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Serum IgA contributes to the comprehension of *Anisakis simplex* associated chronic urticaria

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ABSTRACT

The phenotype of allergic diseases associated with *Anisakis* determines the pattern of cytokines related to antibody production. However, the role of serum IgA and the immunomodulatory mechanisms exerted by active infection of L3 or passive mucosal contact with *A. simplex* specific antigens has not been studied before. We measured serum cytokine by flow cytometry (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, IL-17A, TGF-β1) and antibody levels (IgE, IgG4, IgA) by ELISA against total and excretory-secretory (ES) antigens, Ani s 3,and the group of major allergens Ani s 1, Ani s 7, and Ani s 13 in sera from 10 patients with gastro-allergic anisakiasis (GAA), 11 *Anisakis* sensitization associated chronic urticaria (CU+) as well as 17 non-*Anisakis*-sensitized patients with chronic urticaria (CU-), compared with the urticaria control group (18 subjects). Specific IgE, IgG4 and IgA were high in the GAA, but IgA levels were significantly higher in the CU+ with respect the CONTROL group. We observed higher levels of the ratio IgA/IgG4 in CU+ than GAA group for Ani s 1, Ani s 7, Ani s 13 and ES. Furthermore, chronic urticaria (CU) patients showed significant lower levels of IL-10, IFN-γ and IL-17A than patients without CU. The anti-Ani s 13 IgA/IgG4 ratio correlated positively with pro-inflammatory cytokines and ratios (TNF-α, IL-17A, Th17/Th2, Type1/Type2 and TNF-α/IL-10) in CONTROL group. In general, Anti-*Anisakis* IgA/G4 ratio was high in CU patients. In conclusion, this study demonstrates the importance of serum IgA because it is associated with chronic urticaria independently of *Anisakis* sensitization.

1. Introduction

Diet-associated *Anisakis simplex* larvae are able to trigger allergic symptoms with appearance of different phenotypes of both acute and chronic urticaria. In the first case, acute urticarial reaction is produced when the live parasite penetrates the human gastric mucosa [1]. This clinical entity is named gastro-allergic anisakiasis (GAA) [2]. In some *Anisakis*-sensitized patients, chronic urticaria occurs by a still unknown mechanism [1]. This clinical entity is denominated *Anisakis* sensitization associated chronic urticaria (CU+) [1,3]. Each *Anisakis*-related urticarial phenotype (GAA or CU+) is associated with a different pattern of cytokines related to antibody production [3,4].

Patients with previous parasitism by *A. simplex* (GAA and CU+) displayed higher TGF-β levels than those without (CU-) [5].

Similarly, lower levels of IL-17 were observed in patients with CU+ compared to GAA or controls, with a tendency towards even lower levels in patients with chronic urticaria without *Anisakis* sensitization (CU-) [4].

Fish intake modulates the balance of anti-inflammatory and pro-inflammatory cytokines exerting anti-inflammatory effects. These effects are further modulated by *Anisakis* parasitism in a complex interaction [5].

There are no published studies on the global response to specific *Anisakis* allergens, such as Ani s 3 (tropomyosin-panallergen) and major allergens, such as Ani s 1, Ani s 7, and Ani s 13 in the context of serum antibody levels and their relationship with cytokine levels.

Mucosa-associated lymphoid tissues (MALT), ensure the protection of the mucosal surfaces. This defense is primarily mediated by the IgA isotype, which is abundantly expressed and secreted in the gastrointestinal tract, respiratory tract, vaginal tract, tears, saliva, and colostrum. Peyer's patches and the isolated lymphoid follicles (ILFs) of the gut-associated lymphoid tissues (GALT) contain most of the plasma

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cells of an individual [6]. Serum IgA is monomeric, its concentration is insignificant in comparison to the 3 g of the secretory form (sIgA) produced by an adult human per day and its function remains unclear [7,8]. It is remarkable that the modification of microbiota is one of the main signs in patients with selective IgA deficiency patients [9] and also serum IgA and sIgA may be relevant for the protection against *Anisakis* larvae penetration and with respect to the onset and maintenance of *Anisakis* sensitization associated chronic urticaria [1].

In all previous studies, patients diagnosed with GAA had higher levels of IgE and IgG4 specific to the *Anisakis* antigens than CU+ patients. This was also observed in the case of allergens Ani s 1, Ani s 7, and Ani s 13 [10–12]. However, the role of serum IgA against the *Anisakis* allergens has not been studied before in patients.

Anti-*Anisakis* IgA has been detected in the serum of patients since decades [13,14], and a few studies have reported its serum levels in healthy subjects that are continuously exposed to the parasite by eating fish [15–17]. Serum anti-*Anisakis* crude extract IgA levels were positively correlated with IL-7 and age [17].

We hypothesized that there could be a differential relationship between cytokines and antibody levels against specific *Anisakis* allergens not observed in studies using total crude extract (AkT) or excretory secretory (ES) antigen from third-stage larvae (L3) found in marine fish. In this study, we investigated the immunomodulatory mechanisms exerted by active infection of L3 or passive mucosal contact with *A. simplex* specific allergens or antigenic peptides derived from these allergens which could be generating a local immune mucosal response. We measured serum cytokine and antibody levels against particular allergens: Ani s 3, which is a panallergen, or the group of major allergens Ani s 1, Ani s 7, and Ani s 13 in sera from patients with GAA, CU+, and CU-, compared with the urticaria control group. We compared the results obtained by re-grouping these four initial groups (GAA, CU+, CU-, CONTROL) by the presence (CU: CU+ and CU-) *vs* absence of chronic urticaria (NoCU: GAA and CONTROL) or by the positivity (AK+: GAA and CU+) *vs* negativity to *Anisakis* (AK-: CU- and CONTROL).

2. Methods

2.1. Patients and serum samples

As a cross-sectional study, two main groups of patients were studied: 21 *Anisakis* sensitized patients (8 men and 13 women, 52 $\pm$ 14 years old) and 35 non-*Anisakis* sensitized patients (14 men and 21 women, 39 $\pm$ 13 years old) (eTable 1). The first group encompassed two clinical subgroups: 10 patients with gastro-allergic anisakiasis (GAA) and 11 patients with *Anisakis* sensitization associated chronic urticaria (CU+). All patients were recruited prospectively within the same timeframe and in the same location (Madrid, Spain). GAA was diagnosed when a typical history (acute urticaria/angioedema or anaphylaxis of less than 48 h duration within 48 h of raw or undercooked fish intake) was accompanied by a further positive Skin Prick Test (SPT) and specific IgE against *Anisakis* $>$ 0.35 kU/l by ImmunoCAP™ (Phadia AB, Uppsala, Sweden). SPT was performed using *Anisakis simplex* extract (Lab. ALK-Abelló, Madrid, Spain) following standard protocols and was considered positive with a mean wheal diameter $\geq$ 3 mm. Histamine (1 %) and saline solution 0.9 % NaCl were considered as positive and negative controls, respectively. CU+ patients were included if recurrent wheals were present at least twice a week for at least six weeks [18] and displayed positive SPT and specific IgE against *Anisakis* $>$ 0.35 kU/l by ImmunoCAP™ (Phadia AB, Uppsala, Sweden). Patients were excluded from this study if they presented other conditions known to be associated with CU and if their urticarial reaction was elicited mainly by physical stimuli such as vibrations, pressure, cold or electromagnetic radiation. The second group of non-*Anisakis* sensitized patients was clinically subdivided into 17 chronic urticaria patients without SPT or specific IgE against *Anisakis* (CU-) and 18 healthy subjects without urticaria or currently *Anisakis*-associated symptoms (CONTROL). To minimize the possible bias, the control group was composed of voluntary subjects, sex balanced with respect to the patient groups. There was no follow-up, obtaining a single serum sample per subject. Written consent was obtained from all the study subjects. This project was approved by the Ethics Committee of the University Hospital La Princesa, Madrid with number PI-515–07/04/11.

