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Food protein-induced enterocolitis syndrome in children: what's known? What's new?

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Key words
food protein-induced enterocolitis syndrome; children; pathophysiology; diagnosis; management

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Summary
Food protein-induced enterocolitis syndrome (FPIES) is an under-recognized and frequently misdiagnosed non-IgE mediated food allergy syndrome. Affected infants show gastrointestinal symptoms few hours after ingestion of the incriminating food. Pathophysiology of FPIES has not yet been clearly defined and needs further characterization. The common allergy tests are not helpful for this disorder and tests for food specific IgE are usually negative. A diagnostic oral food challenge (OFC) is the method to confirm the diagnosis of FPIES. This review summarizes what is known about epidemiology, pathophysiology, clinical characteristics and diagnosis and what's new about therapeutic options of FPIES.

List of abbreviations
APT, atopy patch testing; FPIES, food protein-induced enterocolitis syndrome; Ig, immunoglobulin; IL, interleukin; IFN, interferon; MCH1, major histocompatibility complex type 1; OFC, oral food challenge; PBMCs, peripheral blood mononuclear cells; Treg, regulatory T; SPT, skin-prick test; SOTI, specific oral tolerance induction; Th2, T helper 2; TGF-β1, transforming growth factor-β 1; TNF, Tumor Necrosis Factor.

Introduction

Food allergy covers a range of diseases that result from an aberrant immune response to food antigens, which can be either non-immunoglobulin (Ig) E or IgE-mediated. The non-IgE mediated diseases may be caused by direct T cell antigen response and mediated by proinflammatory cytokine (1). Food protein-induced enterocolitis syndrome (FPIES) is an uncommon, non-IgE-mediated food allergy (2).

FPIES begins in the first month of life, resolving in about 38-100% of cases at the age of 3 years (3). The initial reaction generally occurs on first or second exposure to the involved food, usually milk or soya in formula-fed infants (4). In a retrospective chart review of patients seen in the Allergy Section at The Children's Hospital of Philadelphia with International Classification of Diseases Ninth Revision code 558.3 (Allergic Gastroenteritis and Colitis) between 2007 and 2012, 43.5% of patients who had a reaction with milk often react to soya (5). At least in Europe, FPIES caused by fish/shellfish is the most frequent solid-food FPIES (6).

The number of reports about FPIES has increased over the past few years, and reactions to new trigger foods such as green peas,

grains, chicken, barley, rice, vegetables, poultry, fruit, oat, lentils, sweet potatoes, egg, peanuts, and turkey have been described (2,7). However, less than 300 cases have been reported so far in literature and the largest series include only 44 children (8).
Breastfeeding was previously thought to be a protective factor, but some reports documented patients with FPIES who reacted to cow's milk or soya protein passed through the breast milk (9,12). Food sensitization in FPIES that can occur through breast milk is, in fact, also described (13).
A link of FPIES with methicillin-resistant *Staphylococcus aureus* and enterotoxigenic *Escherichia coli* has been also described, suggesting that in some cases, enteral infections can play a role in the progress in FPIES (14).
Affected infants typically demonstrate severe gastrointestinal symptoms that usually occur within the 9th month of life (2). The symptoms, that appear 1 to 4 hours after ingestion of the offending food, are severe protracted vomiting, pallor, diarrhea, lethargy and hypovolemic shock (15,16). Additional findings consist of methemoglobinemia, increased gastric juice leukocyte levels, metabolic acidosis, neutrophilia and thrombocytosis (17,21). All these symptoms and signs are not disease-specific, so FPIES is frequently misdiagnosed and treated as sepsis, surgical abdominal emergency, severe gastroenteritis or metabolic disorders (2).
Several gastrointestinal diseases in children have been attributed to immunologic reactions to food proteins (4). These reactions can involve all the gastrointestinal tract, from mouth to rectum (4).
Even though it is not easy to categorize food protein-induced gastrointestinal disorders in one notion, a consensus conference in 2000 clarified the clinical differences and pathophysiologic process involved in each of these conditions (22). These diseases can be classified as IgE mediated (oral allergy syndrome and immediate gastrointestinal hypersensitivity), non-IgE mediated (food protein-induced enterocolitis, food protein-induced proctocolitis, food protein-induced enteropathy, celiac disease) or mixed (allergic eosinophilic esophagitis, allergic eosinophilic gastritis, allergic eosinophilic gastroenterocolitis) (4).

