Different Fish-Eating Habits and Cytokine Production in Chronic Urticaria with and without Sensitization against the Fish-Parasite Anisakis simplex

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ABSTRACT
Background: Anisakis simplex sensitization has been associated with acute, but also with chronic urticaria. The objective of this study is to characterize chronic urticaria with (CU+) and without sensitization (CU-) against the ubiquitous fish parasite A. simplex in a transversal and longitudinal evaluation.

Methods: 16 CU+ and 22 CU- patients were included and assessed for Urticaria activity score (UAS), fish-eating habits by standardized questionnaire and cytokine production (assessed by flow cytometric bead-based array) of peripheral blood mononuclear cells after stimulation with A. simplex extract or Concanavalin A (Con A). Patients were randomly put on a fish-free diet for three months and UAS, as well as cytokine production were again assessed. A difference of ≥1 in UAS was defined as improvement.

Results: There was no difference in UAS in both groups. Anisakis induced IL-2, IL-4 and IFN-γ production was higher in CU+. Con A induced IL-6 and IL-10 production was higher in CU+. CU+ was associated with higher total fish intake, whereas CU- was associated with oily fish intake. The correlation of UAS was positive with oily fish, but negative with total fish intake.

There was a better UAS-based prognosis in CU+ without diet. Improvement was associated with higher Con A induced IL-10/IFN-γ as well as IL-10/IL-6 ratios. Further, previous higher oily fish intake was associated with improvement.

Conclusions: Our data confirm the different clinical and immunological phenotype of CU+. Our results show a complex relationship between fish-eating habits, cytokine production and prognosis, which could have important consequences in dietary advice in patients with CU. When encountering A. simplex sensitization, patients should not be automatically put on a diet without fish in order to reduce contact with A. simplex products.

KEY WORDS
Anisakis simplex, chronic urticaria, cytokines, diet, phenotype

ABBREVIATIONS
CU, Chronic urticaria; CU+, Anisakis simplex sensitization associated chronic urticaria; CU-, Chronic urticaria without sensitization against A. simplex; PBMC, Peripheral blood mononuclear cells; Con A, Concanavalin A.
INTRODUCTION

Chronic urticaria (CU) is a frequent and disabling illness occurring worldwide in 0.1% of the population and has repeatedly been shown to affect quality of life.1,2 CU has been labelled autoimmune in a high percentage of cases.3,4 But, independently of the possible autoimmune status, multifactorial genesis seems to underlie the appearance of this entity. Therefore different studies attempted at searching for different phenotypes of CU, taking into account possible infectious elicitors, mainly physical stimuli, its association with non-steroidal anti-inflammatory drug intolerance or atopy status.5-7

It is clear that the main shared characteristic in the heterogeneous group of CU is mast-cell activation, and the release of biogenic mediators is responsible for clinical features and the inflammatory reaction. On the other side, the knowledge on previous immunologic mechanisms leading to mast cell activation is scarce in CU, although there is now some evidence to include CU as an inflammatory disorder.8 Some studies have highlighted different pro-inflammatory cytokines to be implicated in severity and missing regulatory features associated with this entity.9-12

Whereas Gastro-allergic Anisakiasis (GAA) is a well established clinical entity, where acute short-lived, IgE-mediated urticaria, angioedema or anaphylaxis accompanies acute Anisakis simplex infection,13,14 a frequent phenotype of CU in our area is its association with sensitization against this fish-parasite (CU+ or A. simplex sensitization associated chronic urticaria).14,15 In our area, this phenotype of CU patients constitutes up to 50% of patients attended for allergological evaluation and in a relevant subgroup of these patients sensitization against A. simplex was explained by previous parasitic episodes by this nematode.15 Detection of specific IgE against A. simplex explains only previous parasitism in a given patient, but is not necessarily linked to a causal or temporal relationship with the onset of chronic urticaria. Thus a previous parasitic episode could be one of the predisposing factors leading to CU together with other mainly unknown eliciting factors.

