Anisakiasis, firstly described in 1960s in the Netherlands, is a fish-borne parasitic disease caused by the consumption of raw or undercooked fish or cephalopods contaminated by third stage (L3) larvae of the Anisakidae family, in particular Anisakis simplex (As), A. pegreffii and Pseudoterranova decipiens. Every year, approximately 20,000 cases of anisakiasis were reported worldwide, over 90% are from Japan and most others in Spain, the Netherlands and Germany, depending on the habits of fish consuming. Live As larvae can elicit i) a parasitic infection of the digestive tract or, occasionally, other organs, causing erosive and/or haemorrhagic lesions, ascites, perforations until granulomas and masses, if larva is not removed; and ii) allergic reactions, as anaphylaxis, acute/chronic urticaria and angioedema. Like other parasite infestations, As larva induces an immune adaptive response characterised by T-lymphocyte proliferation with polyclonal and monoclonal (responsible for As allergic symptoms) IgE production, eosinophilia and mastocytosis. Several As allergens, many of which thermostable, were described. In particular the major allergen Ani s 1 and Ani s 7 could characterized a past or a recent infection. There is a general agreement that an active infection is required to initiate allergic sensitivity to Anisakis. Until now, the only effective treatment for anisakiasis is the endoscopic removal of live larvae and the best protection against anisakiasis is to educate consumers about the dangers of eating raw fish and to recommend avoiding the consumption of raw or inadequately thermally treated marine fish or cephalopods.
Pseudoterranova decipiens. The latter parasite is particularly widespread in Canada and the United States. Nematodes, members of the Anisakis spp., have a complex life cycle that passes through a number of hosts. Adult stages of the Anisakis spp. reside in the stomach of marine mammals, where they are embedded in the mucosa. Unfertilized eggs produced by adult females are expelled through the faeces of marine mammals and become embryonated in seawater, where first-stage (L1) larvae are formed in the eggs. The larvae moult to become free-swimming in the second stage (L2) and are ingested by krill crustaceans, usually Euphausids, in which they mature into the L3 stage. This stage is infective to fish and squid, maintaining the L3 larval form. Through predation, the larvae are transferred between fishes. Upon the host’s death, As larvae migrate from the intestine to the tissues in the coelomic cavity and the muscle tissues, growing up to 3 cm in length. Pseudoterranova spp. larvae are also able to migrate to the fish flesh during their host life. When marine mammals ingest fish or squid containing L3 larvae, the larvae moult twice and develop into adult worms, completing the nematode lifecycle. Humans become infected by eating raw or undercooked parasitised marine fish and cephalopods, thus, representing an accidental host in which the worms cannot survive or reproduce and die in approximately 3 weeks (2).

Anisakiasis was first described as “worm-herring disease” in 1960s in the Netherlands by Van Thiel, who associated different cases of patients suffering from acute abdominal pain with the consumption of lightly salted herrings (3). Until now, several new cases have been reported; of the approximately 20,000 cases of anisakiasis reported worldwide, over 90% are from Japan (approximately 2,000 cases yearly) and most others occur in Spain, the Netherlands, and Germany (4), depending on the habits of consuming raw or undercooked fish.

The presence of live As larvae can elicit two different diseases: i) the parasitic infection (anisakiasis) of the digestive tract or, occasionally, other organs; and ii) allergic reactions with or without digestive symptoms.

Clinical features of anisakiasis

The ingestion of a parasitised fish with a nematode of the Anisakis genus may elicit symptoms within few hours. Four principal clinical syndromes associated to anisakiasis have been described: gastric, intestinal, ectopic (or extra-gastrointestinal), and allergic. The onset of gastric anisakiasis begins within few hours, generally 1 to 2, when a live As larva reaches the human stomach. Here, it adheres to the gastric mucosa by a projection surrounding its mouth and produces proteolytic enzymes, mainly secreted by a dorsal oesophageal gland and other excretory glands around the mouth. These proteases cause erosive and/or haemorrhagic lesions in or near the main lesion, forming a tunnel through the gastric mucosa to the submucosa. This acute phase of the infection elicits severe epigastric pain, vomiting, diarrhea, and a mild fever. Generally, acute symptoms resolve within a few days, but untreated gastric disease can lead to chronic, ulcer-like symptoms lasting for weeks to months. Intestinal anisakiasis is characterised by intermittent or constant abdominal pain starting 5 to 7 days after the larva ingestion. Infected individuals may develop ascites and/or peritoneal signs. Intestinal infection and inflammatory responses mainly occur in the terminal ileum and less commonly in the colon or jejunum. Rare complications include small bowel obstructions, ileal stenosis, intussusception, intestinal perforation, and pneumoperitoneum.

