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## Anti-*Anisakis* sp. antibodies in serum of patients with sepsis and their relationship with $\gamma\delta$ T cells and disease severity

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### ABSTRACT

Immunosuppression in sepsis reduces both  $\alpha\beta$  and  $\gamma\delta$  T cell subsets. *Anisakis* sp. is a parasitic nematode with a high prevalence in Spain. Previous contact with the parasite is related to a decrease in  $\gamma\delta$  T cells. Anti-*Anisakis* antibodies were measured and related to  $\alpha\beta$  and  $\gamma\delta$  T cells in 114 septic patients versus 97 healthy controls. Significant differences were seen with respect to the groups with severe sepsis and septic shock where lower anti-*Anisakis* levels were observed. A similar decrease appeared in the case of specific IgM with significant differences between the groups of control/uncomplicated sepsis versus severe sepsis and septic shock. These differences were also apparent in the case of specific IgA. The lowest IgE levels were detected in the septic shock group. Anti-*Anisakis* IgG levels significantly increased in septic shock groups compared with the controls. We observed positive correlations among anti-*Anisakis* IgA levels and all  $\gamma\delta$  T cell subsets. There were negative correlations among IgA levels and APACHE and SOFA indices. Greater contact with the parasite (IgG) was directly related with septic shock, inflammation and markers of sepsis severity. A lack of protection in the mucosa (IgA and  $\gamma\delta$  T cells) was associated with the disease severity.

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## 1. Introduction

Increasing evidence supports a central role for immunosuppression in sepsis. The initial immune response in sepsis is a hyperinflammatory state (cytokine storm), but it rapidly progresses to a hypoinflammatory response (immunosuppression) (Hotchkiss et al., 2013). This immune dysfunction may lead to increased host susceptibility to secondary opportunistic infections (Otto et al., 2011) or to reactivation of latent infections (Luyt et al., 2007; Limaye et al., 2008).

T cells are classified as  $\alpha\beta$  and  $\gamma\delta$  T cells depending on the type of T cell receptor (TCR) expressed on their surface. While  $\alpha\beta$  T cells are the most frequent in peripheral blood,  $\gamma\delta$  T cells represent only 5–10%. However,  $\gamma\delta$  T cells represent more than 50% of T cells in mucous membranes and are very common among intraepithelial T lymphocytes.  $\gamma\delta$  T cells have characteristics common to both innate and adaptive immune cells, and they are considered to be a first line of defence against common microorganisms (Chien

et al., 2014). We have recently demonstrated that immunosuppression in sepsis reduces both  $\alpha\beta$  and  $\gamma\delta$  T cell subsets, although  $\gamma\delta$  T cells showed the largest decrease and this reduction became more intense when the septic condition became more severe. Similarly, mortality was associated with a significant decrease in  $\gamma\delta$  T cells (Andreu-Ballester et al., 2013). We have also demonstrated a greater expression of IgE against the opportunistic agent *Encephalitozoon cuniculi* in septic patients which was, interestingly, related to a decrease in  $\alpha\beta$  and  $\gamma\delta$  T cells in peripheral blood (Andreu-Ballester et al., 2014). *Anisakis* sp. is a parasitic nematode that cause anisakiosis (Kassai et al., 1988) and whose seroprevalence is highly variable among different Spanish regions, with rates that range from 0.43% (Valiñas et al., 2001) to 22.1% (del Rey Moreno et al., 2006). In spite of the elevated rates of fish consumption in Spain, positivity is more prevalent among subjects who frequently consumed fresh and partially cooked fish than among consumers of frozen, boiled or baked fish (Puente et al., 2008). In a previous study (data not shown) we analysed the levels of anti-*Anisakis* antibodies in the serum of healthy persons. That study showed that previous contact with the parasite was related to a decrease in all  $\gamma\delta$  T cell subsets (Zamora et al., 2017).

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The aim of the present study was to analyse the levels of anti-*Anisakis* antibodies in the serum of septic patients for comparison with healthy persons as well as to determine whether the specific Ig levels were related to  $\alpha\beta$  and  $\gamma\delta$  T cells and the severity of the disease.

## 2. Materials and methods

### 2.1. Study population

In this retrospective study, sera from 114 patients who met the criteria for sepsis in our previous study were analysed (Andreu-Ballester et al., 2013). All patients were admitted to the Emergency Department of the Arnau de Vilanova Hospital and Intensive Care Unit of the Dr. Peset Aleixandre Hospital, both in Valencia (Spain). Sepsis was defined according to internationally established criteria, as well as its different stages: sepsis without organ failure and with organ failure (severe sepsis) (Levy et al., 2003; Dellinger et al., 2004). In addition, patients had to meet the following requirements: they were not suffering from immunodeficiency or autoimmune diseases, they had not been vaccinated in the last 6 months, and they were not undergoing immunosuppressive therapy. The control group (97 subjects) was recruited from relatives of patients admitted to hospital who were not relatives of the septic patients. They were required to have the same characteristics as the patients in addition to not suffering from any acute infectious diseases. Both patient and control groups were informed in writing of the study objectives and gave their authorization to participate in the study. In the case of patients with a significantly altered state of awareness, consent was obtained from their relatives. The Research and Ethics Committee of Arnau de Vilanova Hospital approved the study.

### 2.2. Methods of blood sample analysis

Whole blood was stained using direct immunofluorescence with the following monoclonal antibodies: CD45, CD4, CD8, CD56, CD2, CD3, CD19, TCR $\alpha\beta$  and TCR $\gamma\delta$ . Monoclonal antibodies were conjugated with FITC, phycoerythrin (PE), phycoerythrin-Texas red (ECD), and R-phycoerythrin-cyanine 5 (PC5).

The  $\gamma\delta$  T lymphocyte populations were analysed with PC5 conjugated anti-human  $\gamma\delta$  TCR (Beckman Coulter, Miami, USA (clone: IMM510)). The  $\alpha\beta$  T lymphocyte populations were analysed with PC5 conjugated anti-human  $\alpha\beta$  TCR (Beckman Coulter (clone: IP26A)). Fluorescence analysis was performed using a Beckman-Coulter multiparameter flow cytometry analyzer, Cytomics FC 500, Florida (USA) and later analysed with CXP Software. Miniums of 30,000 events were measured.

The level of C-reactive protein (CRP) was measured in the sera of patients with a heterogeneous enzymatic sandwich immunoassay with an end-point immunofluorescence reading method (Vitros Chemistry Products®, New Jersey, USA).

The erythrocyte sedimentation rate (ESR) was measured in TEST 1 (Alifax, Padova, Italy) using quantitative capillary photometry-based technology.

Procalcitonin was measured with an immunoassay “ECLIA” (electrochemiluminescence immunoassay), automated in a Cobas e601 analyser (Roche Diagnostics, Basel, Switzerland).

Lactate was measured with an ABL800 FLEX (Radiometer Copenhagen, Denmark) blood gas analyser by direct potentiometric electrolyte analysis.

