

Received Date : 25-Jun-2015
Revised Date : 11-Dec-2015
Accepted Date : 12-Dec-2015
Article type : Original

TITLE PAGE

Clinical and immunological characteristics of a pediatric population with food protein-induced enterocolitis syndrome (FPIES) to fish

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Running title:

Fish-induced enterocolitis

Conflict of interests: none

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pai.12529

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ABSTRACT PAGE

González-Delgado P, Caparrós E, Moreno MV, Clemente F, Flores E, Velasquez L, Rubio G, Fernández J.

Clinical and immunological characteristics of a pediatric population with food-protein induced enterocolitis syndrome (FPIES) to fish.

Pediatr Allergy Immunol

ABSTRACT

Background: Food protein-induced enterocolitis (FPIES) is an uncommon, non-IgE mediated food allergy that usually debuts in infancy with profuse vomiting, lethargy and pallor 2-4 hours following ingestion of the offending food. Its immune mechanism is not known. We aimed to describe the clinical features and outcome of children with fish-FPIES as well as to investigate on cellular immune response implicated.

Methods: Prospective and follow-up clinical study of children with FPIES by fish over a period between 2004-2013. Measurement in vitro of both cytokine production in peripheral blood mononuclear cells (PBMCs) and expression of HLA-DR in monocyte-derived dendritic cells, stimulated with fish extracts.

Results: Sixteen children (seven male and nine female) were included, with a mean age of onset at 10 months. Diagnosis was established after a median of 4 reactions. Twelve patients were treated in emergency room, and two were admitted in intensive care. Patch tests were positive in six patients. Skin prick tests (SPTs) and specific IgE to all fish tested were negative. Only three children reached tolerance at a mean age of 4.5 years. Eight children avoided fish because of positive oral food challenge (OFC) after 6 years of age. Other patients have not been challenged because of parent refusal to OFC or a recent diagnosis. TNF- α was increased in patients and a significant elevation of the HLA-DR marker was also observed in these patients vs control donors.

Conclusions: FPIES caused by fish in many cases presents with severe clinical manifestations. Patch test has poor diagnostic value and OFC is the gold standard to test tolerance. The cytokine TNF- α may be implicated in the clinical symptoms. Higher expression of HLA-DR in dendritic cells has also been detected in our patients.

Key words: Cytokines, dendritic cells, fish, food protein-induced enterocolitis syndrome, solid food.

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INTRODUCTION

Food allergy comprises a spectrum of disorders that result from an aberrant immune response to dietary antigens, which can be either IgE-mediated or non-IgE mediated. The non-IgE mediated disorders likely result from direct T cell antigen response, mediated by proinflammatory cytokines that lead to a variety of responses, one of which is food protein-induced enterocolitis syndrome (FPIES) (1). This is a severe childhood food hypersensitivity, typically caused by cow's milk and/or soy and characterized by profuse vomiting and diarrhea, with progression to dehydration and shock in 15% of patients (2). Rice is the most common solid food implicated in FPIES, but recent research shows the involvement of other foods, such as poultry, egg, pea, peanut, oat, barley or fish (3). Reports of FPIES induced by fish are scarce in the literature (4-7). In recent years, both the interest in FPIES and the number of reports has increased considerably, but reports on its pathophysiology remain scarce.

Although the mechanism implicated remains poorly characterized, T cell activation by food allergens and TNF- α production have been proposed to participate in local intestinal inflammation and increase intestinal permeability (8, 9). Humoral immunity has also been explored though not well defined, a suppressed IgA and IgG4 antibody response to casein has been reported in FPIES (10, 11). It has also been suggested that Th2 lymphocytes are increased and Th1 are downregulated or absent after the stimuli (12, 13).

We report the clinical features and follow-up of 16 infants with FPIES due to fish. We aimed to explore the role of innate immune system and cytokines produced to unveil their contribution to the pathophysiology of the entity.