Although less common, the larva penetration through gastric or gut mucosa can lead to its migration into the peritoneal or pleural cavity, mesentery, liver, pancreas and ovary. Chronic infection may present with mesenteric masses (5).

The clinical manifestations of anisakiasis vary depending on the organ where the person was infected and which Anisakis spp. caused the infection. In Japan, a gastric infection occurs primarily, whereas intestinal disease is more common in Europe (6).

In addition to directly visualising the larva(е) embedded in the gastric mucosa, endoscopy may reveal erythema, oedema, severe erosive gastritis, a tumour-like nodule, or ulcerations. Biopsy can show an early inflammatory infiltrate of eosinophils and lymphocytes in the mucosa and submucosa as well as phlegmon formation. Although larvae may be found up to 6 days after the consumption of seafood, if endoscopy is delayed, the worm may degenerate, be eliminated, or pass through the mucosa (resulting in ectopic disease), preventing it from being visualised; the only signs may be thickened gastric folds and inflammation. Chronic infection can result in abscess and/or granuloma formation in response to degenerating larvae. Radiographic findings depend on the site of infection. Thread-like filling defects and mucosal oedema can be observed on barium studies with a gastric infection. Intestinal infection can cause non-specific, irregular bowel-wall thickening with a disappearance of Kerckring folds, mucosal oedema, and luminal narrowing, detectable by
scanning or CT (7,8). CT findings also include lymphadenopathy, focal masses, and/or ascites. Ascitic fluid obtained by paracentesis may demonstrate an eosinophilic predominance. A gastric infection is frequently accompanied by leucocytosis; eosinophilia is more commonly observed in gastric compared to intestinal infections, particularly if the worm remains in place (6). Continual exposure to the offending nematode or massive infestation causes chronic intestinal mucosa and submucosa inflammation or multiple or wide granulomas, simulating a sub-occlusive neoplastic lesion (5,9,10).

In summary, after 4 hours to 6 days, *As* larva penetrates mucosa and submucosa. The excretory/secretory proteases as well as the *As* surface and somatic components induce an immune response, mast cell (IgE-independent) degranulation, immunosuppression, anticoagulant activity, eosinophil chemotaxis and mutagenic effects. These features induce erosive lesions and eosinophilic phleghmon without any damage to the *As* body surface.

After 7–14 days, granulomas, ulcerative lesions and the induction of a hypersensitivity response occur.

After 14 days, *As* larvae die, but a persistent inflammation or granulomas remain. Two situations could occur: the loss of parasite with ulcerative lesions or the ending of a dead larva into a granuloma.

*As* with other parasite infestations, *As* larva induces an adaptive response characterised by T-lymphocyte proliferation with polyclonal and monoclonal (responsible for *As* allergic symptoms) IgE production, eosinophilia and mastocytosis.

### *As* immune stimulation

Different authors aimed to investigate cytokine expression induced by the *Anisakis* parasitism. Cuéllar et al. (11) found low levels of IL-6, IL-10, IL-17, TNF and IFNγ cytokines, mainly indicating a Th1 response, in sera from *As*-sensitised patients after *As* crude extract and mitogen stimulation during a cytomteric bead array (CBA) analysis. Higher values of IL-2 (important for growth and survival of both Th1 and Th2 lymphocytes) were detected after exposure to the crude extract, emphasising the relevant immune stimulation of this extract.

Therefore, *As* is able to induce both a Th1- and Th2-type immune response, with different levels in sensitised patients. González-Munoz et al. (12) found an increased in vitro production of IL-2, IL-4, IL-5, IFNγ cytokines in *As*-sensitised patients with respect to control patients after both crude and thermally treated extract exposure. Moreover, IL-10 levels were higher after crude extract incubation, and there was a correlation between the symptoms and cytokine patterns. Patients with urticaria/angioedema and/or anaphylaxis had higher levels of Th2 cytokines (IL-4, IL-5) and IgE, while patients with predominantly gastrointestinal symptoms had higher levels of IFNγ, which inhibited the IL-4-dependent responses.