The aim of characterizing phenotypes is to search for possible differentiated treatment regimes. Generally these include mainly avoidance of eliciting factors, such as in physical urticaria, drug treatment and possible dietary advice. The last has been scarce in an allergological setting due to the fact that reports on food-induced IgE-mediated elicited CU are anecdotal. On the other side, some studies have highlighted the possible role of a pseudoallergen-free diet in patients with CU.16,17

Sensitization against A. simplex is per se influenced by dietary habits, as raw fish eating has repeatedly been shown to be a risk factor for sensitization and urticaria.18,19 The role of A. simplex as a hidden aller-
Table 1  Fish-eating habits: standardized questionnaire

1. Which are the names of the fish you eat most often?
   This is a question necessary for controlling the necessary
differentiation of oily and non-oily fish. †
2. How often do you eat non-oily fish in one week?
3. How often do you eat oily fish in one week?
   With these data we computed: Total fish-intake: 2 + 3.
4. How often do you eat canned fish in one week? ‡
5. How often do you eat anchovies in vinegar sauce in one
   month or one year?

† Most frequently referred fish consumed by patients were:
   Oily fish: sardine, anchovy, swordfish, tuna, salmon, trout, red
   mullet, bream, seabass, salted cod. Non-oily fish: hake, blue whitt-
ing, sole, halibut.
‡ Most frequently referred canned fish consumed by patients
   were oily fish: tuna, sardine, mackerel.

Cytokine production was measured in supernatants
after stimulation of peripheral blood mononuclear
cells (PBMC), stimulated with A. simplex antigen or
Concanavalin A (Con A).

At study onset patients were randomly selected for
a diet without fishery products for three months or
maintaining their habitual weekly fish intake. UC+ pa-
tients, if included in the group without diet, were ad-
vised to eat previously frozen fish.

After three months all patients were again assessed
for UAS and cytokine production.

Antihistamines were withdrawn five days before
clinical and immunological evaluation. Otherwise, pa-
tients were asked to take the minimum number of an-
thistamines necessary for relief control.

URTICARIA ACTIVITY SCORE
Urticaria activity score (UAS) was assessed as previ-
ously described. 25 Shortly, severity of urticaria in CU
patients was clinically assessed after withdrawing an-
thistamines for 5 days. The mean score of the last
four days was calculated as sum of the wheel number
score (between 0 and 3: 0; 0-9: 10-50; >50) and the
itch severity score (between 0 and 3: no; mild; moder-
ate; severe).

SKIN PRICK TESTS
Skin prick tests (SPT) performed were: SPT with A.
simplex and against the most frequent aeroallergens
in our area: animal dander (cat, dog), house dust
mites (Dermatophagoides pteronyssinus, Dermato-
phagoides farinae), pollen of Cupressus arizonica, Olea
europea, Lolium perenne, weed mix and mould Alter-
naria alternata (ALK-Abello, Madrid, Spain). Fur-
ther, all patients were assessed for other IgE-
mediated food allergies by SPT test against a set of
food-agents, including egg, milk, fish, crustaceans
and vegetables (Laboratories Leti, Barcelona, Spain).

SPT was performed by standard technique and was
considered positive with a mean wheel diameter of 3
mm or more. Histamine at 1% concentration and sa-
line solution 0.9% (NaCl) were positive and negative
controls, respectively. Wheal diameter was measured
15 minutes after treatment.

AUTOLOGOUS SERUM SKIN TEST
ASST was performed as previously described 26 and
could be performed in 23 of the studied patients.
Shortly, whole blood was collected into sterile glass
tubes and allowed to clot for 30 minutes. After cen-
trifugation at 450 g for 10 min, an intradermal 0.05 ml
injection of undiluted serum was applied at the volar
forearm in parallel to the controls: 0.05 ml of normal
saline as negative control and a SPT with Histamine
at 1% concentration as positive control. ASST was con-
sidered positive if the mean wheal of ASST was ≥1.5
mm after 30 minutes, ensuring a positive control at 15
minutes.