2.2. Anti-Anisakis specific IgE, IgG4 and IgA

Specific antibody levels were measured by indirect ELISA. Plates (Costar, Corning, NY, USA) were sensitized with 10 μg/ml of larval CE or ES [19]. Wells were blocked and 100 μl of sera at 1/100 were added and incubated for 2 h. Horseradish peroxidase (HRP) conjugated goat anti-human IgA (Biosource International, Camarillo, CA, USA) or mouse anti-human IgG4-HRP (Clone HP6025) (Southern Biotech, Birmingham, AL, USA) were used for 1 h. Substrate (o-phenylene-diamine, OPD; Sigma-Aldrich, Germany) was added with hydrogen peroxide. The reaction was stopped with sulfuric acid and the plates were read at 490 nm.

For the IgE determination test, sera were added at 1/2 dilution. A murine monoclonal antibody (mAb) against an epsilon human IgE chain (IgG1κ, E21A11, INGENASA, Madrid, Spain) was added, followed by a HRP conjugated goat anti-mouse IgG1 (gamma) (Life Technologies, Grand Island, NY, USA) [14,20].

Antibodies against rAni s 1 (recombinant Ani s 1) and t-Ani s 7 (truncated Ani s 7) allergens were determined by indirect ELISA [10,21] using the Trisakis-170 kit [22].

Anti-*Anisakis* hemoglobin (Ani s 13) antibodies determination by indirect ELISA was carried out in plates (Costar) coated with 100 μl per well of 5 μg/ml nAni s 13 (native Ani s 13) [23]. The rest of the steps were carried out similarly (see above).

The levels of antibodies against *A. simplex* tropomyosin were determined by antigen-capture ELISA using a mAb anti-tropomyosin (Clone TM311; Sigma-Aldrich, Germany) [24]. The wells were coated with the mAb (1.5 μg/well) and were incubated with 500 μg/well of *Anisakis simplex* crude extract. Patient sera (diluted 1:2) were incubated, and a mouse anti-human IgE mAb HRP-labeled (clone B3102E8) (SouthernBiotech, Birmingham, Alabama) was added. The following steps were carried out similarly (see above). All the experiments were performed in duplicate.

2.3. Cytokine measurement

Serum cytokine levels (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, and IL-17A) were quantified using BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 and Human TGF-β1 kits, as indicated by the manufacturer (BD Biosciences, San Jose, CA, USA). All samples were analyzed with a BD FACSCalibur Flow Cytometer™ and the results were expressed in pg/ml using the FCAP Array™ software. The cytokine ratios were also calculated (Table 1).

2.4. Statistics

Median values and interquartile ranges (IQR) were calculated for specific antibody levels and cytokines and compared using the Mann-Whitney *U* test. Spearman's correlation coefficient was used for correlation studies. All statistical analyses were performed using the IBM SPSS Statistics Version 27. Significance was established at $p < 0.05$, 2-tailed.

3. Results

3.1. Serum antibody levels

Specific IgE levels were higher in the GAA group than the other groups (CU+, CU- and CONTROL) for all the antigens tested with the

Table 1
Cytokines and cytokine ratios measured in patients and controls.

Measurements in serum	
Cytokines	IFN-γ, TNF-α, TGF-β1, IL-2, IL-4, IL-6, IL-10, IL-17A
Th1/Th2 ratio	[IFN-γ + TNF-α]/IL-4 [41]
Th1/Th17 ratio	[IFN-γ + TNF-α]/IL-17A [42]
Th17/Th2 ratio	IL-17A/IL-4 [42]
Type 1/Type 2 ratio	[IL-2 + IFN-γ + TNF-α + IL-17A + IL-6]/[IL-4 + IL-10 + logTGF-β1] [42]
Other ratios	IFN-γ/IL-10 [42–46], IL-17A/IL-10 [42,47,48], IFN-γ/IL-4 [41,49–53], IL-6/IL-10 [54], TGF-β/IL-17A [52,55], TNF-α/IL-10 [56]

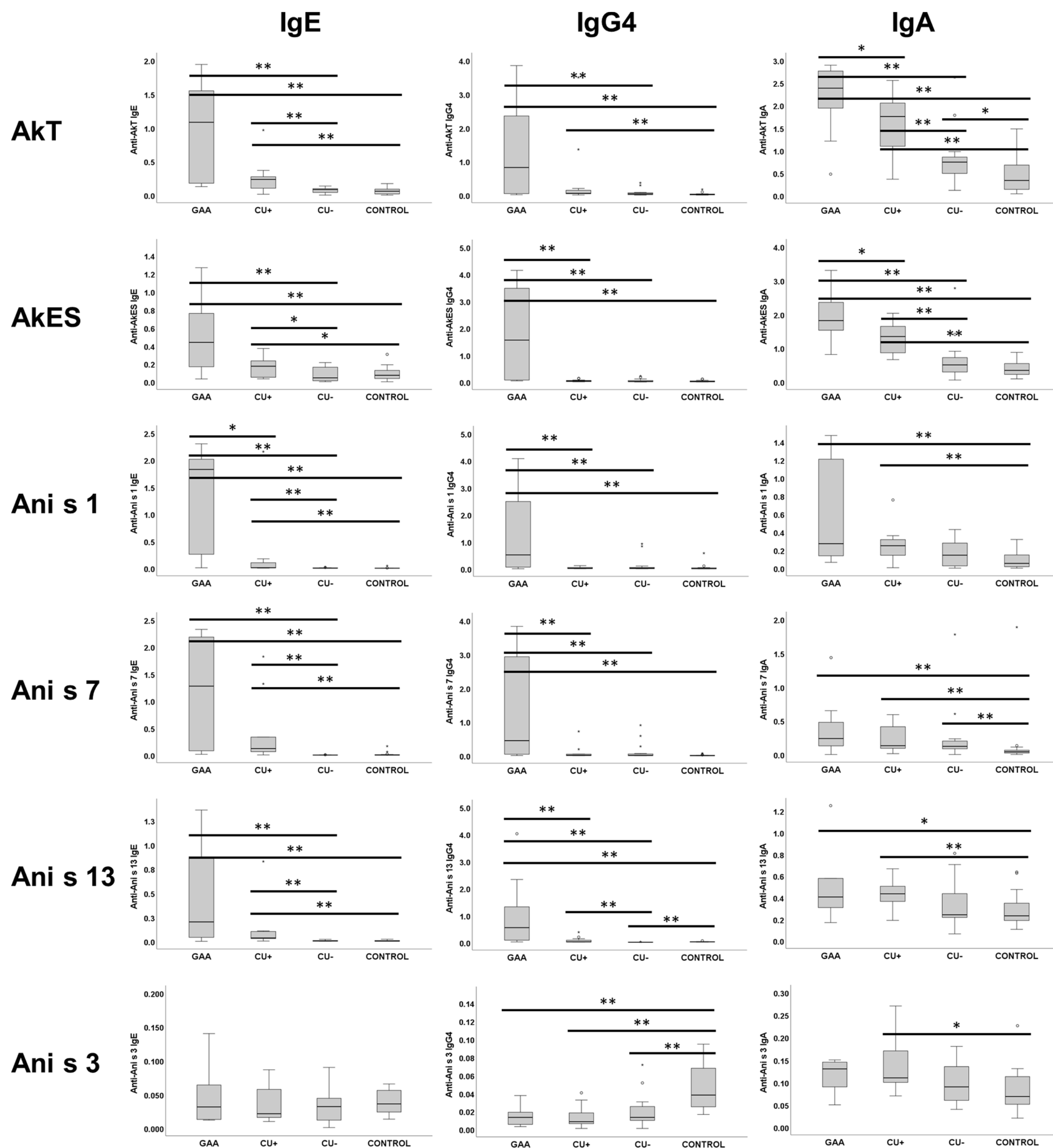

Fig. 1. Antibody levels against specific and complete *Anisakis* antigens in different *Anisakis* patients and control group. (*) $p < 0.05$. (**) $p < 0.01$. AkT: *Anisakis* total antigen; AkES: *Anisakis* Excretory-Secretory antigen; GAA: Gastro-Allergic Anisakiasis; CU+: *Anisakis simplex* sensitization-associated chronic urticaria; CU-: chronic urticaria without sensitization against *Anisakis simplex.*

exception of Ani s 3. Only in the case of anti-Ani s 1 IgE, the differences between GAA and CU+ reached statistical significance ($p < 0.05$) (Fig. 1).