Epidemiology

FPIES is a rare non-IgE-mediated gastrointestinal allergic condition with a mild male predominance (50-60%) (23).
A complete estimation of FPIES epidemiology is limited by the lack of a universally accepted definition and the publication of few prospective population-based case series. Older nonpopulation-based studies reported an average of 1-15 cases per year, but recent studies have reported a number as high as 90 cases per year. The incidence of FPIES to all incriminating food was one in 10,000 infants less than 2 years of age, that closely corresponds to the Australian reported incidence of eosinophilic esophagitis in children of 0.9 per 10,000 (24).
In a recent prospective population-based study in Israel, the incidence of cow's milk FPIES was 0.34% in the first year of life (8). There is a regional variation about involved foods, rates of combined cow milk and soya FPIES, and multiple food group FPIES (23). Eczema and a family history of atopy are frequently present at diagnosis and about 1/10 infants have IgE food allergies and siblings are seldom affected (23,25,26). Acute FPIES is primarily a disorder of young infants, while chronic form usually presents in neonates (23).

Pathophysiology

FPIES is a cell-mediated food hypersensitivity but its exact immune mechanism is not known (1,2).
Although various immunologic alterations have been described in FPIES, little is known about its pathophysiology, and it requires further characterization. An early onset IgE-mediated reaction doesn't seem to be involved: this hypothesis is supported by negative skin prick tests (27). The involvement of antigen-specific T cells and their production of proinflammatory cytokines in the control of intestinal barrier permeability has been suggested, but humoral immune responses can also be interested (4).
The best-known oral tolerance is an immune response involved in the tolerance to food antigens induced via the gut. It is associated with the production of local and systemic immunological tolerance due to induction of regulatory T (Treg) cells, clonal deletion and T cell anergy. Many data supported the role of CD4+CD25+Treg cells in mediating oral tolerance against dietary antigens, by suppression of the effector T cells, a characteristic partly cell contact-dependent. On the other hand, also feeding experiments in mice and indirect evidence as patients with enteropathy, immune dysregulation, polyendocrinopathy, X-linked syndrome, or X-linked autoimmunity-allergic dysregulation syndrome that lack this T-cell subset support this hypothesis (28,29).
Van Sickle et al. suggested a T-cell mediated response because of the in vitro proliferation to food antigen of peripheral blood mononuclear cells (PBMCs) in patients with FPIES as compared with non-allergic controls (30).
Antigen-specific T cells seems to be involved releasing high levels of proinflammatory cytokine Tumor Necrosis Factor (TNF)-α that act synergistically with interferon (IFN)-γ to increase intestinal permeability, contributing to the antigens influx into the submucosa with additional antigen-specific T cells activation (4,28). So, an alteration in intestinal permeability related to TNF-α production by PBMCs characterizes gastrointestinal allergy to cow's milk (4,28,31). Another study reported that lower quantity of cow's milk protein is required to induce TNF-α secretion from PBMCs of patients with active intestinal cow's milk allergy, as compared with patients with skin rather than

gastrointestinal sensitivity or those whose allergy had resolved (32). Moreover, fecal TNF-α levels were augmented in the stool after positive cow's milk challenges in subjects with enterocolitis (33). All these studies suggest that TNF-α has an important function in the pathophysiology of the FPIES (27), although the precise mechanism of this disease is still uncertain.

