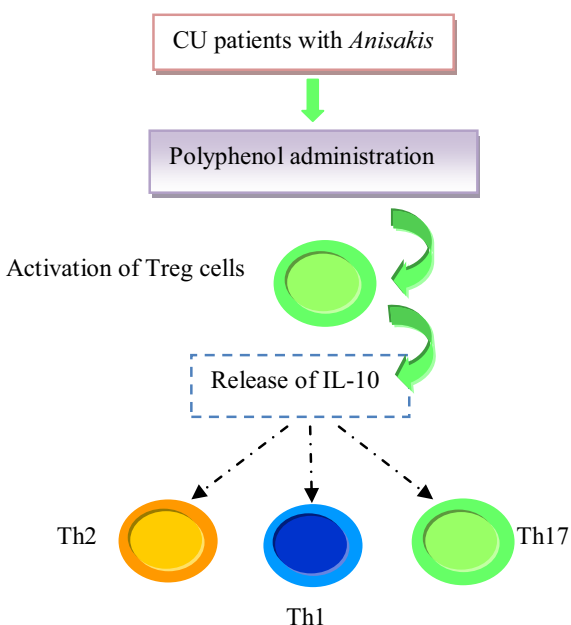


Fig. (4). Hypothetical effects of polyphenol administration to patients with anisakiasis. Polyphenols may expand and activate Treg cells which, in turn, may be able to switch off exaggerated functions of Th1, Th2 and Th17 cells, respectively, in the later phase of anisakiasis.

flavonoids (for example, resveratrol) compounds [33]. In particular, curcumin (a flavonoid) is able to exert adulticidal effects on *Schistosoma* (*S.*) *mansoni* and reduce *Schistosoma*-induced hepatic granuloma in infected animals [34]. Similar results have been reported on the *in vitro* effects of curcumin against Cercariae, pre adults and adults of *S. japonicum* [35]. Quite interestingly, flavonoids from red wine are able to *in vitro* enhance release of NO from human circulating monocytes [36, 37]. In this respect, *in vitro* NO-induced release by genistein has been shown to be cytotoxic for *Schistosoma* [38]. Moreover, fermented grape marcs (FGMs), obtained from Negroamaro and Koschu *Vitis vinifera* cultivars, have been demonstrated to exert anti-inflammatory activities in different settings. In experimental colitis mice, administration of Koschu FGM led to a recovery of colitis (abrogation of colon length shortening) and reduction of IL-1 β and tumor necrosis factor- α contents in colon homogenates [39].

In vitro, FGMs from Negroamaro and Koschu were able to induce and activate Treg cells with release of IL-10 [40]. Moreover, both FGMs reduced human basophil degranulation *in vitro* [41]. These anti-allergic effects have also been confirmed in murine asthma *in vivo* and rat mastocytes *in vitro* [42-44]. On these grounds, it is likely that in the course of chronic anisakiasis administration of polyphenols may be advantageous to patients either with allergic manifestations (e.g., CU) or with gastrointestinal inflammation (Fig. 4).

CONCLUSION AND PERSPECTIVES

In the previous sections, we have clarified the role of Treg cells in helminthiasis, even including Anisakiasis [45]. However, according to current literature other cell types

sharing similar functions with conventional Treg cells should be taken into consideration. Type 1 regulatory T (Tr1) cells, which lack FoxP3 expression, exert a suppressive function via the production of IL-10 and transforming growth factor (TGF)- β [46].

At the same time, Th3 cells secrete TGF- β , thus favoring differentiation of Treg cells [47]. Furthermore, B regulatory cells participate to the anti-inflammatory pathway, thus, regulating the balance between Th1, Th17 and Treg cells, and also converting effector T cells into Tr1 cells [48]. Alternative activated M0 M2 induced by IL-4 or IL-13 also exert anti-inflammatory activity [49].

Placing special emphasis on intestinal dendritic cells (DCs), evidence has been provided that in the presence of retinoic acid, these cells can induce development of Treg cells. It would be very interestingly to verify whether polyphenols may activate DCs as retinoic acid does [50]. Finally, evidence has been provided that ILCs3 [51], when stimulated by IL-1 β , release colony-stimulating factor 2 which, in turn, activates retinoic acid-mediated DC development.

Taking into account this complex regulatory intestinal immune network [52], future research on the host immune response toward *Anisakis* infection should imply a broader investigation on the function of various types of immune cells.

LIST OF ABBREVIATIONS

A	=	Anisakis
BMP	=	Basic major protein
CU	=	Chronic urticaria
DCs	=	Dendritic cells
ECP	=	Eosinophil cationic protein
FGM	=	Fermented grape marc
GAA	=	Gastro-allergic anisakiasis
IFN	=	Interferon
IL	=	Interleukin
ILCs	=	Innate lymphoid cells
M0	=	Macrophages
NO	=	Nitric oxide
PMA	=	Phorbol myristate-acetate
TGF	=	Transforming growth factor;
Th	=	T helper
Tr1	=	Type I regulatory T cells
Treg	=	T regulatory

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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