In a more recent paper (13), cytokine responses in patients with gastro-allergic anisakiasis (GAA) or chronic urticaria with (CU+) and without (CU−) *Anisakis* sensitisation were investigated. IL-10 was low in CU+ and CU−patients and higher, but not statistically significant, in GAA patients. Higher levels of TGFβ, a marker of Th1 response, and IL-17 were found in GAA patients compared to CU+ and CU−patients. The authors correlated TGFβ levels with a previous exposure to *As* and IL-17 values with positive urticaria outcomes rather than to parasite exposure. Because the expression of the two cytokines was similar, we hypothesised that both molecules correlate with a previous *Anisakis* exposure.

In 2010, Daschner et al. (14) investigated the immunological pattern expressed in GAA, prolonged acute urticaria (PROL, 3 days-6 weeks), and CU *As*-sensitised patients by *As*-specific IgE, IgG and IgG4 detection. There were no differences found in the antibody levels between PROL and CU patients, but GAA patients showed significantly higher levels for all the tested immunoglobulins. The authors concluded that there was similar immunological stimulation both in PROL and CU.

Focusing on *As* sensitivity and CU, in a previous study, Daschner et al. (15) analysed the effect of a two-month fish-free diet in an *As*-endemic Spanish region. Among the 65 CU *As*-positive and 11 CU *As*-negative patients, there was a statistically significant symptom improvement (p<0.001) in the first group. Moreover, a clear improvement occurred due to diet in patients presenting with positive specific IgG4 levels to *As*. Because high IgG4 results from continual allergen exposure, CU *As*-positive patients are likely to improve with a fish-free diet when presenting this immunological pattern.

### IgE-mediated allergy to *As*: clinical diagnosis and symptoms

The correct diagnosis of IgE-mediated *As* allergy is based on the following criteria: i) a compatible history, such as typical allergic symptoms following fishery product con-
workers is higher, up to 64% (28), than in the general population, of the 10570 screened individuals, 4.5% had 
As-positive skin prick tests, but only 0.6% experienced 
As allergy symptoms (26).

Anisakis simplex: current knowledge

Considering the 
As body morphology, three groups of possible allergenic proteins can be defined: i) proteases and protease inhibitors secreted during larva penetration, namely the excretory/secretory (ES) allergens; ii) somatic allergens obtained from the 
As whole body; and iii) cuticular allergens, secreted to protect the 
As body from digestive juices.

Depending on the 
As larva’s fate, individuals could be exposed to different 
As allergens. During active penetration of the larva and its subsequent death, patients are exposed to all 
As allergens, while if 
As larva is eliminated intact through the gastrointestinal tract, patients are exposed only to ES allergens. Finally, if the 
As larva that is ingested is already dead, patients are mainly exposed to somatic and cuticular allergens and minimally to ES allergens.

To date, several 
As allergens have been described (from Anis1 to Anis12), but only the first 9 allergens have been identified and characterised. Seven (Anis1, Anis4, Anis5, Anis6, Anis7, Anis8, Anis9) are ES allergens, while two are somatic allergens (Anis2, Anis3). Anis1, Anis2, Anis3 and Anis7 are major allergens, and Anis4, Anis5, Anis6, Anis8, Anis9 and Anis10 are minor allergens. Anis1 is a major ES allergen with a molecular mass of 24 kDa, lacking any significant homology with other known allergens. It is highly specific for 
As-allergic patients; 85% of patients develop IgE to this protein. Interestingly, sera from patients with positive SPT or with serum-specific IgE levels but no clinical allergy to 
As did not bind purified Anis1 in SDS-PAGE immunoblots (27).

Anis7 is a major ES allergen as well, with a molecular mass of approximately 139 kDa and no significant homology with other known allergens, but it is characterised by a repeated cysteine residue-rich motif (28). Rats inoculated intraperitoneally with either live or dead L3 larvae produced IgE, IgA and IgM only against live larvae, even when re-infected with the same type of larvae. Authors suggested that the Ani s 7 allergenicity does not last in dead larvae and that immunisation occurs during the acute phase of infection when the ES allergens are released. In the same study, the authors evaluated the species specificity of Ani s 7 in rats infected with either Anisakis spp. or Pseudoterranova spp. larvae. While the crude extracts of the two nematodes elicited a positive response in both groups of infected rats, Ani s 7 was specific for 
As infection.