METHODS

Study population

We conducted a prospective and follow-up study with patients referred to our Allergy Service over a 9 year period (2004-2013) for evaluation of gastrointestinal symptoms after fish ingestion. All patients fulfilled Powell's criteria of FPIES (14).

The Ethics Committee of the Hospital General Universitario de Alicante approved the project protocol, and parents provided their informed consent before children were included in the study.

Skin prick test (SPT), atopy patch test (APT) and *in vitro* specific Ig E to fish

SPTs were performed at the first and also in monitoring visits with commercial foods extracts of different species of fish: hake, whiting, sole, red sea bream, salmon, monkfish, anchovy and tuna fish (Bial Aristegui, Spain). APT were performed at the first visit and follow-up evaluations with the same extracts used for SPT as previously reported (5). They were applied into Finn chambers, placed on the back and removed after 48h. Reactions were scored by a blinded physician at 72h. Erythema alone was not considered positive. The positive reaction included erythema with infiltration (+), few papules (++), several papules (+++) and vesicles (++++) following the EACIGA2LEN position paper (15).

Serum specific IgE to fish (tuna, sole, salmon, codfish, hake, swordfish) was measured by the ImmunoCAP system FEIA (Thermo Fisher Scientific, Phadia, Scientific Pharmacia Diagnostics, Uppsala, Sweden).

Oral food challenge (OFC)

OFC with the implicated fish was performed when either diagnosis was not clearly established by the clinical history, or to monitor tolerance after a minimum of 18 months since the most recent reaction, always in a hospital setting, according to the guidelines of Powell (14) modified by Sicherer (16). An

intravenous line was placed before the procedure. We administered a total dose of 0.3 g of protein/kg body weight divided into two doses: the first was 25% of the total amount, and the rest of the dose was administered two hours later followed by a 4 h observation period. A CBC (complete blood count) with differential was obtained before the OFC and 6 hours later if OFC was positive. Powell's (14) criteria modified by Sicherer (16) were used to determine positivity of OFC.

Dendritic cell differentiation and maturation assessment

Blood was extracted from healthy donors (control donor group) and fish- allergic patients with confirmed diagnosis at a control visit. Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphocyte Separation Medium (PAA, Washington, USA). Monocytes were separated from PMBC by CD14 immunomagnetic labeling (Miltenyi Biotec, Teterow, Germany) and seeded in RPMI 1640 supplemented with L-glutamine and 10% FBS at a concentration of 0.5×10^6 cells/ml in 6-well plates. Granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) were added both at 100 ng/ml (eBiosciences, San Diego, USA), and cell culture supplemented every two days. At day 6, immature dendritic cells (DCs) were incubated with different fish extracts or LPS (lipopolysaccharide) (100 ng/ml), as a control for maturation state, for 48h. Dose selection was carried out in 3 control donors, with doses tested ranging from 0.2 to 1 mg/ml. The selected dose was 0.5 mg/ml, the maximum dose not inducing any change in DC maturation state. After 48h, cells were washed and detached from plastic and labeled with FITC (fluorescein isothiocyanate) or PE (phycoerythrin) conjugated antibodies against human leukocyte antigen-DR (HLA-DR) (Diaclone, Besacon Cedex, France) and anti-CD86 as markers of DC maturation stage. Cells were subjected to flow cytometry in a FC500 cytometer (Beckman Coulter, California, USA) and analyzed with CXP Analysis software. We calculated the maturation index as mean of expression of the marker per cell ratio between stimulated DCs and non-stimulated cells (17).

***In vitro* PBMCs stimulation with fish extracts and cytokine measurement**

PBMCs were cultured at 0.5×10^6 cells/ml in the presence of fish extracts (0.5 mg/ml) or phytohemagglutinin (PHA) at a concentration of 100 ng/ml, as positive control. PBMCs were left during 48h to be activated as previously reported (18), and supernatants were kept frozen until processing for cytokine quantification. IL-2, IL-4, IFN- γ , IL-10, IL-6 and TNF- α cytokines were measured by ELISA following manufacturer's instructions (R&D Systems, Minneapolis, USA).