### 2.3. *Anisakis* sp. antigen and determination of specific antibodies

L3s of *Anisakis* were extracted from blue whiting (*Micromesistius poutassou*) and were homogenised following sonication and extraction in PBS as previously described (García-Palacios et al., 1996).

ELISA plates (Costar, Corning, NY, USA) were sensitised by the addition of 10  $\mu\text{g mL}^{-1}$  of larval antigen. Human sera at 1/100 in PBS-Tween containing 0.1% BSA were added and incubated. Horseradish peroxidase (HRP) conjugated goat anti-human Igs, (IgM, IgG or IgA; Biosource International, Camarillo, CA, USA) were used (Daschner et al., 2002; Gutiérrez et al., 2002).

For the IgE determination test, sera were added at a 1/2 dilution. A murine monoclonal antibody against an epsilon human IgE chain (IgG1 $\kappa$ , E21A11, INGENASA, Madrid, Spain) was added and incubated, followed by a HRP conjugated goat anti-mouse IgG1 (gamma) (Life Technologies, Grand Island, NY, USA) (Gutiérrez and Cuéllar, 2002; Martínez de Velasco et al., 2006).

The anti-*Anisakis* antibody levels were measured in septic patients at the time of admission without any previous treatment.

In order to carry out the comparison of the quantitative variable mean values, subjects were divided into two groups according to their anti-*Anisakis* antibody levels. Values were considered positive if they were higher than the mean of the O.D. of 211 studied sera obtained for each Ig plus one respective S.D.

### 2.4. Statistical analysis

When normality was assumed (Kolmogorov–Smirnov test), *t* or ANOVA tests were used to compare the quantitative variable mean values. When the hypothesis of normality for the quantitative variable was not accepted, the Mann–Whitney *U* test was used. Correlation studies using Pearson’s Correlation Coefficient were performed to compare Ig levels and T cell subsets.

The frequencies of cell subsets did not show a normal distribution. When values were transformed into Napierian logarithms, they came to the normal distribution with the exception of the B cells ( $P = 0.40$ ). Normal values of the population were calculated using the equation: Exponent (mean Napierian logarithm  $\pm 1.96 \times$  S.D. Napierian logarithm) (Table 1).  $P < 0.05$  was considered significant. Data were analysed using the statistical software SPSS, version 19 (SPSS Inc., Chicago, USA).

## 3. Results

Tables 2 and 3 show the characteristics of patients with sepsis. There were no differences between the mean ages of the septic patients ( $65.5 \pm 20.9$ ) and the control group ( $63.1 \pm 21.2$ ) ( $P = 0.413$ ). Sixty-three out of 97 healthy controls were male (64.9%) and 34 were female (35.1%) versus 67 (58.8%) and 47 (41.2%), respectively, in the group of patients with sepsis.

### 3.1. Anti-*Anisakis* sp. antibody levels according to severity of sepsis

When we determined the anti-*Anisakis* antibodies by ELISA, the levels of total Igs of the control group and the group with sepsis without organ complications were similar. However, significant differences were seen with respect to the groups of patients with severe sepsis ( $P = 0.001$ ) and septic shock ( $P < 0.0001$ ), where lower antibody levels were observed. A similar decrease was observed in the case of anti-*Anisakis* specific IgM. There were significant differences between the groups of control/uncomplicated sepsis versus severe sepsis ( $P = 0.016$ ) and septic shock ( $P = 0.002$ ). These differences also appeared in the case of specific IgA ( $P = 0.004$  and  $P < 0.0001$ , respectively) (Fig. 1A). With regard to IgE, the lowest levels were detected in the case of the septic shock group ( $P = 0.003$ ) (Fig. 1B).

When we measured the levels of anti-*Anisakis* IgG, the results differed from those obtained when the other above-mentioned isotypes were tested. The values of anti-*Anisakis* IgG significantly increased in patients with septic shock compared with the control

**Table 1**  
Normal values and reference range<sup>a</sup> of T cell subsets/mm<sup>3</sup>.

| T cell subset                                        | Mean   | Median | S.D.  | Reference range |
|------------------------------------------------------|--------|--------|-------|-----------------|
| CD3+                                                 | 1487.3 | 1482.0 | 447.3 | 720.5–3070.1    |
| CD3+CD4+                                             | 927.0  | 927.2  | 519.6 | 408.2–2105.2    |
| CD3+CD8+                                             | 583.8  | 580.0  | 586.8 | 236.2–1443.2    |
| CD3+CD56+                                            | 223.1  | 232.1  | 158.8 | 49.3–1008.5     |
| CD3+ $\alpha\beta$                                   | 1363.6 | 1336.2 | 457.4 | 651.7–2853.5    |
| CD3+CD4+ $\alpha\beta$                               | 880.3  | 904.6  | 545.2 | 375.1–2065.8    |
| CD3+CD8+ $\alpha\beta$                               | 432.8  | 455.1  | 797.1 | 137.2–1365.7    |
| CD3+CD56+ $\alpha\beta$                              | 44.6   | 50.0   | 262.8 | 4.3–453.0       |
| CD3+ $\gamma\delta$                                  | 45.6   | 45.2   | 603.2 | 7.0–297.8       |
| CD3+CD4 <sup>−</sup> CD8 <sup>−</sup> $\gamma\delta$ | 31.7   | 35.0   | 769.8 | 4.3–234.0       |
| CD3+CD8+ $\gamma\delta$                              | 11.2   | 10.6   | 899.0 | 1.3–90.6        |
| CD3+CD56+ $\gamma\delta$                             | 5.4    | 6.4    | 59.1  | 0.3–85.1        |
| CD19+                                                | 181.3  | 206.2  | 7.1   | 46.2–710.5      |

<sup>a</sup> Reference range of T and B cells in healthy adult subjects ( $n = 157$ ) were from a previous study (Andreu-Ballester et al., 2012).

**Table 2**  
General characteristics of 114 patients with sepsis in the study (age, sex, scores and biomarkers).

| Criteria/Measurement        | Mean $\pm$ S.D.     |
|-----------------------------|---------------------|
| <b>Age – years</b>          | 65.5 $\pm$ 20.9     |
| APACHE II score             | 13.8 $\pm$ 7.1      |
| SOFA score                  | 4.4 $\pm$ 3.7       |
| C-reactive protein          | 205 $\pm$ 131       |
| Procalcitonin               | 18.5 $\pm$ 23.4     |
| Lactic acid                 | 2.1 $\pm$ 1.5       |
|                             | No. (%) of patients |
| <b>Sex</b>                  |                     |
| Male                        | 67 (58.8)           |
| Female                      | 47 (41.2)           |
| <b>Emergency Department</b> | 61 (53.5)           |
| <b>Intensive Care Unit</b>  | 53 (46.5)           |