CRUDE EXTRACT FROM A. SIMPLEX
Larvae of A. simplex were fragmented and sonicated
and further proteins were extracted in phosphate
buffer. After delipidation with n-hexane and centrifu-
gation at 10,000 rpm at 4 °C during 30 minutes, super-
natant was collected as crude extract and protein con-
ent was quantified by Bradford protein assay. 27

SERUM SAMPLE, PBMCs AND STIMULATION
ASSAY
Blood was also taken after antihistamines were with-
drawn for five days. Serum was stored at -70 °C until
processing and whole blood was immediately proc-
cessed for stimulation assays.

The PBMC were isolated by centrifugation over
Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA)
density gradient, and cell viability was assessed
by means of trypan blue dye exclusion. Cells were
then washed and re-suspended at 1.25 × 10⁶ cells/ml
in RPMI 1640 supplemented with 10% heat-inac-
tivated foetal bovine serum, 10 mM HEPES buffer, 2 mM L-glutamine, and 0.06 g/l of gentamycin.
Cells cultured under stimulation with either Con-
canavalin A from Canavalia ensiformis (Jack bean)
(Con A; Sigma-Aldrich) (50 μg/ml), or A. simplex lar-
val crude extract (500 μg/ml), or with medium alone.
Cells were also co-stimulated by means of the simul-
taneously addition of Con A plus antigen. Cells were
incubated for 72 hours at 37 °C in a humidified incuba-
tor with 5% CO₂. Supernatants were stored at -70 °C
until further processing.

LABORATORY DETERMINATIONS
In all patients we analyzed specific IgE (CAP-System,
PHADIA, Uppsala, Sweden) against Anisakis (Cut-off
point: 0.35 kU/l). Specific IgE against recombinant
Ani s 7 was also determined in order to confirm a previous parasitism.28

IgE AGAINST rAni s 7
rAni s 7 is a polypeptide with a repetitive sequence recognized by mAb UA3. When the anti-Anisakis IgE values for human positive sera in indirect ELISA with rAni s 7 were compared with capture ELISA using the mAbUA3 (which recognizes nAni s 7 allergen) all sera tested were positive by both methods.29,30 This proves that rAni s 7 retained the same IgE reactivity as the nAni s 7 allergen.

Briefly, specific anti-Anisakis IgE antibodies were detected by indirect ELISA, with recombinant Ani s 7 as the target. Wells of the 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were filled with 0.06 μg/well of protein. After incubation of the plates at 4°C overnight and blocking of non-reactive sites, 100 μl of undiluted serum was added to each well and the specific IgE was detected as previously described. Optical densities (ODs) at 492 nm were calculated by subtracting the OD value produced by the same serum in the absence of antigen. The calculated cut-off values for Ani s 7 ELISA were ODs of 0.05.28

CYTOKINE MEASUREMENT
Supernatants of the PBMCs stimulation assays were used for measurement of cytokine production: levels of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A were quantified using a multiplex assay in accordance with the instructions of the manufacturer (BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit; BD Biosciences, San Jose, CA, USA). TGF-β levels in serum were assessed by a single plex assay (BD™ Cytometric Bead Array (CBA) Human TGF-β1 Single Plex Flex Set; BD Biosciences) as indicated by the manufacturer. All samples were analyzed with BD FACSCompTM Flow cytometer™ and the results were expressed in pg/ml using the FCAP Array™ software.

EFFECT OF DIET
Clinical improvement was defined as a difference of UAS at study onset - UAS after three months (after randomization to diet/no diet) ≥1.

STATISTICS
Statistical analysis was performed using SPSS ver. 15.0 for Windows.