Statistical analysis

Statistical analysis was performed using SPSS software version 20 for Windows (SPSS, Chicago, Illinois, USA). The statistical treatment of the calculated data included mean, standard deviation, median and interquartile range (IQR). Comparisons of quantitative variables were performed with the nonparametric (Kruskal-Wallis test and Mann-Whitney test for nonrelated samples). Statistically significant *P*-values were defined as ≤ 0.05 . The box defines the 25% and 75% quartiles, division within the box the median, and whiskers the range.

RESULTS

Patient description

We report on 16 patients with FPIES caused by fish, 7 male and 9 female. A summary of clinical data are presented in Table 1. Thirteen out of sixteen were breastfed at least three months. The mean age at the initial presentation of symptoms was 10 months (range 9-17 months). Time between fish ingestion and onset of symptoms was 2-4 hours. The diagnosis was established after a median of 4 reactions (range 3-5). Twelve patients were treated in the emergency room because of profuse vomiting, and 2 patients were admitted in the intensive care unit as they developed hypotension, dehydration and metabolic acidosis.

None of the patients had adverse reactions after introduction of other foods except one child that presented symptoms of FPIES caused by shellfish at 3 years of age.

The hake, the whiting and the sole were the fish most commonly implicated.

In the initial evaluation, APTs were positive in 6/16, all showing positivity to at least 4 fish species, most commonly hake, sole, monkfish and megrim. When children were re-evaluated two years later, only 2 out of 6 had positive APT, and 4 out of 6 had negative APTs. OFC in these 4 children was positive. SPT and specific IgE to fish yielded negative results in all cases in the initial and follow-up evaluations. One OFC was performed to confirm diagnosis and 22 to ascertain tolerance.

The absolute neutrophil count (ANC) measured after the positive challenge result showed a median increase of 1250 cells/mm³.

Patients were followed up for 2-9 years with serial OFC every 18-24 months. Currently only 3 children have fish regularly, after reaching tolerance at a mean of 4.5 years. Eight children remain intolerant after 6 years of age. Two patients have not been challenged as parents have refused consent to the test. Three children continue with fish-free diet due to a recent diagnosis.

Dendritic cell maturation state in FPIES patients

Maturation of dendritic cells is crucial for the subsequent T-cell activation in order to induce the appropriate immune response. HLA-DR expression increases when DCs mature and so they improve their antigen presentation capability. The expression of HLA-DR in the patient group showed a significantly increased response of the maturation index (median; IQR) to LPS (1.41; 0.4) vs control group (0.86; 0.2) (Figure 1). We didn't find significant differences in CD86 expression between the two groups.

Also, dendritic cells from allergic patients showed a significantly higher increase in expression of the HLA-DR molecule when exposed to sole or tuna extracts (tuna fish $P=0.02$ and sole $P=0.014$) than control group expression (Figure 2a). HLA-DR maturation index (median; IQR) after sole incubation was more increased in patients (1.1; 0.6) than in the control group (0.75; 0.1), as also occurred with tuna (1.1; 0.4 in patients compared to 0.71; 0.4 in the control group) (Figure 2b).

Increased proinflammatory TNF- α production in PBMCs of FPIES patients

Cytokine production in FPIES seems crucial for development determination of the immediate immune response to an allergenic event such as the observed in FPIES. We measured different cytokine production, such as IL-2, IL-4, IFN- γ , IL-10, IL-6 and TNF- α in the supernatant of PBMCs from patients vs control donors after incubation with sole and tuna extracts using PHA as positive control (Figure 3). Patients showed a higher and statistically significant response in TNF- α production to PHA than controls, and also a mild induction of IL-6 but not under statistical significance. None of the other cytokines measured showed an important modification.