**Table 3**  
Details of 114 patients with sepsis in the study (diagnosis, stages of sepsis, organ failure).

|                                | No. (%) of patients |
|--------------------------------|---------------------|
| <b>Diagnosis</b>               |                     |
| Pneumonia                      | 53 (46.5)           |
| Urinary Tract Infections       | 24 (21.0)           |
| Acute Appendicitis             | 6 (5.3)             |
| Acute Cholecystitis            | 6 (5.3)             |
| Acute Cholangitis              | 4 (3.5)             |
| Undetermined                   | 3 (2.6)             |
| Acute Diverticulitis           | 3 (2.6)             |
| Meningitis                     | 3 (2.6)             |
| Enterocolitis                  | 2 (1.8)             |
| Others <sup>a</sup>            | 10 (8.8)            |
| <b>Stages of sepsis</b>        |                     |
| Sepsis                         | 40 (35.1)           |
| Severe Sepsis                  | 50 (43.9)           |
| Septic Shock                   | 24 (21.1)           |
| Positive Cultures              | 48 (42.1)           |
| In-hospital death              | 19 (16.7)           |
| <b>Organ failure</b>           |                     |
| Acute Respiratory Failure      | 43 (37.7)           |
| Acute Renal Failure            | 34 (29.8)           |
| Neurologic                     | 30 (26.3)           |
| Shock                          | 24 (21.1)           |
| Metabolic                      | 22 (19.3)           |
| Acute Hepatic Failure          | 18 (15.8)           |
| Haematologic                   | 18 (15.8)           |
| <b>No. organs with failure</b> |                     |
| 0                              | 44 (38.6)           |
| 1                              | 16 (14.0)           |
| 2                              | 20 (17.5)           |
| 3                              | 18 (15.8)           |
| 4 or more                      | 16 (14.0)           |

<sup>a</sup> One patient had each of the following diagnoses: abscess, cellulitis, pelvic inflammatory disease, gangrene, malaria, intestinal ischaemia, intestinal obstruction, acute pancreatitis, primary spontaneous peritonitis and septic arthritis.

group ( $P = 0.038$ ) (Fig. 1C). Similarly, the percentages of positives for anti-*Anisakis* IgG increased according to the severity of the disease (Fig. 1D).

### 3.2. Correlations among anti-*Anisakis* sp. antibody levels with T cell subsets and severity markers

We observed positive correlations among the anti-*Anisakis* IgA antibody levels and all  $\gamma\delta$  T cell subsets as follows (Pearson's Correlation): CD3+  $\gamma\delta$ , CD3+CD8+  $\gamma\delta$ , CD3+CD56+  $\gamma\delta$  (Fig. 2A). However, there were negative correlations among the anti-*Anisakis* IgA antibody levels and the time before admission, APACHE index and SOFA index (Fig. 2B). Similarly, in the case of the anti-*Anisakis* IgM antibody levels, negative correlations were observed with procalcitonin, APACHE index and SOFA index (Fig. 3B). On the other hand, the anti-*Anisakis* IgG antibody levels showed a positive correlation with lactic acid, procalcitonin, and CRP (Fig. 3A).

### 3.3. Deficit of T cell subsets and anti-*Anisakis* sp. antibodies in septic patients

In previous work, we defined normal reference values in healthy subjects for T lymphocytes for both types of receptors,  $\alpha\beta$  and  $\gamma\delta$ , and their subsets (Andreu-Ballester et al., 2012). A description of the reference ranges for B cell and T cell subsets can be found in Table 1. Based on these values we have analysed the differences in the anti-*Anisakis* antibody levels with respect to the deficit of  $\alpha\beta$  and  $\gamma\delta$  T cells in septic patients (Table 4). When variations of anti-*Anisakis* total Ig levels were analysed, we observed a significant decrease for both CD3+CD8+ and CD3+CD56+  $\gamma\delta$  populations' deficits. This fact was only observed for CD3+CD4+  $\alpha\beta$  cells in the case of anti-*Anisakis* IgM levels. The

lowest levels of anti-*Anisakis* IgA antibodies were observed in patients with deficits of  $\alpha\beta$  (CD4+ and CD8+) and all the  $\gamma\delta$  T cells. These significant differences were more patent in the case of the CD3+CD8+  $\gamma\delta$  and CD3+CD56+  $\gamma\delta$  subsets. No differences were seen for either anti-*Anisakis* IgG or IgE isotypes. Deficiency of  $\alpha\beta$  T cells and  $\gamma\delta$  T cells subsets is shown in Fig. 4.

### 3.4. Relationship between duration of sepsis infection and anti-*Anisakis* sp. antibody levels and T cell subsets

Peripheral blood  $\gamma\delta$  T cell subsets in relation to time since onset of infection/sepsis on admission to Emergency/Intensive Care Unit

