

REGULAR ARTICLE

Serum immunoglobulin free light chain levels are higher in girls than boys during eosinophilic oesophagitis

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ABSTRACT

Aim: Eosinophilic oesophagitis (EO) is an emerging worldwide disease, closely associated with male gender and allergic disorders. This study investigated the distribution of allergy markers in a cohort of children with EO.

Methods: We analysed allergy markers in 91 children (62 males and 29 females) with EO and a control group of 45 age-matched children who had non-EO gastrointestinal allergic symptoms. The markers analysed were serum cow's milk-specific and hen's egg-specific IgE, thymic stromal lymphopoietin (TSLP), thymus-regulated and activation-regulated chemokine (TARC/CCL17) and immunoglobulin free light chain (Ig-fLC).

Results: In the EO group, cow's milk-specific IgE levels were detectable in 41.9% of males and 62.1% of females and hen's egg-specific levels in 25% of males and 26.9% of females. There was no gender difference in increased TSLP or TARC levels. Kappa Ig-fLC were increased in 5.6% of males and 20.8% of females ($p = 0.058$) and lambda Ig-fLC in 1.9% of males and 33.3% of females ($p = 0.000$). No gender differences were found in the control group.

Conclusion: Our findings suggest that serum TSLP might be a potential marker of EO and TARC of non-EO gastrointestinal food allergies. In EO, serum Ig-fLC appeared higher in females, adding another gender difference to the biology of EO.

INTRODUCTION

Eosinophilic oesophagitis (EO) is a disease of the oesophagus that has become increasingly recognised over the last decade. It affects people of all ages, from infancy to adulthood, and is three times more common in males than females. The symptoms of EO include feeding intolerance and gastro-oesophageal reflux disease symptoms in children and symptoms including, but not restricted to, food impaction and dysphagia in adults. It can be diagnosed by the presence of 15 or more eosinophils per high-power field in endoscopic biopsy samples of the oesophageal mucosa and the exclusion of other disorders associated with similar clinical, histological or endoscopic features, particularly gastro-oesophageal reflux disease. In addition to demonstrating eosinophilia, endoscopic mucosal biopsies show severe squamous epithelial hyperplasia. Most of the time, these occur without any gastric and intestinal involvement (1,2). Clinical symptoms and oesophageal abnormalities do

not respond to treatment with high-dose proton pump inhibitor therapy (1), unlike acid reflux oesophagitis. Food allergy is one of the main trigger factors, and mucosal eosinophilia usually responds to eliminating major food allergens (3) or feeding with an amino-acid based formula.

According to recently updated consensus recommendations (2), important questions surrounding allergies mandate future research. In addition to eosinophilic infiltration, the oesophagi of patients with EO exhibit mucosal mastocytosis and mast cell degranulation, with a transcriptome

Abbreviations

EO, Eosinophilic oesophagitis; IgE, Immunoglobulin E; Ig-fLC, Immunoglobulin free light chains; TARC, Thymus- and activation-regulated chemokine; TSLP, Thymic stromal lymphopoietin.

Key notes

- Eosinophilic oesophagitis is an increasingly common condition in children and adults, at least partially related to an allergic background and three times as common in males as females.
- Our study found that immunoglobulin free light chains (Ig-fLC) were novel markers of allergic conditions and participated in symptom pathogenesis.
- Ig-fLC were higher in females than males during eosinophilic oesophagitis, highlighting another gender difference in this emerging worldwide disease.

divergent from the eosinophil-associated transcriptome (4). Patients also demonstrate an increased density of B cells, immunoglobulin E (IgE)-positive mast cells and increased frequency of expression of IgE heavy chains. These suggest the occurrence of both a local immunoglobulin class switch to IgE and IgE production in the oesophageal mucosa (5).

Bhardwaj and Ghaffari carried out a systematic review of the literature on various serum biomarkers of EO, with respect to their correlation with disease activity and response to treatment (6). They reported that higher levels of blood interleukins (IL), namely IL-5 and IL-15, were found in patients with EO and gastroenteritis than in controls, while another study found that EO patients did not show increased IL-5, but did display increased levels of serum eosinophil-derived neurotoxin. A marked dysregulated expression of a select group of mature microRNA molecules in patients with EO suggested that they could potentially serve as biomarkers of EO, alone, or in combination with other markers. Recent reviews of phenotypic (cell surface) markers and serum and tissue-based markers have included a preliminary study to determine whether Siglec-8 could differentiate patients with various eosinophil-associated diseases (7). The conclusion of both reviews was that further research was needed to fully understand and validate the utility of biomarkers for EO (6,7).