DISCUSSION

Classic FPIES begins in early infancy, usually in the first weeks of life after introduction of cow's milk or soy, which are the usual offending foods (19). Solid foods have also been implicated as triggers of FPIES; in fact, rice is the most common solid food that induces FPIES in some countries (20), though many other foods have been reported (3). FPIES induced by solid foods appears later in life, affecting infants of a higher mean age than cow's milk or soy (21). In our series of children of FPIES caused by fish, the mean age of symptoms debut was 9.8 months, reflecting temporal succession of food introduction in diet (21).

Thirteen out of sixteen children were breastfed at least 3 months. Breast-feeding is thought to play a protective role in FPIES (22), but perhaps as Nowak *et al.* state, the only effect is to delay the onset of FPIES (2). The small sample size of our series precludes the establishment of definitive conclusions.

Fish as a causative food was first described in a clinical note 30 years ago (4), and after that no further cases were reported until 2005, when Zapatero *et al.* (5) studied clinical features of 14 infants with FPIES to fish. In 2012, Sopo *et al.* (6) reported a series of 66 children with FPIES, 8 of them caused by fish. In a more recent, retrospective audit through a review database in the UK, Ludman *et al.* (23) identified 8 cases of FPIES caused by fish in a sample of 54 children

with FPIES. The magnitude of FPIES remains unsettled, but because of a low index of suspicion it is likely to be underdiagnosed.

Our patients, as well as in the series of Zapatero *et al.* (5), show multiple and severe reactions before diagnosis was established, most children having treatment several times in the emergency department, and 2 of them even admitted into the intensive care unit. In spite of these factors, the diagnosis was delayed several months with the possibility of FPIES being considered only after alternative causes were discarded by extensive investigations. In most cases the clinical suspicion followed by a careful medical history provided the clue (21).

All the children had symptoms with at least 2 different fish species and most with 3, with hake, whiting and sole the most commonly implicated and usually the first introduced in the diet in our country. We couldn't confirm tolerance to other species as Sopo *et al.* report (6), as parents refused OFC with another fish not implicated in reactions.

Caubet *et al.* (22) reported that 24% of children with FPIES showed specific IgE to the foods implicated. None of the children in our series developed specific IgE during the follow-up period, which is in accordance with the results of Zapatero *et al.* (5). The presence of different phenotypes of the entity may explain this observation.

With respect to the age of tolerance, only 3 children tolerated and regularly consumed fish before turning 5 years old. We detected persistence of symptoms in at least 50% of patients after 6 years of age with the presence of abdominal pain after ingestion in some cases even after 10 years, as stated by other authors in FPIES to meat and fish (22).

The diagnostic value of the APT is not well established in FPIES, with contradictory results in literature (24, 25). Zapatero *et al.* (5) report that only 3 out of 14 patients showed a positive APT which is consistent with findings on our group, with 6 out of 16 patients having a positive APT in the initial evaluation. Moreover, we observed that 4 out of 6 children with a positive APT had negative

results when evaluated 2 years later, although OFC persisted positive in these children, so APT was not useful to predict the result of OFC in these cases. These data are in agreement with the reported by Jarvinen *et al.* (25), who find a poor utility of APT in predicting tolerance development. Probably both the time at which diagnosis is done and the fact that activation capacity of implicated T-specific cells diminishes over time result in a negative APT (26).

We consider the study of the immunological response a crucial aspect in order to find new possible biomarkers for the illness and define intermediaries implicated such as elicited cytokines, which may be responsible for clinical manifestations.