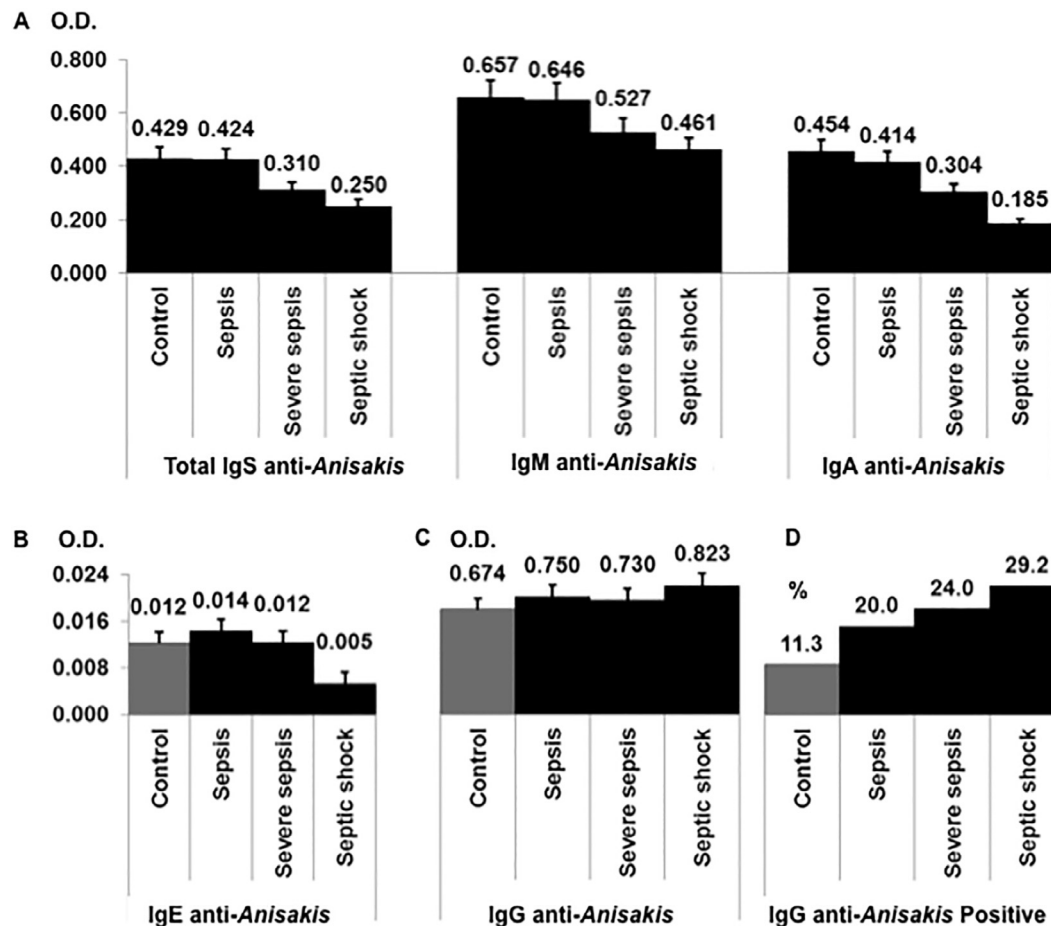


**Fig. 1.** Levels of patients' anti-*Anisakis* sp. antibodies according to severity of sepsis (A–C), and positive (%) IgG anti-*Anisakis* (D). Control ( $n = 97$ ), sepsis ( $n = 40$ ), severe sepsis ( $n = 50$ ), septic shock ( $n = 24$ ). (A) Total IgS anti-*Anisakis*: significant differences between control-sepsis group and severe sepsis group,  $P = 0.001$ ; significant differences between sepsis and septic shock groups,  $P < 0.0001$ . IgM anti-*Anisakis*: significant differences between control-sepsis group and severe sepsis group,  $P = 0.016$ ; significant differences between control-sepsis group and septic shock group,  $P = 0.002$ . IgA: significant differences between control-sepsis group and severe sepsis group,  $P = 0.004$ ; significant differences between control-sepsis group and septic shock group,  $P < 0.0001$ . (B) IgE: significant differences between septic shock and others groups,  $P = 0.003$ . (C) IgG, significant differences between septic shock and others groups,  $P = 0.038$ .

departments are shown in Fig. 5A. When we studied the levels of anti-*Anisakis*-specific antibodies based on the duration of the sepsis infection, a significant decrease was observed in the anti-*Anisakis* IgA values from the third day onwards ( $P = 0.003$ ). This decrease correlated with the decrease in  $\gamma\delta$  T cell subsets (Fig. 5B).

### 3.5. Relationship between anti-*Anisakis* sp. antibody levels and positive cultures of biological fluids

Forty-eight out of 114 septic patients (42.1%) showed positive cultures for microorganisms. The anti-*Anisakis* IgG levels were significantly higher in the group of patients who presented positive haemocultures ( $n = 26$ ) compared with the negative group ( $0.940 \pm 0.295$  versus  $0.702 \pm 0.289$ ,  $P < 0.001$ ) (Fig. 5C). No differences were seen in the case of other positive cultures (urine, exudates, etc.). There were no significant differences in the case of anti-*Anisakis* IgM, IgE and IgA when antibody levels were compared between positive and negative haemocultures. On studying microorganisms after Gram staining, we observed higher serum levels of IgM and IgA in the case of Gram negative (GN) bacteria compared with Gram positive (GP). In this way, the anti-*Anisakis* IgM mean values in GN were  $0.627 \pm 0.301$  versus  $0.465 \pm 0.317$  in GP,  $P = 0.210$ ; and the anti-*Anisakis* IgA mean values in GN were  $0.439 \pm 0.283$  versus  $0.243 \pm 0.163$  in GP,  $P = 0.016$  (Fig. 5D).

### 3.6. Mortality

The rate of mortality from sepsis observed in our study was 16.6% (19/114). The relationship of T cell subset deficit with mortality is described in Table 5.  $\gamma\delta$  T cell deficits, and mainly the CD56+  $\gamma\delta$  T cell subset, were clearly related to mortality. Thus, patients who present a deficit of CD56+  $\gamma\delta$  T cells during the progressing septic infection have a probability of dying almost eight times higher than that of patients without this deficit. There were no significant differences in the case of anti-*Anisakis*-specific antibodies with respect to mortality from sepsis. However, the levels of anti-*Anisakis* IgG were lower in the group of patients who died ( $0.270 \pm 0.151$ ) compared with the group of patients who survived ( $0.350 \pm 0.210$ ) ( $P = 0.061$ ). The same fact was observed in the case of the anti-*Anisakis* IgE levels, which were lower in the group of dead ( $0.007 \pm 0.006$ ) versus survivors ( $0.012 \pm 0.022$ ) ( $P = 0.077$ ).

## 4. Discussion

This paper describes, for the first known time, a relationship between anti-*Anisakis* antibodies and sepsis in human patients. Experimental studies carried out in rabbits showed that anti-*Anisakis* IgG antibodies reached their maximum levels 25–30 days after the infection with larvae (Hong and Lee, 1987; Yang et al., 1991). The presence of IgG against *Anisakis* antigens in inoculated BALB/