Therefore, this study analysed thymic stromal lymphopoietin (TSLP), thymus-regulated and activation-regulated chemokine (TARC/CCL17) and immunoglobulin free light chains (Ig-FLC) in a cohort of EO patients in an attempt to find reliable and noninvasive markers for EO. During EO, the oesophageal mucosa exhibits an overexpression of TSLP, an IL-7-like cytokine produced mainly by nonhaematopoietic cells as fibroblasts, epithelial cells and different types of stromal or stromal-like cells that interact with immune cells, including dendritic cells (8). Expression of TSLP may influence susceptibility to multiple allergic diseases by regulating basophil haematopoiesis and eliciting a population of functionally distinct basophils that promote T helper 2 cytokine-mediated inflammation (9). Interestingly, a nonsynonymous polymorphism in the TSLP receptor gene on Xp22.3 and Yp11.3 is significantly associated with disease in male patients with EO (10,11). TARC is an important molecule in the development and maintenance of allergic diseases, especially the skin (12). Immunoglobulin free light chains (Ig-FLC) may play a role in the development of mast cell-mediated hypersensitivity-like responses (13–15) and are generated and secreted by plasma cells in parallel to complete antibody production. Additional biological functions of Ig-FLCs are demonstrated as proteolytic and hydrolysing activity (16,17).

This study followed initial research on a limited number of patients (18) and aimed to investigate potential new serum markers for allergy in EO in a larger population. These included TSLP, based on its demonstrated role at local levels, TARC as a marker of local allergic reaction and kappa Ig-FLC and lambda Ig-FLC, which are being detected in serum in an increasing range of clinical conditions.

MATERIALS AND METHODS

Ethics statement

This retrospective convenience cohort study was performed in children with EO referred to the paediatric gastroenterology department of the Hospital Necker-Enfants malades and, before that, the Hospital Saint Vincent de Paul, Paris, France. The study moved hospitals when the researcher took up a new paediatric position. Participants were recruited from the ARSENE cohort, a collection of blood and biopsy samples registered within the French Ministry of Health (number DC-2009-955), and all data were anonymised. According to French law, organisations keeping routinely collected paediatric material need to obtain a declaration from the French Ministry of Health and written informed consent from all parents and all children aged 11 years and older. This means that no ethic committee approval was needed for this study.

Study population

The study was carried out in a population of children consecutively followed up for EO in the paediatric gastroenterology department of the Hospital Saint Vincent de Paul, Paris, France, from 17 June 2009 to 3 February 2010 and in the paediatric gastroenterology department of the Hospital Necker-Enfants malades, Paris, France, from 4 February 2010 to 1 September 2010. According to updated international recommendations (2), upper endoscopy showed mucosal eosinophilic infiltration ≥ 15 eosinophils per high-power field in at least two biopsy samples of the lower oesophagus. Gastric and duodenal biopsies were always taken to rule out eosinophilic gastroenteritis. All the children had been previously treated with a proton pump inhibitor, without any improvement, to rule out mucosal eosinophilia related to gastro-oesophageal reflux. The study population therefore consisted of 91 symptomatic children, 62 males with a mean (SD) age of 6.5 (± 4.5) years and 29 females with a mean (SD) age of 6.7 (± 4.6) years. All the children were born in France between February 1996 and December 2011, and 90% were of European descent, 5% of African descent, 4% of Arabic descent and 1% of mixed ancestry. Samples were obtained as part of the routine procedure for assessing their nutritional status and delineating their elimination diet. Serum samples were taken within one month of endoscopy and no dietary treatment or medication was started between the endoscopy and the serum sample. None of the patients were treated with corticosteroids.

The control group comprised children with non-EO gastrointestinal allergies, who showed clinical symptoms such as gastro-oesophageal reflux, vomiting, abdominal pain, constipation, diarrhoea and failure to thrive. They were sampled from the same cohort, with approximately one control ($n = 45$) for every two cases of EO ($n = 91$). Endoscopy was used to rule out EO or any eosinophilic disorder each time clinical or biological features suggested these. The control group, of 25 males (mean age 4.5 years ± 3.3) and 20 females (mean age 6.0 years ± 3.9), showed no

significant difference in age to the study group. All children were born between October 1994 and March 2009, and 100% were of European descent.

The data recorded for each patient included gender, date of birth, date of diagnosis, ethnicity and family atopy, oesophageal biopsy data, together with the clinical and biological parameters (data not shown).