TRANSVERSAL EVALUATION
Prevalences were calculated for sex, atopy status and positive ASST in both studied groups and compared by Chi-square-test. Mean age, UAS, and supernatant TGF-β were calculated in all studied groups and compared by ANOVA (normal distribution of data). Anti-(IL-10, TGF-β)/pro-inflammatory cytokine (IL-6, IL-17, IFN-γ, TNF-α) production ratios were calculated and compared. Previous duration of urticaria, other cytokine levels as well as data on fish-eating habits, specific IgE and total IgE displayed no normal distribution. Here, Median and Interquartile range were calculated for and compared by Mann-Whitney. We further analyzed ratios of pro- and anti-inflammatory cytokine levels by the same methods.

Spearman correlation coefficient was used for correlation studies between cytokine production and fish intake as well as UAS.

EFFECT OF DIET AND PROGNOSIS
Improvement rates were compared in randomized groups (diet/no diet) in CU+ and CU- and compared by Chi-square-test. Further, improvement rates were compared in ASST+ versus ASST-, and atopic versus non-atopic patients.

Initial cytokine production was compared in patients with clinical improvement versus non-improvement. Individual cytokine changes after the clinical trial were assessed by a ratio of their post-/pre-production. Again, Median and Interquartile range were calculated for and compared by Mann-Whitney.

In patients with diet, previous fish-eating habits were compared in patients with improvement versus non-improvement.

Here, a logistic regression model was performed to analyze which factors are associated with improvement. Those variables were included initially in this model, which achieved p < 0.1 in bivariate analysis. Diet was also to be included as a possible explaining factor.

RESULTS
WHAT DIFFERENTIATES CU+ FROM CU-?
Epidemiology, Clinical and Routine Laboratory Data
22 CU- and 16 CU+ patients were included, 26 were female and 12 male. Mean age was higher in CU+ (54.7 ± 12.5 years old) than in CU- (39.3 ± 15.6 years old, p = 0.02).

13/16 patients were atopic in CU+ and 17/22 in CU-. 7/16 were sensitized against pollen in CU+ and 10/22 in CU-. 5/16 were sensitized against HDM in CU+ and 6/22 in CU- (n.s.).

ASST was positive in 3/12 CU+ patients and 4/11
Anisakis simplex and Urticaria

Fig. 1 Fish eating habits in different phenotypes of chronic urticaria. Results after logistic regression analysis including all possible fish-eating variables. Median weekly (total, oily fish, canned fish) and monthly (anchovies in vinegar sauce) portions of fish in chronic urticaria witho (CU-) and with (CU+) previous parasitism by Anisakis simplex.

Table 2 Cytokine production (in pg/ml supernatant) after stimulation of PBMC with either Anisakis simplex extract or Concanavalin A

<table>
<thead>
<tr>
<th>Cytokine induction with A. simplex extract</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IL-17</th>
<th>TNF-α</th>
<th>IFN-γ</th>
<th>TGF-β</th>
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<td>CU-</td>
<td>6.8</td>
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<td>286</td>
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<td>0.14</td>
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<td>(0.7-51.1)</td>
<td>(0-0.1)</td>
<td>(37-576)</td>
<td>(0.3-8.1)</td>
<td>(0-8.5)</td>
<td>(0-1.5)</td>
<td>(0-7.5)</td>
<td>(162-677)</td>
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<td>CU+</td>
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<td>879</td>
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<td>7.2</td>
<td>0</td>
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<td>(37-537)</td>
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<td>(43-1601)</td>
<td>(1.4-41)</td>
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<td>0.25</td>
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<th>IL-17</th>
<th>TNF-α</th>
<th>IFN-γ</th>
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<td>CU-</td>
<td>18.2</td>
<td>1.7</td>
<td>10757</td>
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<td>318</td>
<td>94</td>
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<td></td>
<td>(2.3-163)</td>
<td>(0.06-7.3)</td>
<td>(2153-16100)</td>
<td>(103-371)</td>
<td>(59-730)</td>
<td>(26-334)</td>
<td>(322-2902)</td>
<td>(134-741)</td>
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<td>18152</td>
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<td>482</td>
<td>163</td>
<td>2758</td>
<td>519</td>
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<tr>
<td></td>
<td>(10.6-618)</td>
<td>(1.0-26.5)</td>
<td>(13016-23299)</td>
<td>(231-604)</td>
<td>(202-1283)</td>
<td>(42-522)</td>
<td>(303-5297)</td>
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<td>P</td>
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<td>0.03</td>
<td>0.13</td>
<td>0.26</td>
<td>0.18</td>
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*Median and Interquartile range in pg/ml supernatant. P-values are given after comparing cytokine levels in CU+ versus CU-.*