Similarly, IgG4 levels were also the highest in the GAA group. Nevertheless, between GAA and CU+ groups we observed statistical differences in the case of Ani s 1, Ani s 7, Ani s 13 allergens and ES antigen. Noteworthy, against Ani s 3, the highest levels of IgG4 were observed in the control group ($p < 0.01$) (Fig. 1).

Anti-*Anisakis* IgA levels were highest in the GAA group, followed by CU+, CU- and finally the CONTROL group with minimal levels. However, the difference of IgA levels between the GAA and CU+ groups observed for complete antigens (total antigen and ES) ($p < 0.05$) was not present for any of the specific antigens, Ani s 1, Ani s 7, Ani s 13 and Ani s 3. Specific IgA levels were significantly higher in the CU+ with respect the CONTROL group for all the antigens tested ($p < 0.01$), even for Ani s 3 ($p < 0.05$) (Fig. 1).

After observing lower levels of IgG4 for CU+ patients against all antigens compared to IgA (Fig. 1), we calculated the ratio IgA/IgG4 for each serum and we observed higher levels of this ratio in CU+ than GAA group for Ani s 1, Ani s 7 ($p < 0.05$), Ani s 13 and ES ($p < 0.01$) (Fig. 2). This ratio was the lowest in the CONTROL group for Ani s 3 ($p < 0.01$) (Fig. 2). In particular, the ratio IgA/IgG4 against Ani s 13 was the highest in the CU- patients than the GAA and CONTROL groups ($p < 0.01$) (Fig. 2).

In the groups with chronic urticaria (CU), the levels of anti-Ani s 3 and anti-Ani s 13 IgG4 were significantly lower than in those without CU (NoCU) ($p < 0.01$). Concerning IgE and IgA, no differences were observed grouping sera by CU (eFigure 1).

IgE, IgG4 and IgA levels were higher in the *Anisakis*-positive sera for all the antigens tested ($p < 0.05$) except for anti-Ani s 3 IgE and IgA. In particular, serum anti-Ani s 3 IgA levels were lower in AK+ than AK- ($p < 0.01$) (eFigure 2).

Regarding the ratios, we observed that IgE/IgG4 and IgA/IgG4 values against the allergens Ani s 13 and Ani s 3, were higher in patients with CU than in subjects without chronic urticaria (eFigure 3).

Anisakis-positive patients (AK+) showed significantly higher values of IgE/IgG4 against the specific allergens Ani s 1, Ani s 7 and Ani s 13 ($p < 0.01$) and Ani s 3 ($p < 0.05$) (eFigure 4). Conversely, IgA/IgG4 values against Ani s 13 were lower in AK+ ($p < 0.01$). This tendency to show lower values of specific IgA/IgG4 in AK+ observed for all the antigens tested differed from the results obtained against Ani s 3, which presented higher values in AK+ ($p < 0.01$) (eFigure 4).

3.2. Serum cytokine levels

The highest serum concentrations of IL-10 and IFN-γ were observed in the CONTROL group. The differences reached statistical significance for IFN-γ compared to GAA, CU+ and CU- patients ($p < 0.01$) (Fig. 3A). For IL-10, the differences only reached statistical significance comparing with CU+ and CU- groups ($p < 0.05$ and $p < 0.01$, respectively). Serum concentrations of IL-10 were also lower in the GAA group, but the differences were not statistically significant (Fig. 3A).

GAA and CONTROL groups presented the highest concentrations of IL-17A compared with CU+ and CU- groups ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 3). Consequently, after re-grouping the levels of IL-10, IFN-γ and IL-17A in CU patients (CU+ and CU-) were significantly lower ($p < 0.01$) than in patients without CU (eFigure 5).

Ratio calculations are shown in the Table 1. Th1/Th17, TGF-β/IL17-A and IL-6/IL-10 are higher in CU groups ($p < 0.01$) (Fig. 3B and eFigure 6). However, IL17-A/IL-10 and Th17/Th2 ratios were lower in CU groups ($p < 0.01$) (Fig. 3B and eFigure 6).

3.3. Correlations cytokine and specific antibodies

Correlation analyses were performed between all cytokines and antibodies (eTable 2). To analyze the results, firstly we focused on those with a stronger association ($p < 0.01$) in GAA and CU+ groups.

In GAA, TNF-α showed a negative correlation with anti-Ani s 1 IgG4. TNF-α and Type 1/Type 2 showed a positive correlation with anti-Ani s 1 IgE/IgG4. The ratio IFN-γ/IL-4 was also positively correlated with anti-Ani s 1 IgA in this group of patients.

In CU+, we observed a negative correlation between the following pairs of cytokine *vs* antibody: IL-6 *vs* anti-Ani s 3 IgE; TNF-α and Th1/Th2 *vs* anti-AkT IgG4; TGF-β/IL-17A *vs* anti-Ani s 1 IgG4; TNF-α/IL10 *vs* anti-AkES IgG4.

IL-6, TNF-α, Th1/Th2 and Type 1/Type 2 presented a positive correlation with anti-ES IgE/IgG4 in patients diagnosed with CU+.

Analyzing the other groups with the same criteria of significance ($p < 0.01$), in the CU- group, a positive correlation was observed between IFN-γ and anti-ES IgG4 levels.

Th1/Th2 ratio in CU- and, in general, patients with CU correlated negatively with anti-Ani s 3 IgA.

IFN-γ/IL-10 ratio in CU- correlated positively with anti-Ani s 1 IgG4 levels and negatively in patients without CU (NoCU) with the ratio anti-Ani s 1 IgE/IgG4.