Skin prick test, atopy patch test and food-specific IgE

Skin prick tests and atopy patch tests were carried out with standardised extracts to guarantee the reproducibility of the allergenic activity. When extracts were not available, fresh foods were used. Skin prick tests were carried out using Stallergènes (Paris, France) extracts and considered positive if the wheal was 3 mm larger than the negative controls. Atopy patch tests were carried out using Finn Chambers® (Phoenix, AZ, USA) coated with Stallergènes extracts. However, we had to use fresh milk to test for that specific allergy as a fresh milk extract is not commercially available in France. The test was considered positive if the atopy patch test exhibited a stronger skin reaction than the negative control at 72 h, notably, the presence of clear redness and palpable infiltration or eczema described by Majamaa et al. (19). Food-specific IgE in serum was measured using the Phadia CapRast® (Uppsala, Sweden). Specific IgE values ≥ 0.35 kU/L were considered increased.

Serum thymic stromal lymphopoietin (TSLP)

The human TSLP ELISA Ready-SET-Go!® (eBiosciences, San Diego, CA, USA) was performed according to the manufacturer's protocol.

Serum thymus- and activation-regulated chemokine (TARC/CCL17)

Plates were coated with monoclonal anti-human CCL17/TARC antibody (2 µg/mL; R&D Systems Europe Ltd., Abingdon, UK) and incubated for 18 h at 4°C. All the incubations were carried out at room temperature, and the wells were washed three times with phosphate-buffered saline containing 0.05% Tween 20 between all the incubation steps. The wells were blocked with phosphate-buffered saline containing 0.05% Tween 20 and 1% BSA for one hour under continuous shaking. Standard (Recombinant Human TARC/CCL17; R&D Systems Europe Ltd) or samples were added and incubated for one hour. Biotinylated anti-TARC detection antibody (200 ng/mL; R&D Systems Europe Ltd) was added and incubated for one hour, followed by incubation with poly-HRP-streptavidin (Jackson ImmunoResearch Europe Ltd, Suffolk, UK) for 40 min. Tetramethylbenzidine substrate solution (Thermo Scientific Pierce, Waltham, MA, USA) was added and incubated for 15 min, and the reaction was stopped by adding 5 mol/L H₂SO₄ (Merck, Whitehouse Station, NJ, USA). Absorbance was read at 450 nm. TARC levels were calculated based on the standard curve for each assay plate and compared to cut-off values described in children diagnosed with atopic dermatitis: 1431 pg/mL for birth to

one year of age, 803 pg/mL for two to five years and 510 pg/mL for six years and over (20).

Serum kappa- and lambda immunoglobulin free light chain (Ig-fLC)

Total kappa and lambda Ig-fLC serum concentrations were determined using an ELISA adapted from Abe et al., as described previously (21). No Ig-fLC reference values exist for healthy or allergic children and young adults aged from one to 20 years of age, although there are indications that Ig-fLC levels are increased in certain allergies (13,16). To obtain reference values for Ig-fLC, we analysed Ig-fLC in 250 children of the same cohort with different allergies – skin, gastrointestinal, respiratory and EO – whose mean kappa Ig-fLC level was 13.8 µg/mL (SD 8.0) and mean lambda Ig-fLC level was 14.8 µg/mL (SD 7.0). Ig-fLC concentrations of above average +SD (for both kappa and lambda Ig-fLC 21.8 µg/mL) were considered increased.

Statistical analysis

All analyses were performed using the statistical software package SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Normal distribution was tested using the Shapiro–Wilk test and variances using the Levene's test. When the data were normally distributed and the variances were equal, a two-tailed two-sample *t*-test was used to compare groups. When the data were not normally distributed, a two-tailed Mann–Whitney test was used to compare the groups and a Satterthwaite correction was carried out for differences in group size. Ig-fLC and ages were compared using one-way ANOVA, followed by Bonferroni's multiple comparison test. Percentages were compared using a two-tailed Pearson's chi-squared test. Values of $p < 0.05$ were considered significant.

RESULTS

Skin prick test, atopy patch test and food-specific IgE

Standard allergic procedures showed that 33% of the EO patients had at least one positive skin prick test, compared with 31% of the control group. In addition, 55% had at least one positive atopy patch test, compared with 82% in the control group. Serum food-specific IgE (Fig. 1A) showed a major dominance for cow's milk, detectable in 48.1% of the children in the EO group and 13.3% in the control group ($p < 0.0001$). Just under a quarter of the EO group (24.4%) had detectable levels for hen's eggs, compared with 8.9% in the control group. No gender differences were found in absolute (Fig. 2A+B) or increased levels (Fig. 3) in the EO or the control group.