A relative failure of expression in transforming growth factor-β1 (TGF-β1) receptor can also be involved (34). The regulatory cytokine TGF-β1 acts protecting gut epithelial barrier from penetration of foreign antigens, stimulating extracellular matrix proteins, fibronectin and collagen synthesis and altering the expression of integrins, that enhances the binding between cells and matrix proteins. Moreover, it induces T-cell suppression. In young infants, a reduced activity of TGF-β1 against the barrier-disrupting effect of T-cell cytokines can be involved in the pathogenesis of FPIES. Older children showed increased TGF-β1 level after weaning, justifying the reason why FPIES is only seen in young children (4,34,35). On the other hand, TGF-β blockade also induced IFN-γ production (28,36).

So, the acute FPIES reaction appears to be related with a Th2 skewing of the T-cell cytokine production (28).

Interleukin (IL)-10 amplified expression is linked with the ingestion of the same food when tolerance is reached. So, IL-10 may exert an important function in regulating Th1 and Th2 response, especially at the gut level (37,38). Conversely, IL-4 is an inflammatory cytokine implicated in the gut immune response by blocking the induction of Foxp3 with subsequent generation of inducible regulatory T cells (28,39).

The hypothesis that T helper 2 (Th2) activation with high IL-4 levels can be involved in FPIES pathophysiology, while IL-10 may regulate the Th2 responses involved in the immunotolerance acquisition, was supported by an increase in T-cell IL-4 expression and a decrease in IFN-γ observed in an 8-month-old child with acknowledged FPIES to rice, after a positive challenge with rice; in contrast after acquisition of oral tolerance, an increase in T-cell IL-10 expression was observed after rice challenge 6 months later after a negative challenge (28,40).

The potential involvement of humoral immune response in the pathophysiology of FPIES includes an increase of specific IgA and IgG and a decrease in specific IgG4 antibodies (4,28,31,41). Some authors demonstrated an increase of milk protein-specific IgA in patients with FPIES, not associated with elevated IgG1 or IgG4 antibodies compared with controls. In fact, a lack of milk protein-specific IgG4 may have a role in FPIES, having a protective role in competing with other subclasses that could activate complement (28,41).

Symptoms of food protein-induced enterocolitis syndrome

Signs and symptoms and the natural course of this disease have been explained through many reports, usually single cases or small case series (4,15-18,25,42-47). The clinical features of FPIES range from mild symptoms, such as mild emesis and/or diarrhea, to severe and potentially life-threatening clinical features.

FPIES may be divided into two forms: acute and chronic forms (48). The main characteristics in acute and chronic FPIES are shown in **figure 1**.

FPIES is a non-IgE-mediated reaction that usually presents with acute severe repetitive and profuse vomiting and diarrhea (sometimes bloody) within 1-3 h following the intake of the offending food, with subsequent dehydration and shock in 20% of the cases (17,25,49). Generally, 75% of infants with FPIES are acutely ill, comprising 15-20% with hypotension in need of hospitalization (4). Additional frequent symptoms are cyanosis, pallor, lethargy and hypothermia (4).