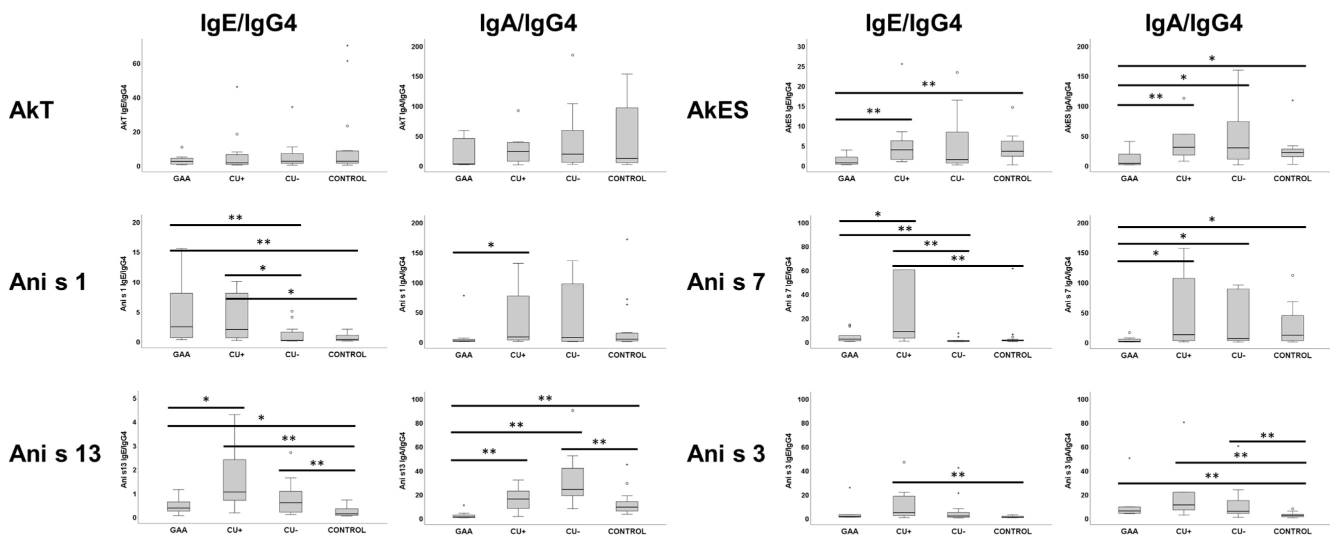

Fig. 2. Ratios of IgE, IgA and IgG4 levels against *Anisakis* antigens in different *Anisakis* patients and control group. (*) $p < 0.05$. (**) $p < 0.01$. AkT: *Anisakis* total antigen; AkES: *Anisakis* Excretory-Secretory antigen; GAA: Gastro-Allergic Anisakiasis; CU+: *Anisakis simplex* sensitization-associated chronic urticaria; CU-: chronic urticaria without sensitization against *Anisakis simplex*.

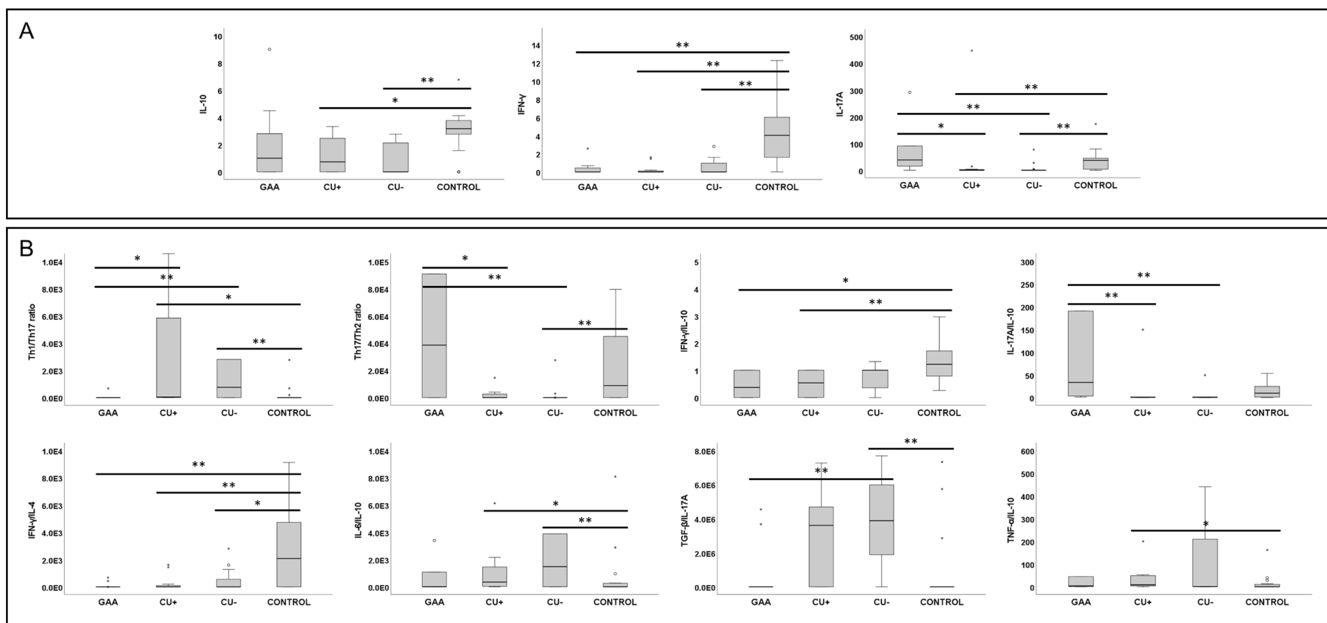

Fig. 3. A) Cytokine concentrations (pg/ml) in sera of different *Anisakis* patients and control group. B) Serum cytokine ratios of different *Anisakis* patients and control group. (*) $p < 0.05$. (**) $p < 0.01$. GAA: Gastro-Allergic Anisakiasis; CU+: *Anisakis simplex* sensitization-associated chronic urticaria; CU-: chronic urticaria without sensitization against *Anisakis simplex*.

In the groups of patients AK+, we observed a negative correlation of anti-Ani s 1 IgA levels and IL-10 concentrations. Likewise, a positive correlation was observed with the ratio IFN-γ/IL-10 ($p < 0.01$). Conversely, in the *Anisakis*-negative group (AK-), anti-Ani s 1 IgA correlated negatively with IL-17A/IL-10 ratio.

In general terms, it is worth noting that IFN-γ levels without dividing in groups showed negative correlation with most of the antibody levels measured but it was significant with anti-AkT IgA, anti-ES IgA, anti-Ani s 3 IgA, anti-Ani s 7 IgA and anti-Ani s 1 IgE ($p < 0.01$). Exceptionally, the IFN-γ levels showed a positive correlation with those of anti-Ani s 3 IgG4 ($p < 0.01$) (Data not shown).

Particularly, this negative correlation of IFN-γ levels was accentuated in the groups without chronic urticaria and it was significant with more different isotypes and *Anisakis* specific antigens. The IFN-γ levels maintained the exceptionality with a positive correlation with those of anti-Ani s 3 IgG4 ($p < 0.01$) (eTable 2).

Regarding the CONTROL group we observed positive correlation with IL-17A, Th17/Th2 ratio and Type 1/Type 2 ratio for the levels of anti-Ani s 13 IgA. In contrast, the correlation was negative with anti-Ani s 13 IgG4 (eFigure 7). Correlations of the IgA/IgG4 against the different antigens with the cytokines were analyzed (eFigure 8). The anti-Ani s 13 IgA/IgG4 ratio confirmed and enhanced the association of serum anti-Ani s 13 IgA with TNF-α, IL-17A, Th17/Th2 ratio, Type 1/Type2 ratio and TNF-α/IL-10 ratio in CONTROL group ($p < 0.01$) (eFigure 8).

4. Discussion

To our knowledge, this is the first study in which simultaneously serum cytokines and human relevant antibody isotypes such as IgE, IgG4 and serum IgA have been measured against *Anisakis* total and ES antigens as well as specific allergens like Ani s 1, Ani s 3, Ani s 7 and Ani s 13 in patients with the main *Anisakis* related disorders.

In GAA and CU+ patients, anti-*Anisakis* IgE, IgG4 and IgA levels are usually increased in comparison to healthy controls because of previous contact with ES antigens released by live larvae [2,14]. Indeed, they are serologically diagnosed by the presence of specific IgE against Ani s 7, which has been proposed as the current gold standard for the *in vitro* diagnosis of anisakiasis [21]. Our results were consistent with all previous studies, but with our dataset we could only differentiate anti-Ani s 1 IgE levels between GAA and CU+, but not using total *Anisakis* extract (anti-AkT IgE) [10]. Our control group showed similar mean levels of anti-*Anisakis* IgE (0.012), and IgA (0.454) measured at 490 nm as a previous study [16].