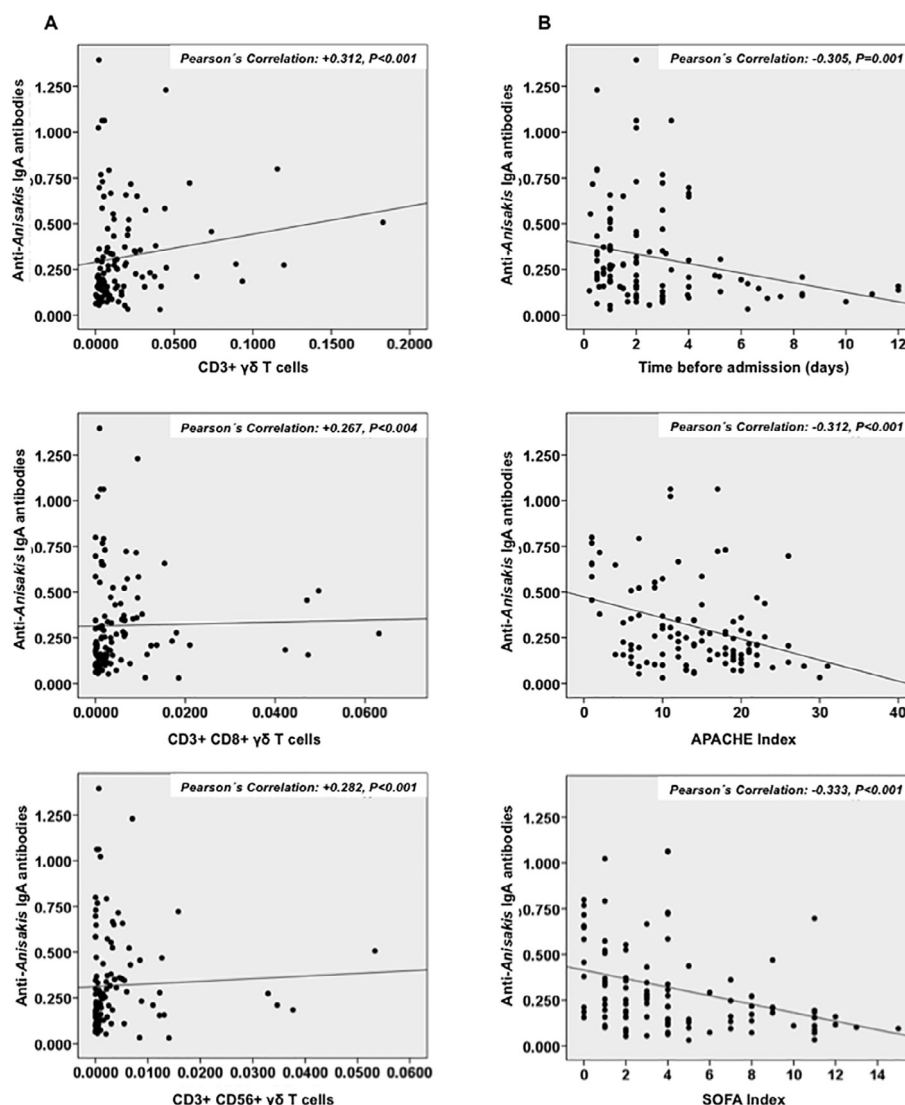


Fig. 2. Anti-*Anisakis* sp. IgA correlations with  $\gamma\delta$  T cell subsets (A) and time before admission, APACHE and SOFA indices (B) in patients with sepsis.

c mice has also been demonstrated (Vericimo et al., 2015). In humans, IgA and IgE responses to larval antigens were a reliable index of infection (Akao et al., 1990). However, Rodero et al. (2006) observed that IgG was the most abundant Ig in sera from patients with *Anisakis* sensitization. In the present study, anti-*Anisakis* IgG levels were higher in relation to the severity of the sepsis, rising to nearly 30% of positives in septic shock patients. This suggests that patients who have previously been exposed to the parasite could present a more severe septic clinical picture. The increase in anti-*Anisakis* IgG in our septic patients could indicate a primary infection or reinfections produced by new contacts with larvae through the ingestion of parasitized fish related with the transient immunodeficiency produced in sepsis. Dowling et al. (2002) suggested the possibility that nematodes which penetrate the gut, such as *Ascaris* and *Strongyloides*, play a role in invasive, non-typhoidal *Salmonella* infections in HIV-positive patients. Salgame et al. (2013) discussed the influence of alternatively activated macrophages and tolerogenic dendritic cells derived from helminth immune responses on the protective responses against infectious diseases. Elias et al. (2006) concluded that intestinal helminth infection is a risk factor for the development of active tuberculosis. George et al. (2012, 2014) showed that helminth infections modulate the Th1 and Th17 responses to mycobacterial-antigens

in latent tuberculosis in a process dependent on IL10. They also previously demonstrated that patients infected with hookworms display microbial translocation. Helminths can induce intestinal mastocytosis, which reduces the expression of tight junction molecules, thereby increasing intestinal permeability and therefore causing endotoxin translocation in infected patients (Farid et al., 2008). Hübner et al. (2013) revised the impact of helminths and their products on endotoxaemia and challenge with bacterial species, although, on the other hand, they suggested that some helminths may prevent excessive inflammatory immune responses, the development of coagulation and improve sepsis pathology. However, other helminths could impair the initial bacterial control, facilitating bacterial replication and systemic translocation, leading to an increased risk of mortality.

It is interesting that anti-*Anisakis* IgG antibodies are directly related with the levels of inflammation markers (CRP and procalcitonin), as well as with a severity marker (lactic acid). This fact suggests a relationship between inflammation markers and previous contacts with *Anisakis* larvae. In addition, higher anti-*Anisakis* IgG levels were associated with a greater severity of the sepsis, and with positive blood cultures. This is probably due to increased  $\gamma\delta$  T cell deficiency in these patients (Andreu-Ballester et al., 2013). These cells are central to the mucosal defence system and their

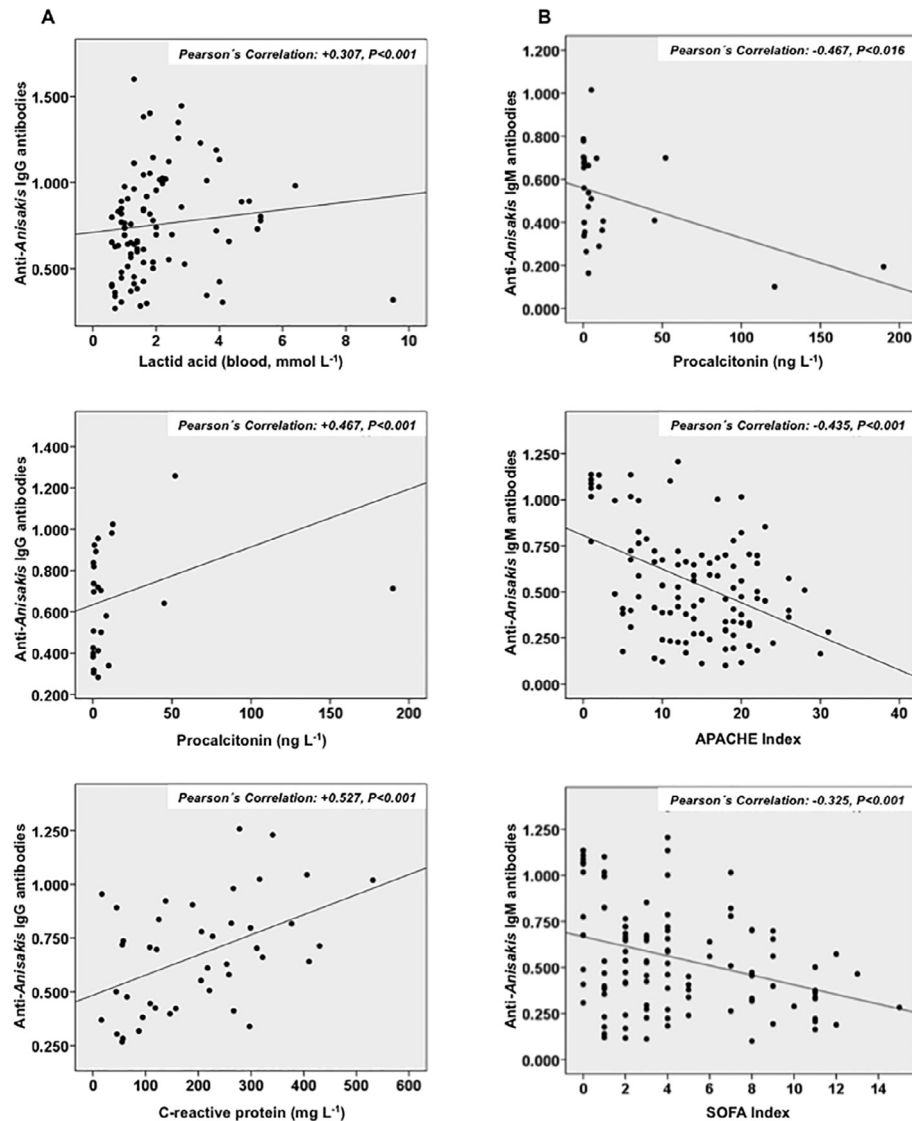


**Fig. 3.** Anti-*Anisakis* sp. IgG (A) and IgM (B) correlations with biomarkers and APACHE and SOFA indices in patients with sepsis. CRP, C reactive protein.