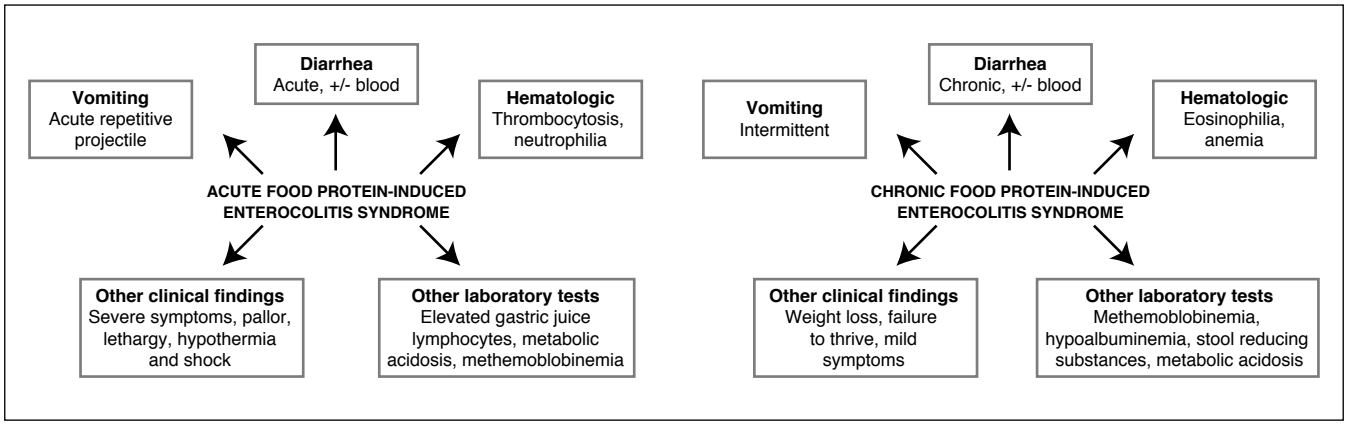
In a recent study counting a great amount of subjects, the main clinical feature was vomiting (100%), followed by lethargy (85%), pallor (67%) and diarrhea (24%) (18). Manifestations resolve usually within 24-48 h of elimination of the causative food (48), even if in the early reports, up to 15-20% of children presented in a hypovolemic shock necessitating fluid resuscitation (4). Anyway, in another recent study, no case of significant hypotension was reported. In this case, all were treated with oral rehydration (8). This difference might be due to under-representation of mild FPIES due to selection bias in previous studies (4).

A recent study demonstrated a mean age of 5.5 months at initial presentation (18).

Chronic FPIES appears if the antigen is being taken regularly and it has been described in young infants fed with cow milk or soya-based formulas (4,15,16,43). Symptoms usually start in the first month of life with intermittent vomiting, persistent diarrhea, poor weight gain and failure to thrive (4). This condition can evolve to acidemia and shock (4). If introduction of the involved food in the diet of the child is delayed, such as in exclusively breastfed infants, the onset of symptoms may occur at an older age (up to 9 months), even if the mother is ingesting the offending foods in her diet (4). However, there are several reports of FPIES onset subsequently early infancy (50). Elimination of the involved food should lead to fast remission of symptoms in some days. The chronic early phase may be followed by an acute phase if the antigen is eliminated from the diet for at least 2-3 days and then reintroduced, with symptoms starting about 1.5-2 h after intake of the offending food (15). Repetitive and severe vomiting usually takes place 1-3 h and diarrhea occurs 5-8 h after ingestion (7).

Methemoglobinemia has been reported in infants with severe reactions and acidemia. Murray and Christie described 35% of 17 cases of PFIES with transient methemoglobinemia (51).

***Figure 1** - Comparison between acute and chronic food protein-induced enterocolitis syndrome: clinical and laboratory findings. Modified from (48).*



These patients appear listless and ashen-gray (4). Methemoglobinemia is due to an elevation of heme oxidation because of increased nitrates in the intestine caused by reduced catalase activity during inflammation (51).

Diagnosis of food protein-induced enterocolitis syndrome

The diagnosis of FPIES is often difficult and delayed because of the lack of classic, unequivocal, typical allergic symptoms and the absence of biomarkers (20,52). Serum food-specific IgE and skin-prick test (SPT) are generally negative (9,18,25,49). But some investigators observed that 4-25% of children with FPIES originally have or develop food-specific IgE (5,8,53,54). Patients with detectable IgE of the incriminating food are called atypical FPIES (17,55). Atypical FPIES has been reported in older children (55) and also in adults (56), generally associated to some specific food, such as fish and shellfish ingestion.

The NIAID Food Allergy Guidelines recommend using the clinical history and oral food challenge (OFC) to diagnose FPIES (57).