in CU- (n.s.).

Median previous duration of urticaria was 20 Interquartile range (IQR) 8-36 months in CU+ and 6 (IQR 2.8-24) months in CU- (p = 0.07).

UAS was 3.6 ± 1.5 in CU+ and 4.0 ± 1.4 in CU- (n.s.).

Median specific IgE was 4.8 (IQR 1.5-11.5) kU/l in CU+. Median total IgE was 117 (IQR 83-414) kU/l in CU+ and 103 (43-161) kU/l in CU- (n.s.). Due to the inclusion criteria, all CU+ displayed IgE-antibodies against Anisakis simplex.

One CU- and one CU+ patient had a positive Hepatitis B serology, one CU- patient showed positive antibodies against Hepatitis C and one CU+ patient suf-
Table 3 Correlation studies

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<td>IL-2</td>
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<tr>
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Spearman correlation coefficient and significance between the production of different cytokines alter stimulation of PBMCs with either Anisakis (a) or Concanavalin A (b).

Farmed non-autoimmune hypothyroidism and was under substitution therapy.

Fish-Eating Habits

Bivariate analysis showed only total fish intake to be significantly higher in CU+ (2.5, IQR 1.6-2.5 versus 1.25, IQR 1-2.5 portions per week; \( p = 0.04 \)).

Figure 1 highlights results on fish-eating habits after regression analysis, which shows CU+ to be highly associated with total fish intake (\( B = +3.2; CI 1.3-8.1; \( p = 0.01 \)), whereas CU- was associated with oily fish (\( B = 0.2; CI 0.03-1.0; \( p = 0.05 \)). Canned fish and anchovies in vinegar sauce are not significantly associated with both urticaria phenotypes.

Cytokine Production

In a general manner, all studied Anisakis-stimulated cytokines were produced in higher quantities in CU+ than in CU-, but highly significant differences could be stated for IL-2, IL-4 and IFN-γ (Table 2).

But also Con A stimulated PBMC were higher in CU+ and reached significant differences for IL-6 and IL-10 (Table 2).

Correlations between cytokines can be seen on Table 3. Overall, cytokine production was frequently positively correlated with each other.

Regression analysis did not yield valuable results of cytokine production explaining CU+ or CU-.
significant results could be stated for the ratios of pro- and anti-inflammatory cytokine production.

**Relationship between Data-Groups**

In patients with CU+, total fish intake was correlated with Con A induced IL-6 and IL-17 production (Rho > 0.5; p < 0.05).

In CU-, total fish intake was associated with *A. simplex* induced IL-2 (Rho 0.46; p < 0.05), but negatively correlated with *A. simplex* induced TNF-α (Rho = 0.2, p = 0.02). Oily fish intake was also associated with *A. simplex* induced IL-2 production (Rho 0.56; p = 0.01). Anchovies in vinegar sauce were negatively associated with TNF-α and IFN-γ (Rho < -0.53, p < 0.02). Canned fish was negatively correlated with *A. simplex* induced TNF-α (Rho = -0.53, p = 0.02). Oily fish intake was also associated with *A. simplex* induced IL-2 production (Rho 0.56; p = 0.01).