In this study, anti-Ani s 3 IgE did not show differences in any of the groups studied, indicating that *Anisakis* tropomyosin is a panallergen and not a specific *Anisakis* antigen. Anti-Ani s 3 IgG4 was significantly increased in the CONTROL group with respect to the GAA, CU+ and CU-. In fact, cross-reactions could be produced not only by the classical tropomyosins from shrimp or insects [25] but also by fish tropomyosins sensitizations in susceptible individuals [26].

CU is considered a multifactorial disease with an uncertain etiology, whose patients develop an immune response mediated by pro-inflammatory cytokines such as IL-6 or IL-17 [5]. Regarding the serum IgA, GAA and CU+ patients generated this isotype specifically against *Anisakis* larvae but interestingly, we observed differences in CU- with CONTROL group for the total extract (AkT) ($p < 0.05$) and Ani s 7 ($p < 0.05$). This fact is indicating that patients diagnosed with CU, even not being positive to *Anisakis*, presented specific serum IgA. Antigens from *Anisakis* larvae are ingested when customarily we eat marine fish. As it was mentioned in the introduction, the major production of IgA is located in the intestine but an impaired or inflamed mucosal barrier may be associated with increased serum levels. Indeed, in a study involving 55 patients with CU there was an increase in gastroduodenal permeability [27]. Additionally, the examination revealed bacterial infiltrations of *Proteobacteria* through the mucus and the intestinal mucosa epithelium [28]. In addition, it is known that IgG and IgA undergo the phenomenon of induced polyreactivity, *in vitro*. However, it was postulated that in an inflammatory context, *in vivo*, local concentrations of free heme, pro-oxidative ferrous ions and reactive oxygen species could modify the antigen binding characteristics of serum antibodies [29]. In the case of our CU patients (CU+ and CU-), but specifically in our CU- group, anti-*Anisakis* IgA detected in serum could be polyreactive antibodies because of their background inflammatory condition. The function of serum IgA in the mucosal system is presently not clear and why low production of IgA increases the risk of developing infections or a systemic immune disease such as CU [30].

Some studies have tried to find serum IgA as biomarker based on the hypothesis that it is associated with levels of activated TGF-β [31]. This cytokine is a co-stimulator for IgA production [32]. In our study the serum levels of this cytokine did not show significant differences between the groups, but a non-significant increase of TGF-β was observed in GAA and CU+ with respect to *Anisakis* negative groups (AK-), as observed in previous studies [4]. Probably the first contact with *Anisakis* by active penetration, necessary for sensitization, is sufficient to induce TGF-β production. However, the serum levels may not be adequate for its determination [5]. In addition, total serum IgA levels are increased with age in a range of 2 mg/dl per year and levels are higher in men than women [33]. Any sexual/age difference in total IgA levels and the subject-selection bias may have affected the present study but we consider our cohort is homogeneous in this respect. (N: 56, mean: 44.14; standard deviation: 14.5 years; 22 men 34 women) (eTable 1).

We also observed low levels of specific IgG4 in patients with CU, thus we calculated the IgA/IgG4 ratio. This novel ratio was surprisingly high in CU patients because their pathology could be the responsible for having higher levels of specific serum IgA than controls. On the one hand, serum IgA has been proposed to play a dual role in the immune system. It is involved in the maintenance of homeostasis and the promotion of anti-inflammatory responses, but it can also induce inflammation [8]. On the other hand, IgG4 appears in the literature as a protective isotype for allergies, but in helminthic parasites a low IgE/IgG4 ratio has been associated with parasite survival. In GAA, anti-*Anisakis* IgE and IgG4 are high, but not in CU+, where IgG4 is low [11]. We detected Ani s 3 and Ani s 13 were the antigens mainly involved in the increase of the ratio IgA/IgG4, maybe to be related with gut exposure as a classical allergen from inactivated (died) larvae in fish. In addition, Ani s 13 as heme rich protein, could be stimulating poly-reactive IgA liberating free heme groups during digestion [23,29].

In our CU patients, serum levels of IL-10, IFN-γ and IL-17A were significantly lower than in patients without CU (GAA and CONTROL groups). It is worth mentioning that GAA is accompanied by acute urticaria in presence of the live larvae and is a transient condition. Sera were obtained long after the acute episode, when GAA patients were not suffering an inflammatory state because the patients had previously expelled the larvae and they were asymptomatic at that time [3]. However, CU patients were suffering from active urticaria.

Th1 lymphocytes play a key role in the activation of B cells. They recognize antigens presented on the surface of macrophages and subsequently initiate the activation of these cells via the release of cytokines, such as IFN-γ or TNF-α. This cascade of events enhances the efficacy of macrophages in combating pathogens [34].

Helminthic infections induce a powerful stimulation of Th2 lymphocytes that activate undifferentiated B cells by means of cytokines such as IL-4, IL-10 or TGF-β. These cytokines stimulate their proliferation and induce isotype switching to IgG and IgE. These immunoglobulins constitute the effector molecules of the humoral response [35].

The Th2 subset is associated with the growth and persistence of parasites in the body, facilitating their evasion from the immune system. These cells increase the proliferation of B cells, in turn inhibiting the Th1 response. Consequently, in our patients, serum IFN-γ levels were inversely correlated with the antibody response with the exception of anti-Ani s 3 IgG4. McSorley et al. (2011) observed from two clinical trials that *Necator americanus*-induced hookworm disease in patients with celiac disease altered the Th1/Th2/Th17 balance by regulating the inflammatory response in the mucosa. Patients infected during the trial with hookworm larvae, showed anti-inflammatory responses in the duodenum. The basal Th1 and Th17 pro-inflammatory responses (IFN-γ and IL-17) were suppressed by the effect of IL-10 induced by the parasites. [36]. In our study, *Anisakis* positive groups (AK+: GAA and CU+) were expected to present higher levels of Th2 cytokines such as IL-10. However, as it was observed previously [4], control subjects presented more serum IL-10 than patients with chronic urticaria (CU+ and CU-), because they may be consuming this cytokine in the serum or it may not be released into the blood for, at the moment, still unknown reasons.

Recently, in addition to clinical trials with helminthic therapy, a new idea based on the intestinal microbiota has been proposed. It is intended to modify the gut microbiota with *Firmicutes* and *Bacteroides,* which are abundant in healthy subjects and are decreased in patients with CU. The authors aim to increase Tregs and decrease Th17 cells in blood and skin [37].

In the *Anisakis* negative groups (CU- and CONTROL), even though there are lower levels of specific antibodies anti-Ani s 13 IgA, these antibodies are correlated with pro-inflammatory cytokines as IL-17A, Th17/Th2 ratio and Type 1/Type 2 ratio. This fact may be indicating that dietary residues of dead L3 larvae of *Anisakis* could be associated with a pro-inflammatory environment. In this case IgA is not related to the classically view of IgA as a protective and anti-inflammatory isotype. Serum IgA immune complexes stimulate the production of pro-inflammatory cytokines by human macrophages and decreases the activation threshold of Toll-like receptors [38]. Recently, high levels of serum IgA were detected in patients with other inflammatory diseases [39,40].