Nevertheless, if medical history suggests that the children have experienced hypotensive episodes or multiple reactions to the same food, the diagnosis can be based on a history and lack of symptoms when the culprit food is removed from the alimentation (58). The original diagnostic criteria, proposed by Powell, were based on the fact that exposure to the causative food causes repetitive vomiting and/or diarrhea within 4 h, excluding other causes for these symptoms; symptoms are restricted to the gastrointestinal tract; the removal of the causative protein from the diet of the children causes remission of symptoms; and a standardized OFC or isolated re-exposure causes typical symptoms (43). In 2013 Miceli Sopo et al. (59) suggested criteria to support the clinician to establish a diagnosis of FPIES, which consist of the following: less than 2 years of age at first episode (not mandatory); repetitive pallor, vomiting and lethargy within 2-4 h, and generally lasting less than 6 h, after the intake of the causative food; lack of symptoms that advocate an IgE-mediated response; remission of the symptoms due to the elimination of causative food; relapse of symptoms within 2-4 h of re-exposure to the same trigger food.

Classic allergy testing such as prick skin testing or in vitro testing for food specific IgE are not useful to diagnose FPIES as they are negative.

Atopy patch testing (APT) is a useful tool for a delayed, non-IgE-mediated reaction to food. It is performed by dissolving small quantities of causative food proteins in saline and placing the mixture in aluminum Finn chambers (27). Fogg MI et al. (27) tried to establish if the APT could be able to detect the presence or absence of FPIES in nineteen infants. The results of APT and oral food challenge (OFC) were compared and used to determine specificity and sensitivity of the APT. APT predicted OFC results in 28/33 instances. 16 cases of FPIES were confirmed by OFC and in these 16 cases the APT was positive to the suspected food. The APT was found positive in 5 instances where the OFC was negative. All 12 patients with a negative APT had a negative OFC to the suspected food. So APT appears to be a promising diagnostic tool for the diagnosis of FPIES (27) Jarvinen et al. (60) evaluated APT in 38 children with FPIES and undergoing OFC. They estimated sensitivity of 11.8% and specificity of 85.7%. However, to date, the diagnostic utility of this test remains still unclear (58).

Although the diagnosis of FPIES is based on clinical features, in the acute cases of FPIES, laboratory exams can document neutrophilia and thrombocytosis, peaking about 6 h post-ingestion, and, in the more severe cases, methemoglobinemia and meta-

bolic acidosis can also occur. In chronic cases, hypoalbuminemia, anemia, and also eosinophilia can be present. Radiologic evaluation or other procedures can demonstrate nonspecific abnormalities. Further studies looking at the phenotypes of FPIES are required to recognize clinical subtypes, and to understand the predisposing factors for developing FPIES (48).
The majority of cases of FPIES appear in infants and resolve by school age, but FPIES can occur at any age and persist into teenage and adult years (61).
The presence of specific IgE antibodies to the trigger food defines an "atypical form" of FPIES. This is characterized by a reduced possibility of developing tolerance and a probable evolution to typical IgE-mediated hypersensitivity. Indeed, the shift from non-IgE-mediated milk-protein induced enterocolitis syndrome to IgE-mediated milk allergy has been described (62), even if it is infrequent.

Differential diagnosis of food protein-induced enterocolitis syndrome

A detailed clinical assessment associated to laboratory findings, is required to lead the clinicians to diagnose FPIES.
In acute forms of FPIES, the differential diagnosis comprises (52):
- sepsis or other infectious diseases
- acute gastrointestinal episodes
- surgical emergencies (necrotizing enterocolitis or pyloric stenosis in neonates)
- food allergies (allergic proctocolitis, anaphylaxis, IgE-mediated food allergy).

In chronic forms of FPIES, the differential diagnosis comprises (52):
- malabsorption syndromes (such as celiac disease) (63)
- metabolic disorders (amino acid metabolism disorders, organic acidemias, urea cycle defects, some inherited energy metabolism disorders)
- primary immunodeficiencies (64)
- psychosocial conditions (Munchausen by proxy syndrome, food aversion)
- neurological conditions
- infectious conditions (e.g. HIV, Salmonella, Yersinia)
- coagulation defects
- other types of non-IgE-mediated food allergy.