Thymic stromal lymphopoietin (TSLP)

Serum TSLP measurements were conducted in 77 of the 91 EO patients (55 males and 22 females) and 37 of the 45 control group patients (22 males and 15 females), as insufficient blood samples were available for some patients. There were missing values in the EO group for seven males (mean age 4.3 ± 3.0 years) and seven females (mean age

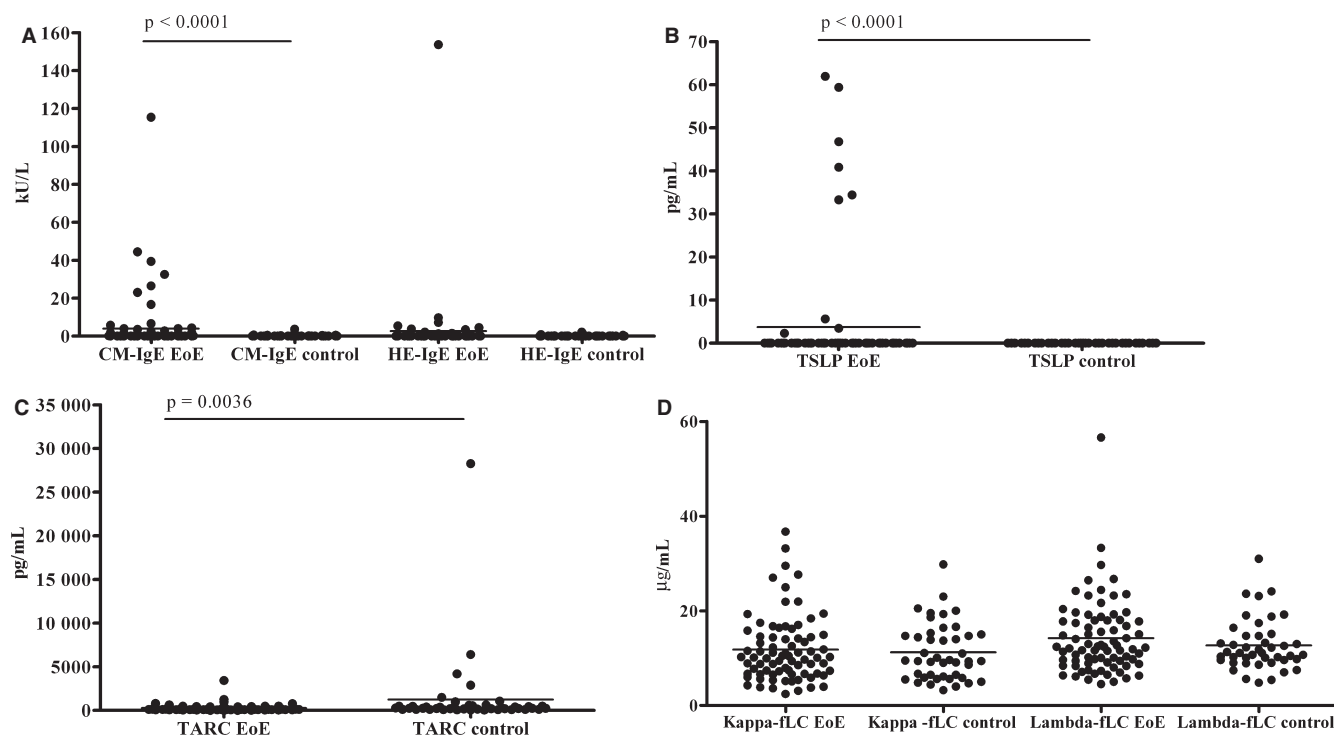


Figure 1 Overall serum levels of cow's milk (CM)-specific immunoglobulin E (IgE) and hen's egg (HE)-specific IgE (A), thymic stromal lymphopoietin (B), thymus- and activation-regulated chemokine (C) and kappa (κ) immunoglobulin free light chains (Ig-fLC) and lambda (λ) Ig-fLC (D) of the eosinophilic oesophagitis study group and control group. Data are represented as individual data points and the mean value (black line). p-value by two-tailed Mann–Whitney test.

3.9 $\pm$ 3.3 years) and in the control group for three males (mean age 5.2 $\pm$ 5.8 years) and five females (mean age 4.0 $\pm$ 1.9 years). Detectable levels (Fig. 1B) were found in nine of the 77 EO patients (11.6%), but none of the control group ($p < 0.001$). No gender differences were found in absolute (Fig. 2C+D) or increased levels (Fig. 3) in either the EO (A) or the control group (B).

Thymus- and activation-regulated chemokine (TARC/CCL17)

TARC levels were measured in the serum of 86 of the 91 patients in the EO group (59 males and 27 females), as there were missing values in three males (mean age 4.0 $\pm$ 2.5 years) and two females (mean age 4.0 $\pm$ 3.0 years). There were no missing values in the control group. Serum TARC levels (Fig. 1C) in the control group were significantly higher than in the EO group ($p = 0.0036$). However, no differences were seen between the genders with regard to absolute (Fig. 2E+F) or increased levels (Fig. 3A+B).