**Table 4**

Differences among means of anti-*Anisakis* sp. antibodies according to deficits in  $\alpha\beta$  and  $\gamma\delta$  T cell subsets in all patients with sepsis, using the Mann–Whitney *U* test.

| T cells subset              | Deficit | N (%) | Total Ig           |       | IgG   |       | IgM                |       | IgE   |       | IgA                |       |
|-----------------------------|---------|-------|--------------------|-------|-------|-------|--------------------|-------|-------|-------|--------------------|-------|
|                             |         |       | Mean               | S.D.  | Mean  | S.D.  | Mean               | S.D.  | Mean  | S.D.  | Mean               | S.D.  |
| CD3+ $\alpha\beta$ 83       | NO      |       | 0.351              | 0.202 | 0.741 | 0.281 | 0.567              | 0.257 | 0.017 | 0.033 | 0.352              | 0.252 |
|                             | YES     |       | 0.332              | 0.205 | 0.762 | 0.317 | 0.550              | 0.306 | 0.009 | 0.014 | 0.305              | 0.272 |
| CD3+CD4+ $\alpha\beta$ 74   | NO      |       | 0.377              | 0.219 | 0.737 | 0.284 | 0.620 <sup>a</sup> | 0.280 | 0.016 | 0.030 | 0.370 <sup>a</sup> | 0.272 |
|                             | YES     |       | 0.315              | 0.192 | 0.767 | 0.319 | 0.520 <sup>a</sup> | 0.295 | 0.009 | 0.014 | 0.289 <sup>a</sup> | 0.260 |
| CD3+CD8+ $\alpha\beta$ 60   | NO      |       | 0.365              | 0.208 | 0.813 | 0.310 | 0.568              | 0.289 | 0.015 | 0.028 | 0.364 <sup>a</sup> | 0.290 |
|                             | YES     |       | 0.312              | 0.197 | 0.706 | 0.297 | 0.543              | 0.298 | 0.008 | 0.010 | 0.275 <sup>a</sup> | 0.237 |
| CD3+CD56+ $\alpha\beta$ 25  | NO      |       | 0.318              | 0.186 | 0.758 | 0.301 | 0.548              | 0.286 | 0.012 | 0.023 | 0.294              | 0.230 |
|                             | YES     |       | 0.404              | 0.248 | 0.752 | 0.332 | 0.580              | 0.321 | 0.009 | 0.010 | 0.404              | 0.363 |
| CD3+ $\gamma\delta$ 48      | NO      |       | 0.347              | 0.208 | 0.749 | 0.313 | 0.549              | 0.296 | 0.014 | 0.026 | 0.329 <sup>a</sup> | 0.227 |
|                             | YES     |       | 0.323              | 0.199 | 0.767 | 0.300 | 0.563              | 0.292 | 0.008 | 0.009 | 0.301 <sup>a</sup> | 0.315 |
| CD3+CD4-CD8- $\gamma\delta$ | NO      |       | 0.342              | 0.202 | 0.749 | 0.304 | 0.540              | 0.295 | 0.016 | 0.028 | 0.330 <sup>a</sup> | 0.222 |
|                             | YES     |       | 0.332              | 0.206 | 0.764 | 0.311 | 0.569              | 0.292 | 0.008 | 0.009 | 0.306 <sup>a</sup> | 0.305 |
| CD3+CD8+ $\gamma\delta$ 42  | NO      |       | 0.368 <sup>b</sup> | 0.208 | 0.771 | 0.318 | 0.583              | 0.314 | 0.012 | 0.018 | 0.340 <sup>b</sup> | 0.240 |
|                             | YES     |       | 0.283 <sup>b</sup> | 0.185 | 0.731 | 0.287 | 0.506              | 0.247 | 0.012 | 0.025 | 0.278 <sup>b</sup> | 0.305 |
| CD3+CD56+ $\gamma\delta$ 84 | NO      |       | 0.351 <sup>b</sup> | 0.233 | 0.768 | 0.282 | 0.565              | 0.304 | 0.016 | 0.024 | 0.387 <sup>b</sup> | 0.254 |
|                             | YES     |       | 0.332 <sup>b</sup> | 0.193 | 0.753 | 0.316 | 0.551              | 0.290 | 0.010 | 0.020 | 0.293 <sup>b</sup> | 0.268 |

<sup>a</sup>  $P = 0.040$ .

<sup>b</sup>  $P = 0.005$ .