Management of food protein-induced enterocolitis syndrome

To date, the elimination of the causative food from the diet of the little patient is the only therapy for FPIES.
The intake of food and nutritional status should be monitored and advice on added protein, micronutrients and kcal should be provided when necessary (65).
Sicherer (50) has underlined that the management of FPIES is based on the symptoms and on recent knowledge of its immunopathogenesis that is yet incomplete. **Table 1** shows the management of acute and chronic FPIES.
The treatment of acute forms of FPIES is mainly supportive with continued fluid resuscitation. Patients need monitoring until they become hemodynamically stable (35).
Intravenous fluids and steroids should be used in acute and severe forms of FPIES with profuse and repetitive vomiting, bloody and severe diarrhea, hypotension, pallor, hypotonia and lethargy (66). The role of epinephrine has still not been elucidated, and the consistency of the reports on the epinephrine usage - in treating hypotension when intravenous fluids and steroids fail (50,67,68) or in cases with severe lethargy and without

Table 1 *- The management of acute and chronic food protein-induced enterocolitis syndrome (FPIES) (66,67).*

Acute FPIES	**Chronic FPIES**
rehydration fluids: • in the mild form of FPIES: oral rehydration fluids • in moderate to severe form of FPIES: intravenous fluids (20 ml/kg boluses of isotonic saline)	removal of offending food from child's diet
intravenous steroids: methylprednisolone 1 mg/kg (max 60-80 mg)	if dehydration: intravenous fluids
in severe form of FPIES or in case of PFIES unresponsive to fluids: vasopressors for hypotension	in case of cow's milk-FPIES: use soy alternative (following a supervised oral food challenge), casein hydrolysate, or elemental formula
in case of acidemia: bicarbonate	in case of acidemia: bicarbonate
in case of methemoglobulinemia: methylene blue	in case of methemoglobulinemia: methylene blue
supplemental oxygen	

hypotension (59) - is debatable, because the studies enrolled a small number of patients without any controls. Some investigators reported the rapid resolution of symptoms (such as vomiting and lethargy) within 10-15 min after the use of ondansetron hydrochloride, a serotonin 5-HT3 receptor antagonist utilized mostly as an antiemetic, (0.2 mg/kg/dose) in 5 children above 3 years of age diagnosed with FPIES, who had an adverse reaction during the OFC (69). In chronic FPIES with dehydration it is often necessary to use bicarbonate to correct acidosis and methylene blue for methemoglobulinemia, unlike acute forms in which these treatments are rarely necessary (66). The critical cases of chronic FPIES should be rehydrated with either oral or intravenous fluids, and temporary bowel rest and parenteral nutrition can be used in the most severe forms (66).

The clinical treatment of a patient with FPIES includes also 3 key points: recurrence of acute episodes for the unintentional intake of offending food; dietary introduction of at-risk foods; acquisition of tolerance with periodic re-evaluations (66).

Recurrence of acute FPIES episodes due to unintentional intake of offending food should be carefully taken into consideration. If it occurs, the patient should seek medical attention (50), but if symptoms start in the absence of medical assistance, the oral intake of fluids and steroids is recommended and it is supportive to give patients an action plan, explaining symptoms and recommending treatment of acute reactions (66).

The primary recommended managements are steroids and intravenous fluids. In mild-to-moderate cases, oral rehydration should be sufficient (66).

Regarding the dietary introduction of at-risk foods, it has been recommended not to introduce foods that cannot be tolerated (typically cereals, cow's milk, poultry and legumes) during the first year of life, in addition to that identified as offending food. Then, they can be introduced in the diet for the first time in hospital, performing an oral food challenge (66).