In a further study (29), IgE levels to rAni s 7 decreased more rapidly than those to rAni s 1; the authors suggested that rAni s 1 might be a marker of a previous infection, while high rAni s 7 IgE levels may indicate a recent infection. Ani s 2 and Ani s 3, paramyosins and tropomyosins, respectively, present a high degree of homology with other myosins. In particular, Ani s 2 is highly cross-reactive
with Blomia tropicalis and Dermatophagoides spp., while Ani s 3 presents a wide range of cross-reactivity with tropomyosins of major and minor house dust mites, crustaceans, molluscs, cockroaches and insects (30). Asturias (31) investigated patients’ response to Ani s 3 in those with a clear history of an allergic reaction following the ingestion of As-contaminated food, in patients with possible As allergy and in patients hypersensitive to mites. No sera from As-allergic patients reacted to Ani s 3, while 13% of the patients with a possible As allergy reacted to Ani s 3. Moreover, pre-incubation of sera with other tropomyosins completely inhibited the Ani s 3 binding in As immunoblotting. Thus, Ani s 3 cannot be regarded as a relevant allergen in As sensitisation.

Dead or live As larva: which is necessary to induce As allergy?

Currently, to avoid the parasitism risk, the European Community recommends cooking fish at 60°C for at least 10 minutes or freezing at -20°C for at least 24 h; the USA Food and Drug Administration (FDA) agency demands the same cooking conditions but freezing at -20°C for at least 7 days. Discrepancies could be explained considering the higher resistance of Pseudoterranova spp., typically diffused in Canada and the northern USA.

Many As allergens have been found to resist heating and freezing. Therefore, some authors demonstrated the in vitro IgE reactivity of As-allergic patients to thermally treated As extracts (32,33). As allergens may also be present in the fish flesh near the larvae (20); therefore, parasite allergens can be present in edible fish muscle and might cause allergic symptoms irrespective of larvae ingestion.

Recently, As allergens and in particular Ani s 4 were quantified in fresh or different thermally treated fish muscle using IgG dot blot analysis. Ani s 4 appeared to suggest the presence of As larvae in fish flesh (33). Tejada et al. (34) submitted hake steaks artificially parasitised with As larvae to different treatments (chilling, freezing, heating at 86.3°C and microwave cooking). By scanning electron microscopy, no apparent changes in the frozen larvae or disruptions in the cuticle were found. Few studies are available on oral challenges with thermally treated As larvae. In one study, 11 As-allergic patients with positive tests and allergic symptoms after heating marinated fish were submitted to double-blinded, placebo-controlled oral challenges with lyophilised As larvae (35). The ultimate dose corresponded to 100 larvae, and five patients were also challenged with an aqueous extract corresponding to 105 or 210 larvae. None of the patients experienced a positive reaction during or after the challenge. In another study (36), 5 As-allergic patients with one or more nematodes detected by gastroscopy in the stomach were submitted to two different single-blinded challenges versus placebo. The first challenge was performed with 11 As larvae frozen at -20°C for 48 h, while the second was performed with the offending seafood after freezing at -20°C for 48 h. All patients tolerated both challenges without any allergic or gastric symptoms. Patients were advised to consume deep-frozen fish at least once a week. After 6 months, no reaction had been reported, even if SPT and serum-specific IgE to As remained positive. Alonso-Gomez et al. (37) challenged 22 As-allergic patients with up to 20 frozen larvae without reporting any allergic reactions. The patients were followed-up for more than two years, in which patients consumed deep-frozen fish without problems.

There is a general agreement that in the majority of cases, an active infection is required to initiate allergic sensitivity to Anisakis, even if a prior sensitisation via exposure to thermally resistant As allergens, including dead larvae, could not be excluded.

Therapy and prevention

Until now, the only effective treatment for anisakiasis is the endoscopic removal of live larvae, as they naturally die after approximately three weeks in the human body. When eosinophilic granulomas occur, surgical removal is necessary to avoid a subocclusive emergency. Arias-Diaz et al. (38) studied the in vitro activity of different concentrations of albendazole against As larvae under different pH levels. Albendazole dose-dependently reduced the survival of the larvae, but acidic pH media significantly reduced its efficacy.

The lower prevalence of anisakiasis in certain Asiatic populations who season raw fish with aromatic plants, such as perilla or ginger, prompted several authors to investigate the biocidal effects of natural products. Navarro et al. (39) studied different monoterpenic derivatives from several essential oils, concluding that α-pinene significantly reduced the lesion-treated rats. Hierro et al. (40) demonstrated that citral damaged As L3 larvae. However, the best protection against anisakiasis is to educate consumers about the dangers of eating raw fish and to recommend avoiding the consumption of raw or inade-
treated fish consumption. Unfortunately, the current regulations do not protect the consumers against allergic hazards from ingesting killed parasites, but research has indicated, in the majority of cases, the safety of thermally treated fish consumption.

References