Regression analysis including cytokine results showed only Con A induced IL-6 to be associated with CU+, but with a negligible odds ratio (Odds ratio 1.0003 CI 1.000-1.0006; p = 0.05).

UAS was not associated with the production of specific cytokines, but in CU+ UAS was positively correlated with higher *A. simplex* induced IL10/IL17 (Rho 0.63; p = 0.02) as well as IL10/IFN-γ (Rho 0.51; p = 0.05) ratios, and in CU- positively with IL10/TNF-α (Rho 0.48; p = 0.04) but negatively with TGF-β/TNF-α ratio (Rho -0.51; p = 0.03).

UAS was also associated with fish-eating habits. Regression analysis demonstrates UAS to be positively associated with oily fish intake (B = 0.89; p = 0.02), but negatively with total fish intake (B = -0.47; p = 0.03) (Fig. 2).

**WHICH FACTORS AFFECT THE PROGNOSIS OF CU?**

**Bivariate Analysis**

We could not find a positive effect of diet on improvement of CU+, there was rather a tendency to a better outcome if patients were not under diet (p = 0.07). No such effect was detected in CU-. There was no different outcome with respect to positive ASST.

When analysing only CU+, improvement was associated with atopy (mainly when HDM sensitized, p = 0.03). No such effect was found in CU-.

Initial high Con A induced IL-10/IFN-γ (p = 0.009) as well as IL-10/IL-6 (p = 0.07) ratios were associated with improvement.

When comparing individual post- and pre-trial cytokine production, in CU-, improvement was associated with higher *Anisakis* induced TNF-α (p = 0.04), IFN-γ (p = 0.01) as well as Con A induced IFN-γ production (p = 0.04), whereas Con A induced TGF-β production was lower (p = 0.01). In CU+, improvement was associated with a higher *Anisakis* induced TGF-β production (p = 0.04).

**Multivariate Analysis**

Regression analysis showed that both diet, but also previous higher total fish intake were associated with a worse prognosis, whereas previous higher oily fish intake was associated with a better prognosis (Fig. 3a).

Again, when analysing only patients who were put on diet, these improved when they have previously been eating higher amounts of oily fish and lower amounts of total fish (Fig. 3b).

**DISCUSSION**

Our results confirm that CU+ and CU- have, besides the presence of specific IgE, some different clinical and immunological characteristics. CU+ patients were older than CU-. This fact is in concordance with previous studies, which showed *A. simplex* sensitization and parasitism to be associated with raw fish eat-
Explaining variables for clinical improvement of chronic urticaria. Improvement is defined as a minimum decline of Urticaria activity score of 1 after the trial. Those variables were included initially in this model, which achieved $p < 0.1$ in bivariate analysis (fish-eating habits, diet, atopy status). a) Here diet was also included as a possible explaining factor and achieves significance for a worse prognosis. b) Analysis of only those patients who were put under diet without fishery products.

Table: Odds ratio and 95% C.I.

- **Total fish**: No improvement, $p = 0.03$, Improvement, $p = 0.02$
- **Oily fish**: No improvement, $p = 0.04$, Improvement, $p = 0.04$
- **Atopy**: No improvement, $p = 0.14$, Improvement, $p = 0.14$

**Fig. 3** Explaining variables for clinical improvement of chronic urticaria. Improvement is defined as a minimum decline of Urticaria activity score of 1 after the trial. Those variables were included initially in this model, which achieved $p < 0.1$ in bivariate analysis (fish-eating habits, diet, atopy status). a) Here diet was also included as a possible explaining factor and achieves significance for a worse prognosis. b) Analysis of only those patients who were put under diet without fishery products.