FPIES is assumed to be a cell-mediated disorder (27), although for now there is a lack of evidence about the implication of allergen-specific T cells (28). The disorder may result partly from development of the cytokine TNF- α , a pro-inflammatory molecule that induces intestinal permeability and is secreted by monocytes, NK cells or by food-sensitized T lymphocytes (8). It has been shown that cells rapidly produce other pro-inflammatory cytokines, such as IL-6, together with TNF- α , in response to *in vitro* milk challenge in non-IgE mediated allergic patients (13). Also, mucosal biopsies from children with challenge-proven cow's milk FPIES show not only an increased expression of TNF- α , but also decreased expression of TGF- β , a cytokine that induces T cell suppression, promotes B cell switching to IgA production, and preserves epithelial barrier function (9). In accordance with these findings, our results show that in children with FPIES, stimulation of PBMCs with fish extracts induced higher amounts of TNF- α than in control donors. This cytokine has also been observed to increase in DCs treated with milk extracts and in the presence of T cells (29). In our PBMCs cultured with fish extracts, IL-6 levels were mildly increased, but observed differences were not statistically significant. Both cytokines, TNF- α and IL-6, may participate in the clinical manifestations observed in patients with FPIES after food exposure. We didn't detect in culture significant levels of other cytokines, such as IL-10, IL-2, IFN- γ or IL-4. Determination of cytokines at the time of the symptoms may have additional diagnostic value.

Accepted Article

Little is known about the participation of the innate immune system in the development of FPIES. Dendritic cells constitute the basic bridge between innate and adaptative immunity. So it is important to know whether these cells respond differently to stimuli in these patients activating when exposed to food allergens as if detecting some pathogenic extract. In this sense, HLA-DR expression was significantly elevated in our patients' dendritic cells in the presence of two different white and blue fish extracts (sole and tuna fish, respectively). Similarly, Nagata *et al.* (30) reported an increase in expression of HLA-DR in CD4+ T cells in small intestinal mucosa of food-sensitive enteropathy. Moreover, our results show that there were an increased number of markers per cell after LPS treatment in patients, which could imply a heightened possibility of sensitization to an allergen compared to the control group. Similar results have been published with Prp3 in peach-allergic patients, showing mild dendritic cell maturation profile (17).

In this sense, the increased level in TNF- α along with measurements of dendritic cell activation may constitute potential biomarkers for diagnosis.

This could also be a useful tool to evaluate the status of the patients, if future studies confirm these findings with larger series of children.

One of the limitations of the study is the small sample size of our series due to the uncommonness of the entity. Also, measurement of cytokines performed at acute phase may give more precise information of the immunological events. An additional control group tolerant to fish but with FPIES to other foods could support our findings.

To summarize, we report the clinical features, follow-up and immune profile of 16 patients diagnosed with FPIES. We observed a food allergen-dependent higher expression of HLA-DR in dendritic cells of patients compared to control donors. The increased production of TNF- α by PBMCs may indicate an altered immune response elicited in these patients after fish ingestion.

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Table 1. Characteristics of the study population.

Nº patient	Sex	Breast feeding (mo)	Age at first reaction (mo)	Nº episodes	Symptoms	ED/ICU	Fish implicated	SPT/sIgE	Age achieving tolerance (mo)	Patch test
1	m	-	10	5	V, L	3/-	Ha, W, S, P	-/-	66	-
2	f	3	10	4	V, L, D, H	3/1	Ha, W, P	-/-	no	-
3	f	4	10	3	V, L, D	3	Ha, W, S	-/-	no	+
4	m	4	11	4	V, L, D	-	Ha, W, M	-/-	NC	+++
5	f	4	10	3	V, L, D	2	Ha, W, An	-/-	60	++
6	f	-	9	4	V, L, D, H	3/1	Ha, W, S	-/-	no	-
7	m	1	9	4	V, L, D	3	Ha, W	-/-	no	-
8	m	4	9	3	V, L	1	Ha, W	-/-	36	-
9	f	6	9	5	V, L, D	-	Ha, W, M	-/-	no	-
10	m	4	9	4	V, L	3	Ha, W, M	-/-	NC	-
11	f	7	10	5	V, L, D	3	Ha, W, S	-/-	no	++
12	f	5	9	4	V, L, D	-	Ha, W, S	-/-	no	++
13	m	11	9	4	V, L	2	Ha, W	-/-	no	-
14	f	5	10	4	V, L, D	2	Ha, W	-/-	no	-
15	f	3	9	4	V, L	-	Ha, W, M	-/-	no	+
16	m	4	17	3	V, L	2	Ha, W	-/-	no	-