In conclusion, this study demonstrates the important implication of serum IgA in the clinical phenotypes of *Anisakis* related diseases. Anti-*Anisakis* ES, Ani s 1, 7 and 13, but not Ani s 3, IgA/G4 ratios are increased in chronic urticaria with respect to GAA and CONTROLS. This ratio was particularly high in the case of Ani s 13 in chronic urticaria patients without *Anisakis* sensitization (CU-). We detected a clear low level of serum IL-17A and IFN-gamma in CU. In addition, in the AK+ groups, IgA/G4 ratio (targeting ES, Ani s 1, 7 and 13) allows us to differentiate patients with GAA from those with CU+. Our data indicate that anti-*Anisakis* serum IgA plays an important role in the recognition of *Anisakis* allergens, either through diet or through active infection of third stage larvae, shedding new light on the immunological mechanisms of *Anisakis*-related urticaria phenotypes.

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CRediT authorship contribution statement

Juan González-Fernández: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Laura Ullate:** Investigation. **Virginia Fernández-Fígares:** Investigation. **Marta Rodero:** Investigation. **Alvaro Daschner:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Carmen Cuéllar:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2024.111602.

References

[1] A. Daschner, C. Cuellar, M. Rodero, The Anisakis allergy debate: does an evolutionary approach help? Trends Parasitol. 28 (1) (2012) 9–15, https://doi.org/10.1016/j.pt.2011.10.001.

[2] A. Daschner, A. Alonso-Gómez, R. Cabañas, J.-M. Suarez-de-Parga, M.-C. López-Serrano, Gastroallergic anisakiasis: Borderline between food allergy and parasitic disease—Clinical and allergologic evaluation of 20 patients with confirmed acute parasitism by Anisakis simplex, J. Allergy Clin. Immunol. 105 (1) (2000) 176–181, https://doi.org/10.1016/s0091-6749(00)90194-5.

[3] A. Daschner, C.Y. Pascual, Anisakis simplex: sensitization and clinical allergy, Curr Opin. Allergy Clin. Immunol. 5 (3) (2005) 281–285, https://doi.org/10.1097/01.all.0000168795.12701.fd.

[4] A. Daschner, M. Rodero, C. de Frutos, A. Valls, F. Vega, C. Blanco, C. Cuellar, Different serum cytokine levels in chronic vs. acute Anisakis simplex sensitization-associated urticaria, Parasite Immunol. 33(6) (2011) 357-362. 10.1111/j.1365-3024.2011.01282.x.

[5] A. Daschner, V. Fernandez-Figares, A. Valls, C. de Frutos, M. Rodero, F.M. Ubeira, C. Cuellar, Different fish-eating habits and cytokine production in chronic urticaria with and without sensitization against the fish-parasite Anisakis simplex, Allergol. Int. 62 (2) (2013) 191–201, https://doi.org/10.2332/allergolint.12-OA-0504.

[6] Y. Li, L. Jin, T. Chen, The Effects of Secretory IgA in the Mucosal Immune System, Biomed Res. Int. 2020 (2020) 2032057, https://doi.org/10.1155/2020/2032057.

[7] K.W. Leong, J.L. Ding, The unexplored roles of human serum IgA, DNA Cell Biol. 33 (12) (2014) 823–829, https://doi.org/10.1089/dna.2014.2639.

[8] S.K. Davis, K.J. Selva, S.J. Kent, A.W. Chung, Serum IgA Fc effector functions in infectious disease and cancer, Immunol. Cell Biol. 98 (4) (2020) 276–286, https://doi.org/10.1111/imcb.12306.

[9] J. Fadlallah, H. El Kafsi, D. Sterlin, C. Juste, C. Parizot, K. Dorgham, G. Autaa, D. Gouas, M. Almeida, P. Lepage, N. Pons, E. Le Chatelier, F. Levenez, S. Kennedy, N. Galleron, J.-P.-P. de Barros, M. Malphettes, L. Galicier, D. Boutboul, A. Mathian, M. Miyara, E. Oksenhendler, Z. Amoura, J. Doré, C. Fieschi, S.D. Ehrlich, M. Larsen, G. Gorochov, Microbial ecology perturbation in human IgA deficiency, Sci. Transl. Med. 10 (439) (2018), https://doi.org/10.1126/scitranslmed.aan1217.

[10] C. Cuellar, A. Daschner, A. Valls, C. de Frutos, V. Fernandez-Figares, A.M. Anadon, E. Rodriguez, T. Garate, M. Rodero, F.M. Ubeira, Ani s 1 and Ani s 7 recombinant allergens are able to differentiate distinct Anisakis simplex-associated allergic clinical disorders, Arch. Dermatol. Res. 304 (4) (2012) 283–288, https://doi.org/10.1007/s00403-012-1206-8.

[11] A. Daschner, V. Fernandez-Figares, M. Rodero, A. Valls, C. de Frutos, F.M. Ubeira, C. Cuellar, Specific IgG4: possible role in the pathogenesis and a new marker in the diagnosis of Anisakis-associated allergic disease, Scand. J. Immunol. 79 (2) (2014) 120–126, https://doi.org/10.1111/sji.12129.

[12] J. Gonzalez-Fernandez, A. Daschner, N.E. Nieuwenhuizen, A.L. Lopata, C. de Frutos, A. Valls, C. Cuellar, Haemoglobin, a new major allergen of Anisakis simplex, Int. J. Parasitol. 45 (6) (2015) 399–407, https://doi.org/10.1016/j.ijpara.2015.01.002.

[13] N. Akao, T. Ohyama, K. Kondo, Immunoblot analysis of serum IgG, IgA and IgE responses against larval excretory-secretory antigens of Anisakis simplex in patients with gastric anisakiasis, J. Helminthol. 64 (4) (1990) 310–318, https://doi.org/10.1017/s0022149x00012359.

[14] A. Daschner, C. Cuellar, S. Sanchez-Pastor, C.Y. Pascual, M. Martin-Esteban, Gastro-allergic anisakiasis as a consequence of simultaneous primary and secondary immune response, Parasite Immunol 24 (5) (2002) 243–251, https://doi.org/10.1046/j.1365-3024.2002.00458.x.

[15] V. Zamora, C. Garcia-Ballesteros, C. Benet-Campos, F. Ballester, C. Cuellar, J.C. Andreu-Ballester, Anti-Anisakis sp. antibodies in serum of healthy subjects. Relationship with alphabeta and gammadelta T cells, Acta Parasitol 62(1) (2017) 97-103.10.1515/ap-2017-0011.

[16] J.C. Andreu-Ballester, V. Zamora, C. Garcia-Ballesteros, C. Benet-Campos, F. Lopez-Chulia, C. Tormo-Calandin, C. Cuellar, Anti-Anisakis sp. antibodies in serum of patients with sepsis and their relationship with gammadelta T cells and disease severity, Int. J. Parasitol. 48(6) (2018) 483-491. 10.1016/j.ijpara.2017.11.007.

[17] C. Cuellar, M. Rodero, J. Perez-Griera, L. Galindo-Regal, F. Lopez-Chulia, C. Garcia-Ballesteros, J. Carlos Andreu-Ballester, Association between anti-Anisakis simplex antibodies and interleukin-7 levels, Int. Immunopharmacol. 111 (2022), https://doi.org/10.1016/j.intimp.2022.109134.