The third key point is the acquisition of tolerance that depends on the involved food. In fact, children with FPIES caused by cow's milk have a good chance of acquiring tolerance at the age of 18-24 months. But insufficient data are available to date for other involved foods, so that it is suggested that an OFC should be performed about 1 year after the last acute episode (66).

Future therapy will also be based on the increased understanding of pathogenesis of FPIES, on different phenotypes, and on the use of more effective management for acute episodes.

New therapeutic strategies in food protein-induced enterocolitis syndrome

To date, the elimination of the causative food from diet of the little patient is the only therapy for FPIES (70). For children with IgE-mediated cow's milk or egg allergy, instead, Specific Oral Tolerance Induction (SOTI) is one of the therapeutical options (53). In addition, some children with IgE-mediated cow's milk or egg allergy may tolerate well-cooked causative food (71,72). SOTI is maybe of limited benefit in FPIES cases, due to its rare persistence after 3-4 years of age. Because of FPIES is a non-IgE mediated food allergy syndrome, should not benefit from cooking of the causative food, which can decrease protein allergenicity destroying the conformational epitopes (73). In fact, a case of egg FPIES with adverse reaction after ingestion of a baked cake prepared with eggs supported this hypothesis (74). Nevertheless, Miceli Sopo et al. (70) did not confirm this theory observing that 4/7 children with FPIES tolerated the well-cooked (baked) causative food.

Contrasting data about the utility of cooking causative food suggests that this aspect should be more elucidated and investigated.

If FPIES pathogenesis is only based on cell-mediated response, tolerance versus well-cooked causative food could not be possible in patients with active FPIES, as conformational epitope denaturation due to high temperature should not obstruct antigen-receptor link, as for IgE-mediated allergies (70). In fact, the recognition by T lymphocytes of antigenic peptides exposed (after protein degradation) on cellular surface of dendritic cells by major histocompatibility complex type 1 (MCH1) receptors is not specific for allergen and independent from its structural conformation (75).

However, a number of factors could suggest a probable function for specific IgE in the pathogenesis of FPIES (**table 2**).

Several expert authors have postulated that FPIES may also be IgE-mediated (4,78). These observations, together with the observations of Miceli Sopo S. et al (70) that part of their children with FPIES tolerated the well-cooked (baked) causative food, makes the question suitable for further analysis. In addition, finding specific IgE for ovalbumin, that is a thermolabile egg protein, in one of their children, makes this hypothesis consistent. In addition, these authors postulate that phenotypes of FPIES mediated by specific IgE against the causative food could exist. So, it can be concluded that some of these children could tolerate the causative food if well-cooked due to the degradation of the conformational causative epitope.

Conclusions

In conclusion, FPIES is an unrecognized non-IgE-mediated food allergy usually caused by cow's milk and soya in children. Early recognition of symptoms of FPIES and removal of the offending food is imperative to prevent misdiagnosis and mismanagement of symptoms that might mimic others conditions (such as viral illness or sepsis) and lead to failure to thrive when food is chronically present in the diet.

Although there are advances in knowledge of FPIES, a better characterization of this important and often misunderstood dis-

Table 2 *- Hypothesis of probable function of specific IgE in pathogenesis of FPIES.*

in the same patient, FPIES with undetectable IgE for the causative food may co-occur with food IgE-mediated allergy for other foods (76)
a number of FPIES develop to IgE-mediated gastrointestinal anaphylaxis (77)
in the literature is described a case with symptoms of a pathology similar to FPIES, seemingly not IgE-mediated, that had specific IgE detect only at duodenal site (78)
some studies report that 4-25% of children with FPIES initially have or develop food-specific IgE (5,8,20,54)
although some children present symptoms before 2 h from the intake of the causative food, the time of symptoms occurrence is generally 2 h; it is neither distinctive for cell-mediated responses, which habitually take place later, nor of classic IgE-mediated responses; time of symptoms occurrence in FPIES appears to be intermediate between non IgE-mediated and IgE-mediated reactions (70)

ease is urgent, in order to identify biomarkers and to develop new treatment strategies.