Abbreviations: m= male, f= female, mo= months, V= vomiting, L= lethargy, D= diarrhea, H= hypotension, Ha= hake, W= whiting, S= sole, P= perch, An= anchovie, M=monkfish, ED= Emergency Department, ICU= Intensive Care Unit, SPT= Skin Prick Test, sIgE= specific IgE to fish, Nc= Not challenge

Figure 1

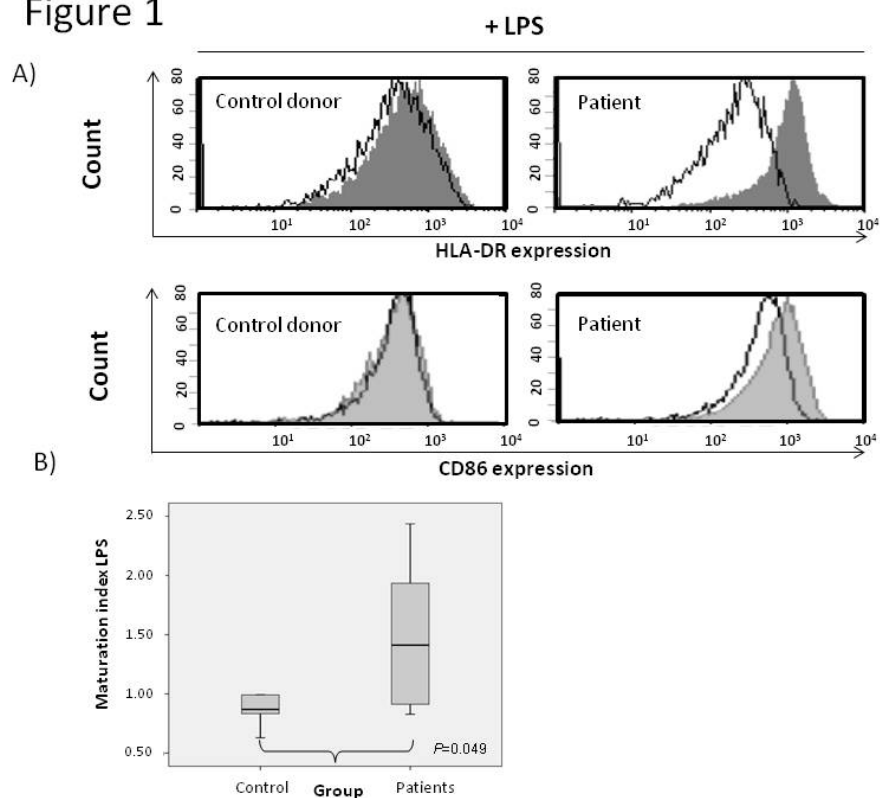