Kappa and lambda immunoglobulin free light chain (Ig-fLC)

Serum kappa and lambda Ig-fLC were analysed in 78 of the 91 EO patients (54 males and 24 females) and in 44 of the 45 control group patients (25 males and 19 females). Kappa and lambda Ig-fLC levels showed no differences between the EO and control groups (Fig. 1D). However, in the EO group, kappa Ig-fLC tended to be higher in females

(14.1 μ g/mL) than males (10.8 μ g/mL, $p = 0.056$) and lambda Ig-fLC levels were significantly higher in females (18.3 μ g/mL) than males (12.4 μ g/mL, $p = 0.002$) (Fig. 2G). These gender differences were not observed in the control group (Fig. 2H). Increased levels showed similar results (Fig. 3A) in the EO group. Kappa Ig-fLC levels were less frequently increased in males (5.6%) than females (20.8%) ($p = 0.058$), and this gender difference was significant for lambda Ig-fLC, at 1.9% for males and 33.3% for females ($p = 0.000$). Again, no gender differences were found in the increased levels in the control group (Fig. 3B).

DISCUSSION

EO is a relatively newly discovered entity, which is recognised in children and adults and is more dominant in males (1). Mediators such as IL-5, IL-13 and eotaxin, cellular infiltrates (eosinophils, mast cells and lymphocytes) and the association with allergic disorders all indicate a T helper 2-associated disease, and allergy testing seems to be of limited use (22). Current treatments include dietary restrictions, and new potential is offered by corticosteroids with targeted immunomodulation (23).

This study focuses on newly explored serum markers for allergies in children diagnosed with EO. The children with EO in our study were comparable to bigger EO series in terms of age, gender ratio, race and standard allergy markers such as skin prick tests and measurements of specific IgE. The 45 children in the control group were