[18] T. Zuberbier, A.H. Abdul Latiff, M. Abuzakouk, S. Aquilina, R. Asero, D. Baker, B. Ballmer-Weber, C. Bangert, M. Ben-Shoshan, J.A. Bernstein, C. Bindslev-Jensen, K. Brockow, Z. Brzoza, H.J. Chong Neto, M.K. Church, P.R. Criado, I.V. Danilycheva, C. Dressler, L.F. Ensina, L. Fonacier, M. Gaskins, K. Gaspar, A. Gelincik, A. Gimenez-Arnau, K. Godse, M. Goncalo, C. Grattan, M. Grosber, E. Hamelmann, J. Hebert, M. Hide, A. Kaplan, A. Kapp, A. Kessel, E. Kocaturk, K. Kulthanan, D. Larenas-Linnemann, A. Lauerma, T.A. Leslie, M. Magerl, M. Makris, R.Y. Meshkova, M. Metz, D. Micallef, C.G. Mortz, A. Nast, H. Oude-Elberink, R. Pawankar, P.D. Pigatto, H. Ratti Sisa, M.I. Rojo Gutierrez, S.S. Saini, P. Schmid-Grendelmeier, B.E. Sekerel, F. Siebenhaar, H. Siiskonen, A. Soria, P. Staubach-Renz, L. Stingeni, G. Sussman, A. Szegedi, S.F. Thomsen, Z. Vadasz, C. Vestergaard, B. Wedi, Z. Zhao, M. Maurer, The international EAACI/GA(2)LEN/EuroGuiDerm/APAAACI guideline for the definition, classification, diagnosis, and management of urticaria, Allergy 77(3) (2022) 734-766.10.1111/all.15090.

[19] M.J. Perteguer, R. Raposo, C. Cuellar, In vitro study on the effect of larval excretory/secretory products and crude extracts from Anisakis simplex on blood coagulation, Int. J. Parasitol. 26 (1) (1996) 105–108, https://doi.org/10.1016/0020-7519(95)00111-5.

[20] R. Gutierrez, C. Cuellar, Immunoglobulins anti-Anisakis simplex in patients with gastrointestinal diseases, J. Helminthol. 76 (2) (2002) 131–136, https://doi.org/10.1079/JOH2001104.

[21] A.M. Anadon, E. Rodriguez, M.T. Garate, C. Cuellar, F. Romaris, T. Chivato, M. Rodero, H. Gonzalez-Diaz, F.M. Ubeira, Diagnosing human anisakiasis: recombinant Ani s 1 and Ani s 7 allergens versus the UniCAP 100 fluorescence enzyme immunoassay, Clin. Vaccine Immunol. 17 (4) (2010) 496–502, https://doi.org/10.1128/CVI.00443-09.

[22] L. de Las Vecillas, P. Munoz-Cacho, M. Lopez-Hoyos, V. Monttecchiani, V. Martinez-Sernandez, F.M. Ubeira, F. Rodriguez-Fernandez, Analysis of Ani s 7 and Ani s 1 allergens as biomarkers of sensitization and allergy severity in human anisakiasis, Sci. Rep. 10 (1) (2020) 11275, https://doi.org/10.1038/s41598-020-67786-w.

[23] J. Gonzalez-Fernandez, L. Rivas, J.R. Luque-Ortega, R. Nunez-Ramirez, P. Campioli, T. Garate, M.J. Perteguer, A. Daschner, C. Cuellar, Recombinant vs native Anisakis haemoglobin (Ani s 13): Its appraisal as a new gold standard for the diagnosis of allergy, Exp. Parasitol. 181 (2017) 119–129, https://doi.org/10.1016/j.exppara.2017.08.010.

[24] J. Gonzalez-Fernandez, B. Veleiro, A. Daschner, C. Cuellar, Are fish tropomyosins allergens? Ann Allergy Asthma Immunol. 116 (1) (2016) 74–76, https://doi.org/10.1016/j.anai.2015.09.017, e5.

[25] N.E. Nieuwenhuizen, A.L. Lopata, Anisakis - a food-borne parasite that triggers allergic host defences, Int. J. Parasitol. 43 (12–13) (2013) 1047–1057, https://doi.org/10.1016/j.ijpara.2013.08.001.

[26] T. Ruethers, A.C. Taki, S. Karnaneedi, S. Nie, T. Kalic, D. Dai, S. Daduang, M. Leeming, N.A. Williamson, H. Breiteneder, S.S. Mehr, S.D. Kamath, D. E. Campbell, A.L. Lopata, Expanding the allergen repertoire of salmon and catfish, Allergy 76 (5) (2021) 1443–1453, https://doi.org/10.1111/all.14574.

[27] S. Buhner, I. Reese, F. Kuehl, H. Lochs, T. Zuberbier, Pseudoallergic reactions in chronic urticaria are associated with altered gastroduodenal permeability, Allergy 59 (10) (2004) 1118–1123, https://doi.org/10.1111/j.1398-9995.2004.00631.x.

[28] T. Lu, Y. Chen, Y. Guo, J. Sun, W. Shen, M. Yuan, S. Zhang, P. He, X. Jiao, Altered Gut Microbiota Diversity and Composition in Chronic Urticaria, Dis. Markers 2019 (2019) 6417471, https://doi.org/10.1155/2019/6417471.

[29] E.N. Gorshkova, M. Lecerf, I.V. Astrakhantseva, E.A. Vasilenko, O.V. Starkina, N. A. Ilyukina, P.A. Dimitrova, J.D. Dimitrov, T.L. Vassilev, Induced antigen-binding polyreactivity in human serum IgA, Immunobiology 227 (3) (2022) 152213, https://doi.org/10.1016/j.imbio.2022.152213.

[30] P.E. Conrey, L. Denu, K.C. O'Boyle, I. Rozich, J. Green, J. Maslanka, J.B. Lubin, T. Duranova, B.L. Haltzman, L. Gianchetti, D.A. Oldridge, N. De Luna, L.A. Vella, D. Allman, J.M. Spergel, C. Tanes, K. Bittinger, S.E. Henrickson, M.A. Silverman, IgA deficiency destabilizes homeostasis toward intestinal microbes and increases systemic immune dysregulation, Sci. Immunol. 8 (83) (2023) eade2335, https://doi.org/10.1126/sciimmunol.ade2335.

[31] L. Ten Klooster, C.H. van Moorsel, J.M. Kwakkel-van Erp, H. van Velzen-Blad, J. C. Grutters, Immunoglobulin A in serum: an old acquaintance as a new prognostic biomarker in idiopathic pulmonary fibrosis, Clin. Exp. Immunol. 181 (2) (2015) 357–361, https://doi.org/10.1111/cei.12636.

[32] P.H. Kim, M.F. Kagnoff, Transforming growth factor-beta 1 is a costimulator for IgA production, J. Immunol. 144 (9) (1990) 3411–3416, https://doi.org/10.4049/jimmunol.144.9.3411.

[33] W.J. Kim, I.S. Choi, C.S. Kim, J.H. Lee, H.W. Kang, Relationship between serum IgA level and allergy/asthma, Korean J. Intern. Med. 32 (1) (2017) 137–145, https://doi.org/10.3904/kjim.2014.160.

[34] E.A. Wierenga, M. Snoek, H.M. Jansen, J.D. Bos, R.A. van Lier, M.L. Kapsenberg, Human atopen-specific types 1 and 2 T helper cell clones, J. Immunol. 147 (9) (1991) 2942–2949, https://doi.org/10.4049/jimmunol.147.9.2942.

[35] A. Sher, R.T. Gazzinelli, I.P. Oswald, M. Clerici, M. Kullberg, E.J. Pearce, J. A. Berzofsky, T.R. Mosmann, S.L. James, H.C. Morse 3rd, Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection, Immunol. Rev. 127 (1992) 183–204, https://doi.org/10.1111/j.1600-065x.1992.tb01414.x.

[36] H.J. McSorley, S. Gaze, J. Daveson, D. Jones, R.P. Anderson, A. Clouston, N. E. Ruyssers, R. Speare, J.S. McCarthy, C.R. Engwerda, J. Croese, A. Loukas, Suppression of inflammatory immune responses in celiac disease by experimental hookworm infection, PLoS One 6 (9) (2011) e24092.