Figure 2

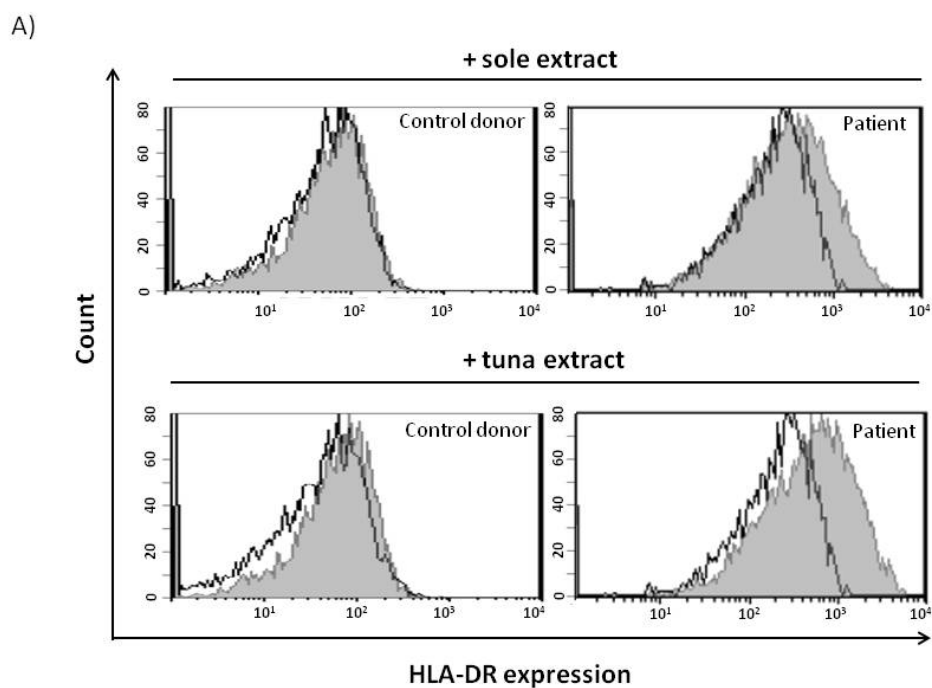


Figure 2

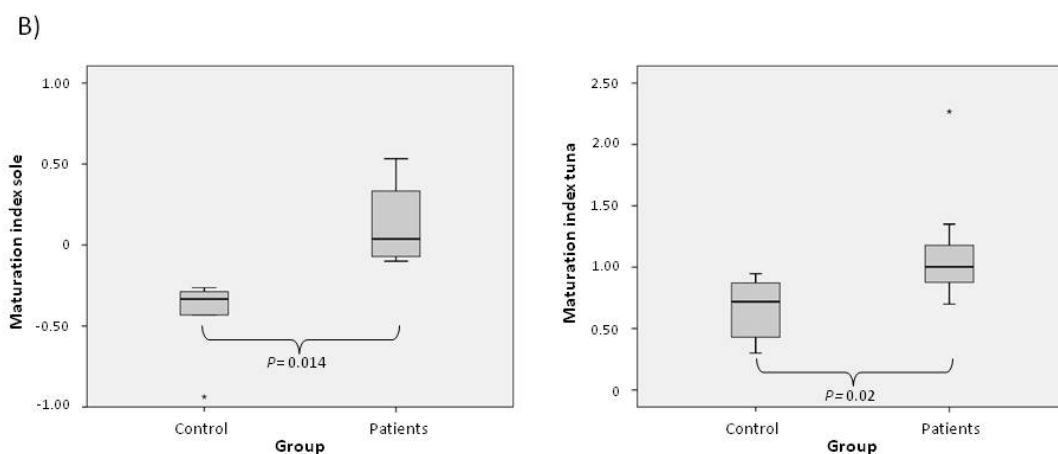
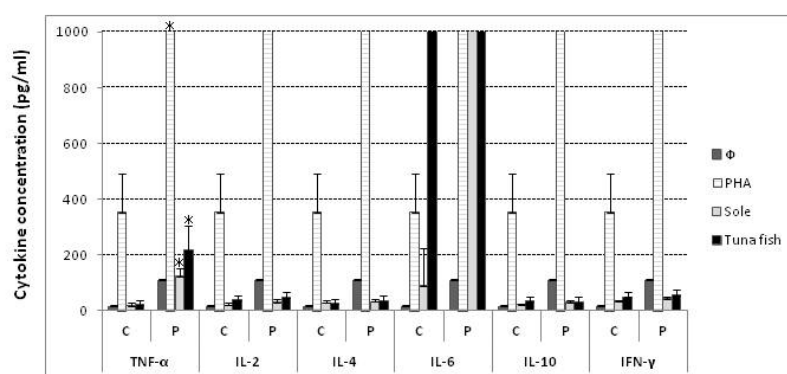


Figure 3



	Fish	Control donors	Patients
TNF- α	Tuna*	8 $\pm$ 13	217 $\pm$ 25
	Sole*	6 $\pm$ 10	30 $\pm$ 13
IL-6	Tuna	91 $\pm$ 133	2549 $\pm$ 1161
	Sole	1116 $\pm$ 1193	2588 $\pm$ 1461

* $P<0.05$