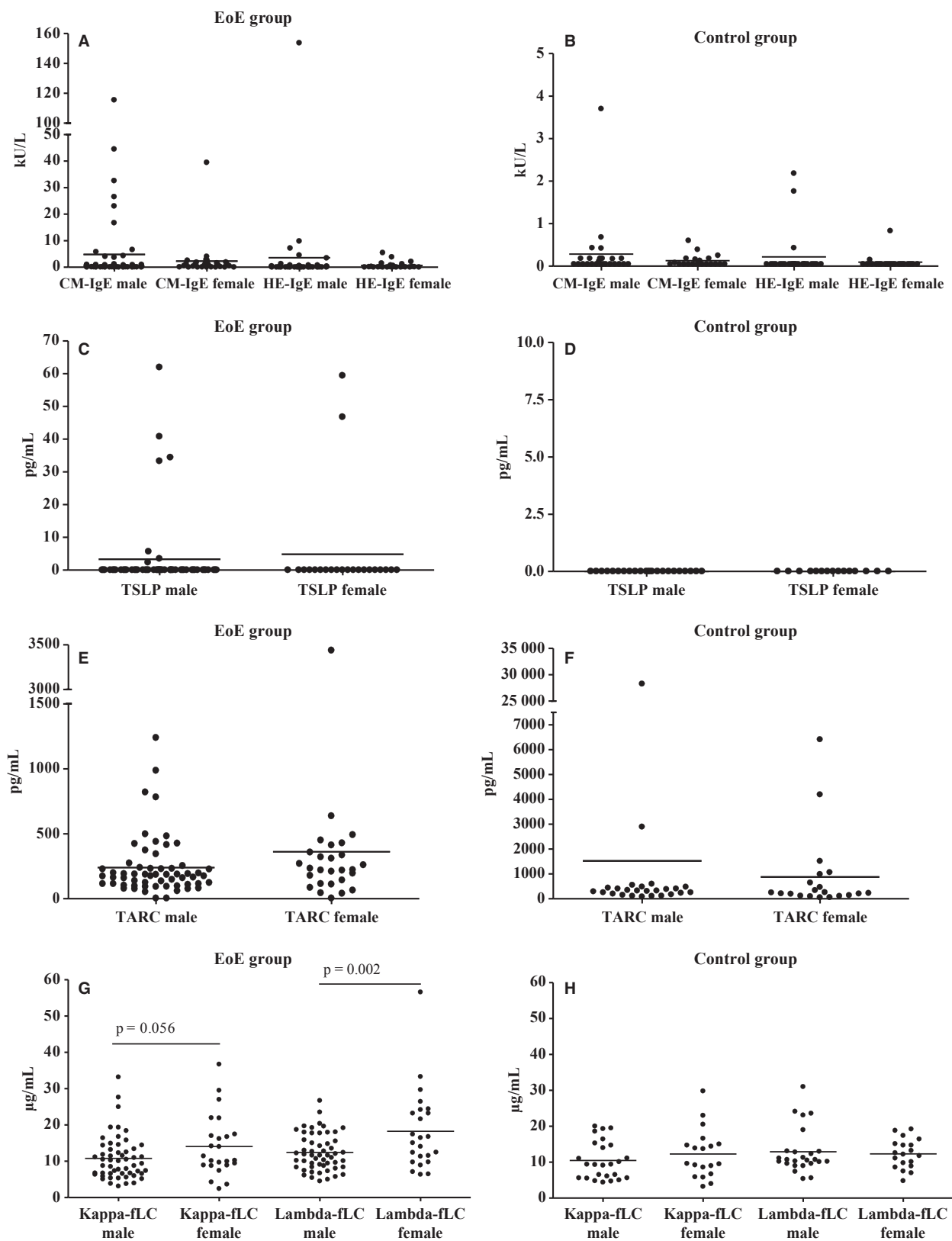


Figure 2 Serum levels of cow's milk (CM)-specific immunoglobulin E (IgE) and hen's egg (HE)-specific IgE of eosinophilic oesophagitis (EO) (A) and control (B) group, thymic stromal lymphopoietin of EO (C) and control (D) group, thymus- and activation-regulated chemokine of EO (E) and control (F) group and kappa (κ) immunoglobulin free light chains (Ig-fLC) and lambda (λ) Ig-fLC of EO (G) and control (H) group, split according to gender distribution. Data are represented as individual data points and the mean value (black line). p-value by two-tailed two-sample *t*-test.

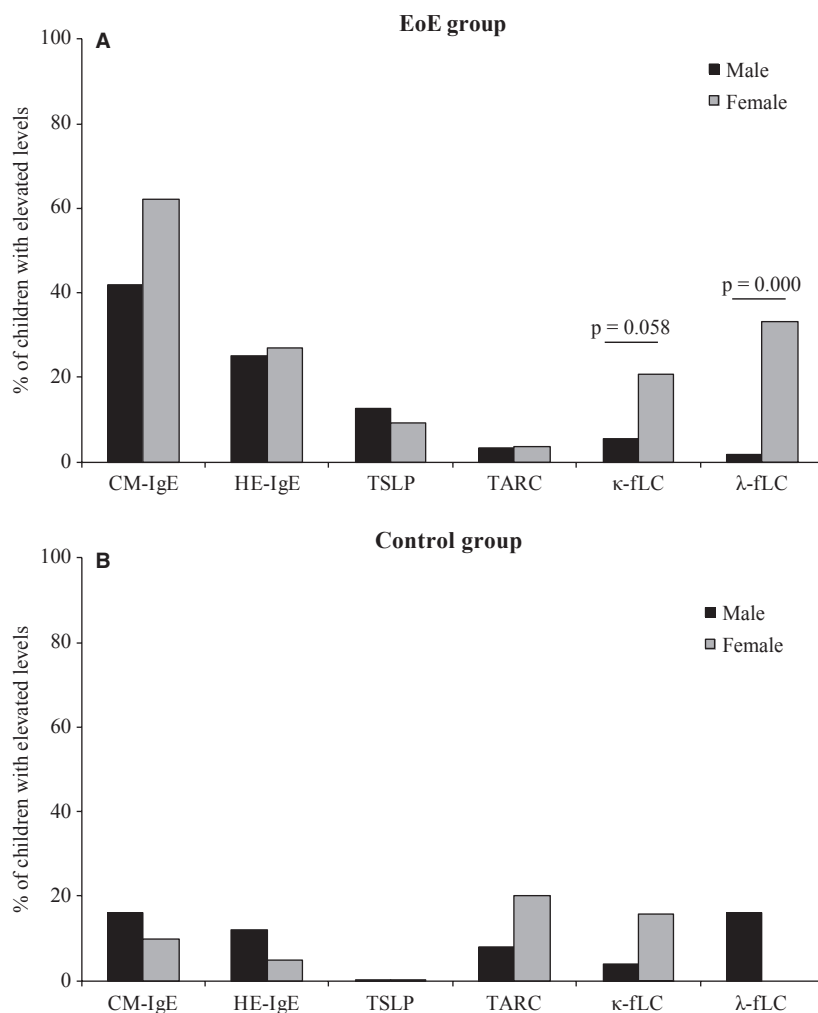


Figure 3 Percentage of children split according to gender distribution with increased serum levels of cow's milk (CM)-specific immunoglobulin E (IgE), hen's egg (HE)-specific IgE, kappa (κ) immunoglobulin free light chains (Ig-fLC), lambda (λ) Ig-fLC, thymus- and activation-regulated chemokine and thymic stromal lymphopoietin according to reference values in the eosinophilic oesophagitis (A) and the control (B) group. Bars represent the percentage of males (grey) and females (black) with increased levels. p-value by two-tailed Pearson's chi-squared test.

diagnosed with gastrointestinal allergies without EO and showed a normal gender distribution and no significant age differences to the EO group.