ing habits (in our region anchovies in vinegar sauce) in older populations. We cannot otherwise rule out that urticaria in its acute or chronic phenotype appears, like in other immune conditions, after a certain threshold of accumulated contact with *A. simplex* antigen. As we included in our CU+ only those patients who also displayed IgE against *Ani s 7* and thus suffered previous parasitism, the different age-patterns confirms that previous parasitism is really at least one causal factor leading to the appearance of CU+. The recognition of this allergen is sufficient to prove a previous parasitic episode and known pan-allergens,
such as tropomyosins or paramyosins from other sources, which have their equivalent as *Ani s 2* and *Ani s 3*, have not been proven to elicit clinically relevant cross-reactions after parasitism by *A. simplex* and thus were not in the scope of this study.20

Raw fish-eating habits have been associated both with sensitization against *A. simplex* and GAA as well as CU+.18,31 Our study did not find a significant difference in the frequency of raw fish eating (anchovies in vinegar sauce), but two reasons could be discussed: first, perhaps the low number of studied patients. Second, we know that social alarm about *A. simplex* infections and legal requirements concerning control of parasites in fish to be consumed raw in the last decade have highly impacted the population with respect to fish-preparation and this fact could impact on our results.

A previous study showed that raw fish eating was the main risk factor for IgE production against *A. simplex* and less the quantity of total fish intake.18 Our own results show that total fish intake is associated with CU+, compared to CU-. This is due to the higher probability of contact with live larvae due to the second risk factor, undercooked fish, which leads to detectable specific IgE.13,32

More interestingly, the amount of oily fish-intake is independently associated with CU-. Further, UAS was both dependent on the amount and type of fish intake. One possibility explaining UAS to be positively correlated with oily fish intake is the already known presence of biogenic amines mainly in oily fish, which are partly responsible for UC- and severity.33

On the other side oily fish intake has previously been shown to be associated with a decreased production of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α,11,12 a fact that would not fit with these data in the transversal evaluation. But independently of fish-eating habits, cytokine production shows the same seemingly counterintuitive direction, with UAS correlating with higher Con A induced IL-10/IL-17 as well as IL10/IFN-γ and *Anisakis* induced IL-10/TNF-α ratios. Similarly, IL-10 production by mitogen has been verified in previous studies involving patients with CU.10,11 We could not verify IL-6 production to be associated with the severity of urticaria, as proposed in another study.12 But the negative association of UAS and the *Anisakis* induced TGF-β/IL-6 ratio in CU renders emphasis to these cytokines in a plausible direction. Here IL-6 could be interpreted as a marker of systemic inflammation as well as a marker of disease activity in CU.12 On the other side of the anti-pro-inflammatory axis, TGF-β could be implicated in dampening the inflammatory reaction, lowering UAS. We should otherwise not forget that we are not assessing a control population and that our results could be specific when analysing CU.

How can we interpret these data? As we have seen, pro- as well as anti-inflammatory cytokine production is highly correlated with each other, when stimulated with the same agent. Thus, in CU it seems that the significant factor leading to activity of urticaria is missing in our analysis and only epiphenomena are detected. Biological processes are marked by a steady tendency towards a regulation of disequilibrium as in inflammation. It is thus possible, that we find associations, such as in this case, which seem counterintuitive, but fit well when cytokines with antagonistic properties are found on the same axis, such as association with higher versus lower UAS. Expectedly, the production of several cytokines by *A. simplex* is higher in CU+ patients reaching statistical significance for IL-2, IL-4 and IFN-γ. Interestingly, not only Th2- associated IL-4, but also Th1-associated IFN-γ is higher here. GAA in humans, as well as acute parasitism in a murine model is associated with a simultaneous Th1 and Th2 type response, which parallels our findings.34-36 But also several Con A stimulated cytokines were higher in CU+, with significant results for IL-6 and IL-10. This is interesting, because it reflects an overall higher reactivity of cytokine secreting cells in patients with previous parasitism, not only for specific but also for unspecific stimuli. Thus, in patients in whom previously an active parasitism had stimulated their cells in vivo with specific antigens, these cells are specifically stimulated in vitro with the antigens from the parasite resulting in a Th1/Th2 response typically for helminth infections. These responses were previously demonstrated by us and other authors in human and animal models.34-36 On the other side, total fish intake was higher in CU+ patients, but oily fish intake was lower. We could hypothesize that intake of ω3 PUFA could be lower in CU+ patients and consequently their anti-inflammatory effects were reduced.