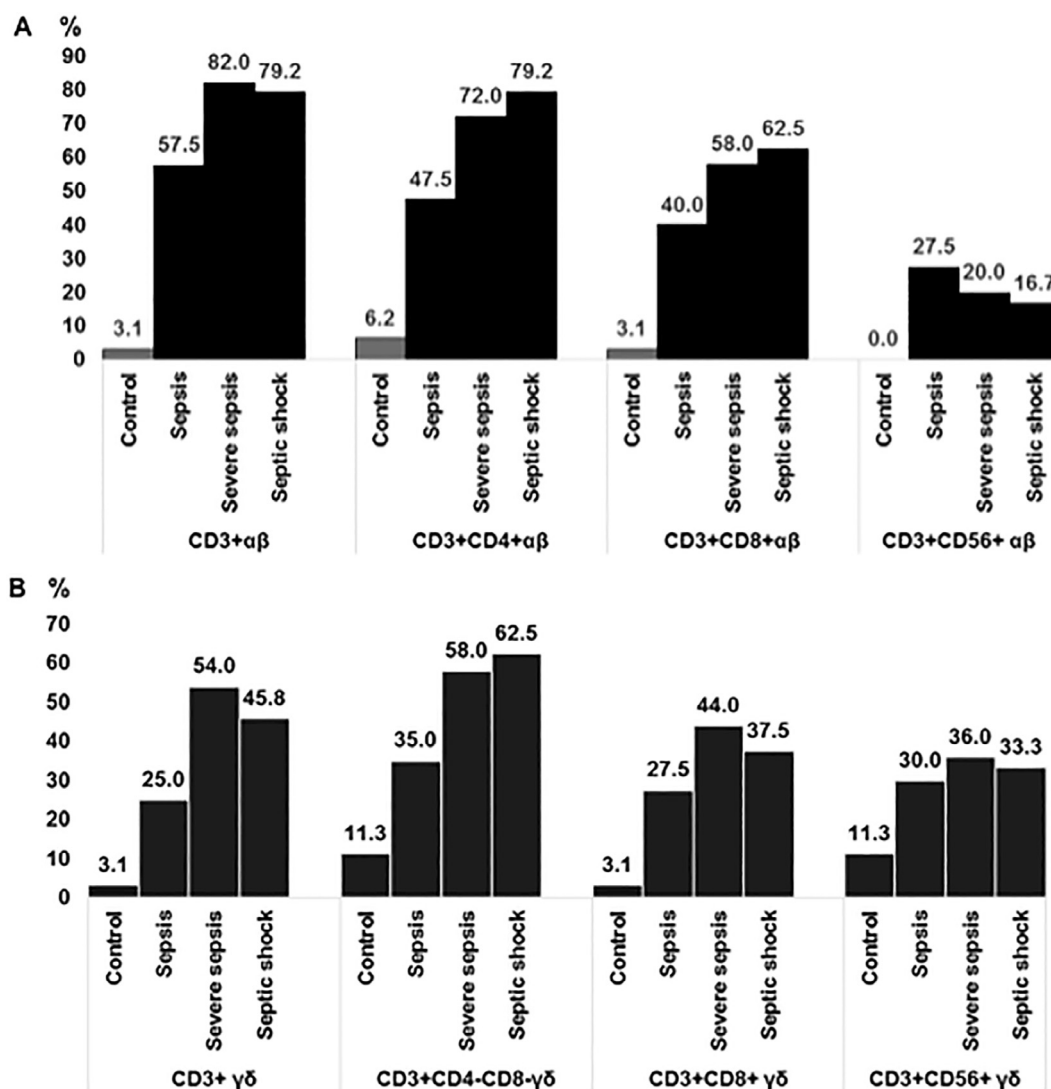


**Fig. 4.** Deficiency of  $\alpha\beta$  T cell (A) and  $\gamma\delta$  T cell (B) subsets according to the severity of sepsis in patients (percentages). Control ( $n = 97$ ), sepsis ( $n = 40$ ), severe sepsis ( $n = 50$ ), septic shock ( $n = 24$ ). There were significant differences between the control group and all septic patients ( $P < 0.001$ ), and between uncomplicated sepsis and severe sepsis-septic shock ( $P < 0.05$ ), except CD3+CD56+  $\alpha\beta$  and  $\gamma\delta$  T cells.

deficiency could facilitate invasion by larvae that try to penetrate the mucosa.

It has been shown that human  $\gamma\delta$  T cells (V $\delta$ 2+ T cells) induced IgG, IgA and IgM, but not IgE production by B cells (Petrasca and Doherty, 2014). IgA is the most frequent Ig present in mucosa, where more pathogens try to invade organisms and more  $\gamma\delta$  T cells are accumulated. Mucosal IgA responses are impaired in  $\gamma\delta$  T cell-deficient mice, suggesting that  $\gamma\delta$  T cells are involved in the regulation of synthesis of IgA from B cells (Fujihashi et al., 1996). Toyabe et al. (2001) found that  $\gamma\delta$  T cells in IgA nephropathy patients are IgA-specific switching cells. The direct relationship found in our study between anti-*Anisakis* IgA and  $\gamma\delta$  T cells confirm the roles of these cells in the regulation by  $\gamma\delta$  T cells of IgA synthesis from specific B cells against *Anisakis* antigens.

Previously, we demonstrated that  $\gamma\delta$  T cells were related to the severity and poor prognosis of septic patients.  $\gamma\delta$  T cells showed the largest decrease and this reduction became more intense when the septic process became more severe (Andreu-Ballester et al., 2013). As expected the deficiency of subpopulations of T cells increased with the severity of sepsis. Similarly, it is logical to think that the fewer  $\gamma\delta$  T cells there are, the less

anti-*Anisakis* IgA production and the less protection against the parasite thus the poorer prognosis there will be. These facts have been observed in our study (Fig. 1A), where the decrease in anti-*Anisakis* IgA antibodies was higher than that observed in the case of the other specific isotypes as the process worsens. The anti-*Anisakis* IgA levels observed in the septic shock group were 50% lower than those observed in the group of septic patients without organ complications. Therefore, it is not surprising that there is an inverse relationship between the sepsis severity indices (APACHE and SOFA) and the anti-*Anisakis* IgA and IgM. The patients with deficits of  $\gamma\delta$  T cells would not be able to produce enough IgM and IgA responses to combat infection or reinfection with the parasite. This could further worsen the prognosis for the septic infection outcome.

One curious observation is that the duration (h) of infection in septic patients from the onset of symptoms until they were treated in the Emergency/Intensive Care Unit was inversely related to their anti-*Anisakis* IgA levels. That is, the greater the duration of sepsis, the lower the IgA levels. This duration was related in our previous study with the lowest number of  $\gamma\delta$  T cells, as well as the severity of the disease and a poorer prognosis.