The study population is part of a large cohort of children with different allergic diagnoses, such as atopic dermatitis, gastrointestinal allergy and asthma, who have been investigated since June 2009 in the legal framework of the ARSENE cohort. The aim was to further investigate new potential serum markers for allergies, including TSLP, TARC and kappa Ig-fLC and lambda Ig-fLC.

Polymorphisms in the gene encoding the cytokine TSLP are associated with the development of multiple allergic disorders in humans, suggesting that TSLP is a critical regulator of allergic diseases (9). Despite being found in the mucosa of oesophageal samples, TSLP rarely appears in detectable levels in serum, suggesting that it acts locally with virtually no release into the blood stream. However, TSLP levels were not detectable in the control group, and

therefore, TSLP might be considered as a potential marker for EO. TARC is a mediator of allergic inflammation, especially atopic dermatitis (12,20), and was detectable in all serum samples, but at lower mean levels than those observed in eczema (12). Of the three children with increased TARC levels in the EO group, only one exhibited active atopic dermatitis with a SCORing Atopic Dermatitis (SCORAD) score of 55.5. We observed significantly higher levels of TARC in the control group than EO group, and these occurred in children with isolated gastrointestinal allergy symptoms, an observation also reported by Oba et al. (24). Serum TARC may therefore provide a useful marker of gastrointestinal food allergies in young children.

Serum Ig-fLC appeared clearly higher in females than males, adding a possible third gender difference to the biology of this disease, in addition to the gender ratio distribution of EO and the nonsynonymous polymorphism in the TSLP receptor gene associated with the disease in

male patients (10). No differences in Ig-fLC levels between males and females were found in other allergies, such as skin allergy (48 males and 27 females), gastrointestinal allergy (23 males and 19 females) or respiratory allergy (four males and three females), as shown in Figure 4.

This study compares a group of children with EO with another group of allergic children without EO. No healthy controls were available in this age range, so the Ig-fLC serum levels were analysed with reference to literature data. Concentrations of serum Ig-fLCs are known to be low at birth and rapidly increase from the first week of life until low normal levels are reached by one year of age. The concentration then gradually increases until approximately 20 years of age, when levels of $13.2 \pm 3.8 \mu\text{g/mL}$ for kappa Ig-fLC and $10.6 \pm 3.1 \mu\text{g/mL}$ for lambda Ig-fLC are found (25). Interestingly, these levels are much lower than the highest ones reached in the same age range in our EO study group (for both kappa and lambda Ig-fLC $21.8 \mu\text{g/mL}$). According to Solling et al. (25), in the five- to nine-year age group, the mean age of our different groups, concentrations of Ig-fLCs are steady. Increased serum kappa and lambda

Ig-fLC have been described in children with a severe form of atopic dermatitis until three years of age (16). Local increases in Ig-fLC are also possible, such as in the nasal mucosa during rhinitis, suggesting a role in nasal hypersensitivity (26). Increased serum IgE or Ig-fLC levels have also been found in cow's milk allergy mouse models, depending on the food allergen used (27,28). Although TSLP, TARC and Ig-fLC seem to play roles in either EO or non-EO gastrointestinal allergies, none of these can currently be used as diagnostic markers, owing to the overlap in levels between EO and non-EO patients.

The role of Ig-fLC in EO is not yet understood. Other immunoglobulins were measured in the same cohort described in this study (29). These found that IgM and IgG, but not IgA, were significantly higher in females than in males. This might indicate that the increased levels of Ig-fLC reflect increased immunoglobulin production in females during EO. On the other hand, Ig-fLC can mediate mast cell activation in an antigen-specific manner (15,30). These studies showed that Ig-fLC can bind to mast cells and that cross-linking the mast cell surface proteins with