TNF-α, IL-17 and TGF-β values were those cytokines to demonstrate most similar production in both CU phenotypes after specific or unspecific stimulation. TNF-α up-regulation has been shown in skin of different types of urticaria.37 Increased TNF-α as well as IL-10 production was also detected in patients with chronic idiopathic urticaria.11 Further TNF-α is significantly increased in sera form chronic idiopathic urticaria patients and is one of the implicated factors contributing to the skin lesions seen in CU, with higher IL-17 secretion when ASST was positive.10 TGF-β, which has positive as well as negative effects on mast-cell function and survival, has been proposed as a pro- as well as anti-inflammatory cytokine.7 A previous study showed higher circulating serum TGF-β levels to be associated with the chronic urticaria phenotype in *Anisakis* sensitized subjects.38 As we were not able to show difference of this cytokine between CU+ and CU-, it is possible that circulating TGF-β levels do not reflect the local production.

Thus, it can be argued that in CU, previous parasitism has no measurable effect on those cytokines pre-
viously known to be produced in higher quantities, whereas cytokines of the Th1/Th2 axis are differentially stimulated by Anisakis in CU+, and anti-/pro-inflammatory IL-10 and IL-6 are unspecifically stimulated in CU.

The clinical relevance of CU+ as a differential entity was further assessed by measuring the clinical and immunological effect of a diet without fishery products, which also parallels absence of contact with A. simplex products.

Improvement was unexpectedly not associated with diet in CU+. Rather, the opposite effect was stated: patients who continued to eat fish had a better UAS outcome. Our results show a complex relationship between fish-eating habits, cytokine production and prognosis, which extends to the whole group of CU patients. Improvement is not only related to the continuation of fish consumption, but also to previous fish-eating habits with higher oily fish consumption associated with improvement, but an opposite effect of total fish intake. Higher initial anti-/pro-inflammatory cytokine ratios showed further a significant association with improvement. In CU+ TGF-β production changed to higher values in those who improved. However, CU-patients showed changes towards higher pro-inflammatory cytokine and lower TGF-β production in those who improved.

These results highlight again the fact that cytokine production and their possible anti- or pro-inflammatory properties and the influence on UAS depend on the urticaria phenotype studied and possibly on other multiple factors. Together, possible factors affecting the immunological and clinical phenotype of CU as well as the prognosis with or without diet will not only depend on contact with A. simplex products, but more importantly on other factors associated with fish-eating habits, which again affect the ratio of pro- and anti-inflammatory cytokines.

Taken together, by phenotyping CU with respect to previous parasitism, we can propose an interesting model, where a complex interaction of A. simplex associated balance between Th1/Th2, but also a pro- and anti-inflammatory balance (IL-10/TGF-β: IL17/IL-6) modulated also by fish intake is associated not only with the phenotype of CU, but also with the prognosis and the outcome when put under a fish free diet.

Dietary intervention should therefore not only take into account the possible missing eliciting factors, but also the possible missing protective factors. When encountering A. simplex sensitization, patients should not be automatically put on a diet without fish in order to reduce contact with A. simplex products. Future studies could now complete this analysis using biomarkers of fish-consumption in order to strengthen our findings based on a questionnaire.

ACKNOWLEDGEMENTS

The study was funded by grants from Fundación Sociedad Española de Alergología e Inmunología Clínica (SEAIC) 2009 and Fundación Mutua Madridense 2009, Spain.

The authors thank Lorena Vega Píris and Francisco Rodríguez Salvanés (IIS-Hospital de la Princesa, Madrid, Spain) for help with statistical analysis, and Laura Fernández Gámez and Duarte-Miguel de Sousa Teixeira for technical support.

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