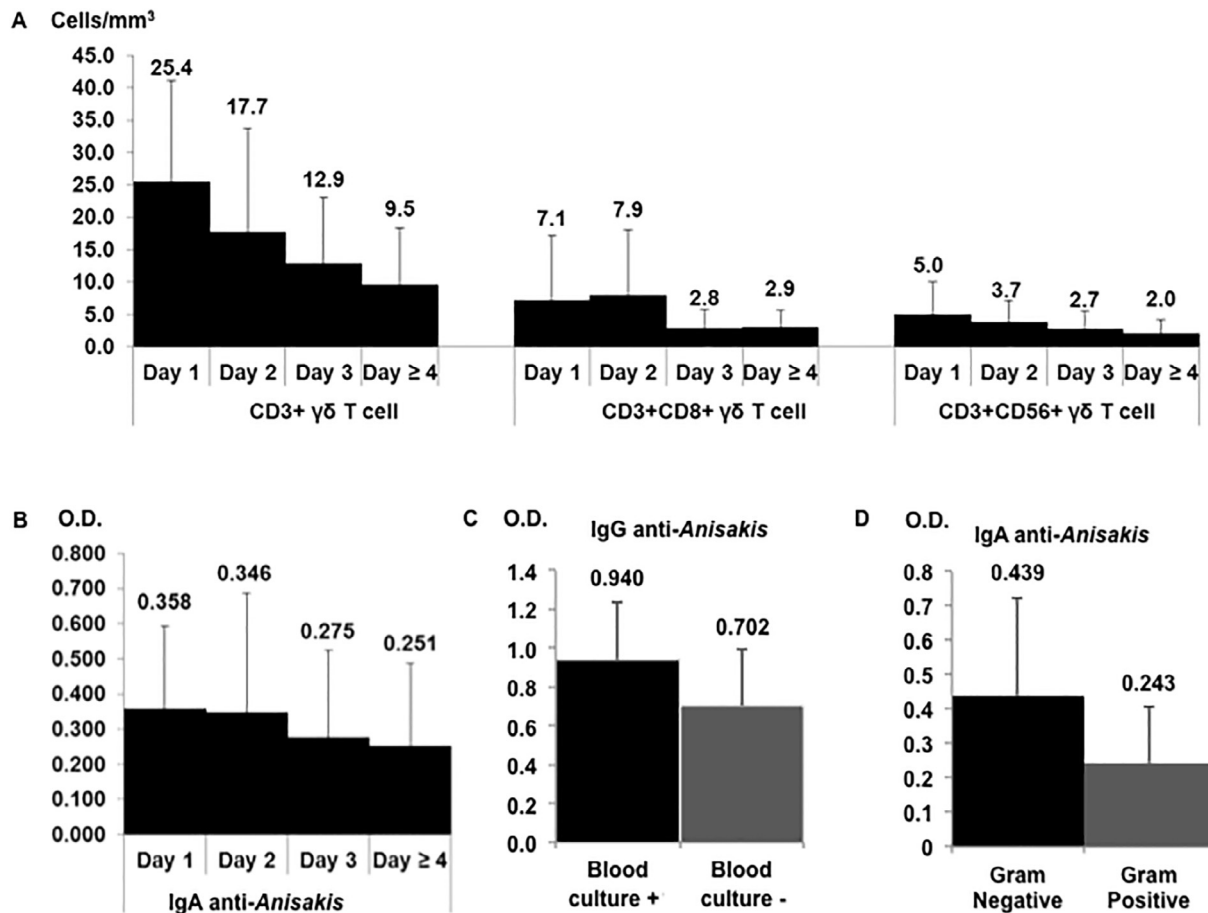


**Fig. 5.**  $\gamma\delta$  T cell subsets and IgA anti-*Anisakis* sp. antibodies in relation to time since onset of infection/sepsis on admission of patients, and anti-*Anisakis* sp. antibodies in relation to blood culture and Gram stain. (A) Peripheral blood  $\gamma\delta$  T cell subsets in relation to time since onset of infection/sepsis on admission to Emergency/Intensive Care Unit departments (Arnau de Vilanova Hospital/Dr. Peset Aleixandre Hospital, Valencia, Spain) (all septic patients). Values are expressed as means (cells/mm<sup>3</sup>), and T-bars denote 95% confidence intervals. The numbers of patients in each group were as follows: 1 day,  $n = 43$ ; 2 days,  $n = 27$ ; 3 days,  $n = 16$ ; and 4 or more days,  $n = 28$ . CD3+  $\gamma\delta$  T cell: differences between Day 1 and Day 3 ( $P = 0.050$ ), Day 1 and Day  $\geq 4$  ( $P = 0.008$ ); CD3+CD8+  $\gamma\delta$  T cell: differences between Days 1–2 and Day 3 ( $P = 0.035$ ), Days 1–2 and Day  $\geq 4$  ( $P = 0.042$ ); CD3+CD56+  $\gamma\delta$  T cell: differences between Day 1 and Day  $\geq 4$  ( $P = 0.045$ ). (B) IgA anti-*Anisakis* sp. antibodies in relation to time since onset of infection/sepsis on admission to Emergency Department/Intensive Care Unit departments (Arnau de Vilanova Hospital/Dr. Peset Aleixandre Hospital, Valencia, Spain). Differences between Days 1–2 and Day  $\geq 4$  ( $P = 0.003$ ). (C) IgG anti-*Anisakis* sp. in relation to blood culture ( $P < 0.001$ ). (D) IgA anti-*Anisakis* sp. in relation to Gram stain ( $P = 0.044$ ).  $P$  value: Mann–Whitney  $U$  test. Values of Igs are expressed as means (O.D.) and T-bars denote 95% confidence intervals.

**Table 5**  
Relationship between mortality and T cell subset deficits in all patients with sepsis.

| Cells                       | Dead/Deficit (%) | Dead/No deficit (%) | OR (95% CI)       | $P$ value |
|-----------------------------|------------------|---------------------|-------------------|-----------|
| CD3+ $\alpha\beta$          | 16/83 (19.3)     | 3/31 (9.7)          | 2.23 (0.60–8.26)  | 0.271     |
| CD3+CD4+ $\alpha\beta$      | 16/74 (21.6)     | 3/40 (7.5)          | 3.40 (0.93–12.49) | 0.067     |
| CD3+CD8+ $\alpha\beta$      | 12/60 (20.0)     | 7/54 (13.0)         | 1.68 (0.61–4.63)  | 0.451     |
| CD3+CD56+ $\alpha\beta$     | 4/25 (16.0)      | 15/89 (16.9)        | 0.94 (0.28–3.14)  | 1.000     |
| CD3+ $\gamma\delta$         | 12/48 (25.0)     | 7/66 (10.6)         | 2.81 (1.02–7.79)  | 0.038     |
| CD3+CD4+CD8+ $\gamma\delta$ | 14/58 (24.1)     | 5/56 (8.9)          | 3.25 (1.08–9.73)  | 0.043     |
| CD3+CD8+ $\gamma\delta$     | 10/42 (23.8)     | 9/72 (12.5)         | 2.19 (0.81–5.92)  | 0.128     |
| CD3+CD56+ $\gamma\delta$    | 18/84 (21.4)     | 1/30 (3.3)          | 7.91 (1.01–62.08) | 0.023     |

OR, Odds Ratio; CI, confidence interval.

In conclusion, the levels of anti-*Anisakis* IgG antibodies were seen to have increased in patients with a more severe disease status. This increase in IgG was directly related to procalcitonin levels, CRP, lactic acid and the positive blood culture of microorganisms in sepsis.

The rest of the specific isotypes tested, especially IgA, declines as the septic process worsens. This decrease was higher in patients with more severe sepsis. There is a direct relationship between levels of anti-*Anisakis* IgA and  $\gamma\delta$  T cells. The decreases in anti-*Ani-*

*sakis* IgA levels were associated with a delayed admission to hospital, and severity of sepsis, as well as an increase in APACHE and SOFA scores. Thus, we believe that the greater contact with the parasite (IgG) was directly related to septic shock, inflammation and severity markers of sepsis. The lack of protection in the mucosa (IgA and  $\gamma\delta$  T cells) was associated with the disease severity.

As aforementioned, immunosuppression in sepsis reduces  $\gamma\delta$  T subsets with the largest decrease in the more severe septic infection, leading to increased host susceptibility to infections. How-

ever, in the present study, the time elapsed since the onset of sepsis symptoms and the determination of anti-*Anisakis* antibodies was less than 10 days (data not shown), confirming that contact with the parasite was prior to the septic infection. In addition, the advanced age of septic patients is related to a deficiency of  $\gamma\delta$  T cells. For this reason, it is logical to assume that a low frequency of  $\gamma\delta$  T cells in these patients would favour a previous infection by the parasite. So, not only is  $\gamma\delta$  T cell deficiency related to the severity of sepsis, but also the levels of anti-*Anisakis* antibodies.

In this study, we show a direct relationship between the deficiency of both  $\gamma\delta$  T cells and anti-*Anisakis* IgA, as well as the duration of the septic infection. This means that previous parasitic infection with *Anisakis* sp., demonstrated by detectable levels of specific IgG, could decrease the  $\gamma\delta$  T cell population in the gut mucosa and thus the IgA anti-*Anisakis* production, leading to lower protection against the parasite and a worse prognosis for the septic infection. Furthermore, this study allowed us to confirm that exposure to *Anisakis* sp. antigens, as has been shown for others helminths, caused a transient immunodeficiency state and predisposed patients to a higher risk of developing sepsis pathology and if so, a more severe septic clinical picture.

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