[37] M. Kristo, L. Lugovic-Mihic, M. Munoz, M. Rupnik, A. Mahnic, P. Ozretic, M. Jaganjac, D. Cesic, M. Kuna, Gut microbiome composition in patients with chronic urticaria: a review of current evidence and data, Life (Basel) 13 (2023), https://doi.org/10.3390/life13010152.

[38] I.S. Hansen, W. Hoepel, S.A.J. Zaat, D.L.P. Baeten, J. den Dunnen, Serum IgA immune complexes promote proinflammatory cytokine production by human macrophages monocytes, and Kupffer cells through FcalphaRI-TLR cross-talk, J. Immunol. 199 (12) (2017) 4124–4131, https://doi.org/10.4049/jimmunol.1700883.

[39] D. Abdukhakimova, K. Dossybayeva, Z. Almukhamedova, Z. Mukusheva, M. Assylbekova, D. Zhangabylova, K. Nurgaliyeva, N. Boluspayeva, K. Kulmangabetova, L. Hasanova, M. Tanko, D. Poddighe, Serum immunoglobulin A (IgA) levels in children affected with Juvenile Idiopathic Arthritis, Heliyon 9 (7) (2023) e17479.

[40] S. Tsuge, I. Mizushima, M. Horita, H. Kawahara, H. Sanada, M. Yoshida, Y. Takahashi, T. Zoshima, R. Nishioka, S. Hara, Y. Suzuki, K. Ito, M. Kawano, High serum IgA levels in patients with IgG4-related disease (IgG4-RD) are associated with mild inflammation, sufficient disease-specific features, and favorable responses to treatments, Mod. Rheumatol. (2023), https://doi.org/10.1093/mr/road056.

[41] S.L. Zhao, Z.H. Mo, H.H. He, L.L. Zhao, Y.H. Xie, Imbalance of T-helper 1/T-helper 2 cytokines and impaired glucose tolerance among patient with acute coronary syndrome, J. Cancer Res. Ther. 14 (Supplement) (2018) S480–S485, https://doi.org/10.4103/0973-1482.194346.

[42] A. Mouzaki, M. Rodi, N. Dimisianos, A. Emmanuil, D. Kalavrizioti, R. Lagoudaki, N. C. Grigoriadis, P. Papathanasopoulos, Immune Parameters That Distinguish Multiple Sclerosis Patients from Patients with Other Neurological Disorders at Presentation, PLoS One 10 (8) (2015) e0135434.

[43] Y. Ala, M.K. Pasha, R.N. Rao, P.L. Komaravalli, P. Jahan, Association of IFN-gamma : IL-10 Cytokine Ratio with Nonsegmental Vitiligo Pathogenesis, Autoimmune Dis 2015 (2015) 423490, https://doi.org/10.1155/2015/423490.

[44] Y.H. Ho, Y.C. Lu, H.C. Chang, S.Y. Lee, M.F. Tsai, Y.T. Huang, T.Y. Hsu, Daily intake of probiotics with high IFN-gamma/IL-10 ratio increases the cytotoxicity of human natural killer cells: a personalized probiotic approach, J. Immunol. Res. 2014 (2014) 721505, https://doi.org/10.1155/2014/721505.

[45] O.M. Espindola, L.C. Oliveira, P.M. Ferreira, A.C. Leite, M.A. Lima, M.J. Andrada-Serpa, High IFN-gamma/IL-10 expression ratio and increased frequency of persistent human T-cell lymphotropic virus type 1-infected clones are associated with human T-cell lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis development, Intervirology 58 (2) (2015) 106–114, https://doi.org/10.1159/000371766.

[46] B. Wei, C. Sheng Li, Changes in Th1/Th2-producing cytokines during acute exacerbation chronic obstructive pulmonary disease, J. Int. Med. Res. 46(9) (2018), 3890-3902. 10.1177/0300060518781642.

[47] J. Liu, H. Zhao, Y. Wang, Y. Shao, L. Zhang, M. Xing, Impacts of simultaneous exposure to arsenic (III) and copper (II) on inflammatory response, immune homeostasis, and heat shock response in chicken thymus, Int. Immunopharmacol. 64 (2018) 60–68, https://doi.org/10.1016/j.intimp.2018.08.021.

[48] J.M. Lee, H.J. Han, W.K. Choi, S. Yoo, S. Baek, J. Lee, Immunomodulatory effects of intraoperative dexmedetomidine on T helper 1, T helper 2, T helper 17 and regulatory T cells cytokine levels and their balance: a prospective, randomised, double-blind, dose-response clinical study, BMC Anesthesiol. 18 (1) (2018) 164, https://doi.org/10.1186/s12871-018-0625-2.

[49] V.R. Askari, V. Baradaran Rahimi, S.A. Rezaee, M.H. Boskabady, Auraptene regulates Th(1)/Th(2)/T(Reg) balances, NF-kappaB nuclear localization and nitric oxide production in normal and Th(2) provoked situations in human isolated lymphocytes, Phytomedicine 43 (2018) 1–10, https://doi.org/10.1016/j.phymed.2018.03.049.

[50] S. Deloer, R. Nakamura, M. Kikuchi, T. Moriyasu, Y.D.J. Kalenda, E.S. Mohammed, M. Senba, Y. Iwakura, H. Yoshida, S. Hamano, IL-17A contributes to reducing IFN-gamma/IL-4 ratio and persistence of Entamoeba histolytica during intestinal amebiasis, Parasitol. Int. 66 (6) (2017) 817–823, https://doi.org/10.1016/j.parint.2017.09.011.

[51] Y. Kim, S.H. Kang, T.H. Hong, M.L. Cho, H.J. Han, S.J. Kwon, J. Lee, Effects of dexmedetomidine on the ratio of T helper 1 to T helper 2 cytokines in patients undergoing laparoscopic cholecystectomy, J. Clin. Anesth. 26 (4) (2014) 281–285, https://doi.org/10.1016/j.jclinane.2013.11.018.

[52] H. Lu, Y. Huang, H. Xin, C. Hao, Y. Cui, The expression of cytokines IFN-gamma, IL-4, IL-17A, and TGF-beta1 in peripheral blood and follicular fluid of patients testing positive for anti-thyroid autoantibodies and its influence on in vitro fertilization and embryo transfer pregnancy outcomes, Gynecol. Endocrinol. 34 (11) (2018) 933–939, https://doi.org/10.1080/09513590.2018.1459546.

[53] Y. Wang, W. Yu, C. Shen, W. Wang, L. Zhang, F. Liu, H. Sun, Y. Zhao, H. Che, C. Zhao, Predictive Value of Serum IFN-gamma inducible Protein-10 and IFN-gamma/IL-4 Ratio for Liver Fibrosis Progression in CHB Patients, Sci. Rep. 7 (2017) 40404, https://doi.org/10.1038/srep40404.

[54] E.M. Bovee, M. Gulati, A.H. Maas, Novel Cardiovascular Biomarkers Associated with Increased Cardiovascular Risk in Women With Prior Preeclampsia/HELLP Syndrome A Narrative Review, Eur. Cardiol. 16 (2021) e36, https://doi.org/10.15420/ecr.2021.21.

[55] N. Joshi, R.W. Minz, S. Anand, N.V. Parmar, A.J. Kanwar, Vitamin D deficiency and lower TGF-beta/IL-17 ratio in a North Indian cohort of pemphigus vulgaris, BMC Res. Notes 7 (2014) 536, https://doi.org/10.1186/1756-0500-7-536.

[56] F.Y. Azizieh, K. Al Jarallah, D. Shehab, R. Gupta, K. Dingle, R. Raghupathy, Patterns of circulatory and peripheral blood mononuclear cytokines in rheumatoid arthritis, Rheumatol. Int. 37 (10) (2017) 1727–1734, https://doi.org/10.1007/s00296-017-3774-6.