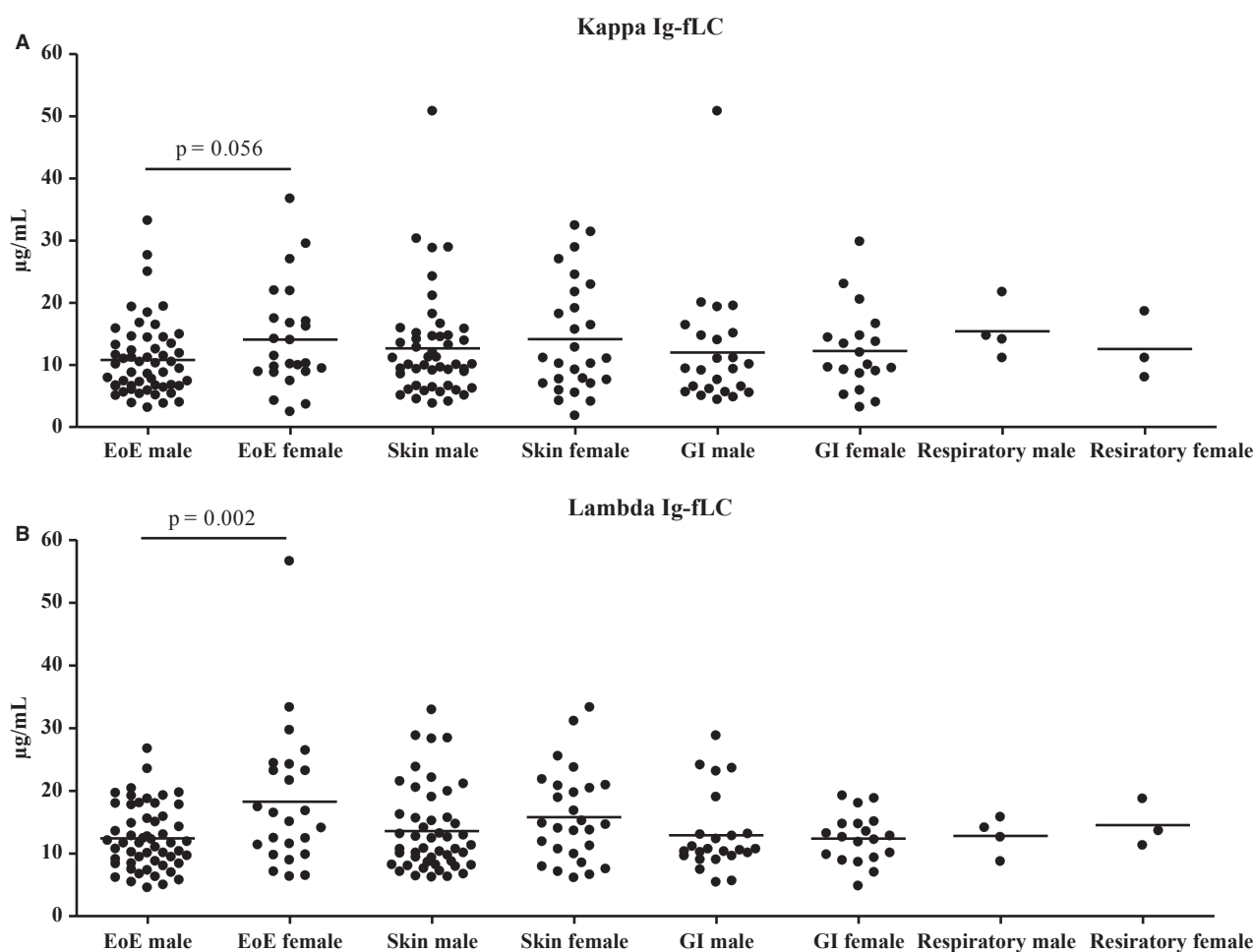


Figure 4 Serum levels of kappa (κ) immunoglobulin free light chains (Ig-fLC) (A) and lambda (λ) Ig-fLC (B) in eosinophilic oesophagitis, skin allergy, gastrointestinal allergy and respiratory allergy. Data are represented as individual data points and the mean value (black line). p-value by two-tailed two-sample *t*-test.

Ig-fLC-induced activation and degranulation of the mast cells. Increased levels of mast cells have recently been identified in the oesophaguses of EO patients (4). Mast cells may be particularly important in disease pathogenesis as they produce an abundance of cytokines that activate eosinophils as well as molecules that directly promote tissue remodelling. Ig-fLC might play an additional role in the local mastocytosis and mast cell degranulation in the oesophaguses of EO patients. Future studies should further delineate if Ig-fLC may contribute to the pathogenesis of EO.

In conclusion, our study of 91 children with EO identified gender distribution as a characteristic of the disease. The gender differences observed in the Ig-fLC plasma levels suggest that the process leading to the disease might include a particular immune process in females that is different from the process in males.

CONFLICT OF INTEREST

Knipping, Knippels and Garssen are employees of Nutricia Research. Colson is employee of Nutricia France.

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