Conflict of interest

The authors declare that they have no conflict of interest.

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Vitamin D receptor variants and uncontrolled asthma

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Summary
Background. *Asthma is a common childhood respiratory disease, affecting around 20% of Irish children. In other populations, vitamin D receptor (VDR) polymorphisms have been associated with asthma risk. We aimed to investigate the association between 2 VDR polymorphisms and uncontrolled paediatric asthma.* ***Methods.*** *44 asthmatic children and 57 healthy volunteers were studied. The VDR TaqI gene variant in exon 9 (T/C) (rs731236) and ApaI (rs7975232) in intron 8 (C/T) were determined, using TaqMan® Assays. The lung function, serum 25-hydroxyvitamin D (25OHD) levels and other biomarkers of allergy, immunity, airway and systemic inflammation were assessed.* ***Results.*** *The distribution of T and C alleles and genotype frequencies differed significantly between asthmatics and controls for both polymorphisms ($p < 0.05$). A significant association was found between both TaqI (OR = 2.37, 95% CI (1.27 - 4.45), $p = 0.007$) and ApaI polymorphisms, and asthma risk (OR = 2.93, 95% CI (1.62 - 5.3), $p = 0.0004$). No association was observed between genotypes and 25OHD levels, lung function and other biomarkers, with the exception of Interleukin-10 (IL-10) and white blood cells count (WBC). IL-10 levels were lower in asthmatics with TC genotype for TaqI polymorphism ($p < 0.01$) and were higher in patients with TT genotype for ApaI ($p < 0.01$). WBC were higher in patients with TC and CC genotypes for TaqI ($p < 0.05$) and lower in TT genotype for ApaI ($p < 0.05$).* **Conclusion.** *TaqI and ApaI polymorphisms are associated with asthma in Irish children. Further studies are warranted to investigate the importance of decreased IL-10 levels in paediatric asthmatics with specific genotypes.*

Introduction

Asthma is a chronic heterogeneous respiratory disease with both genetic and environmental components. It is the most common chronic disease in Ireland, affecting about 20% of children. Vitamin D receptor (VDR) polymorphisms are associated with asthma and allergy susceptibility (1,2). VDR is widely expressed in human lungs throughout the full epithelial layer, as is 1a-hydroxylase (3), which is responsible for the formation of the active vitamin D metabolite, 1a-calcitriol ($1\alpha,25(OH)_2D_3$). Vitamin D might influence the regulation of adaptive and innate immune functions, and the proliferation and differentiation of many cell types (4), and it may influence airway remodelling (5).
Vitamin D deficiency (VDD) is common worldwide, particularly in children (6,7), and several medical conditions are associated with low serum levels of 25-hydroxyvitamin D (25OHD), including asthma (8,9). It remains controversial whether vitamin D supplementation, which is broadly recommended for bone health, has significant effects in children with asthma. In our recent study of Vitamin D supplementation in paediatric asthma in Ireland, 50%

of the children were vitamin D deficient (25OHD levels < 50 nmol/L) (10), but Vitamin D supplementation led to improved asthma control only in selected children, suggesting that VDR genetic variants may influence the effects of Vitamin D on the asthmatic airway. Some children who did well during the study have low vitamin D levels at the baseline, and some have high (25OHD levels > 50 nmol/L), but only 44% of patients achieved 25OHD levels over 100 nmol/L after supplementation. We used blood samples from the study mentioned above (10) for our genetic investigations.
The VDR binds to its ligand $1\alpha,25(OH)_2D_3$. It belongs to the nuclear receptors family of trans-acting transcriptional regulatory factors and it shows a sequence similarity to the thyroid's and steroids' hormone receptors. The VDR gene is known as a pleiotropic gene, and is associated with numerous conditions - such as autoimmune, inflammatory, and allergic diseases, including asthma. The gene maps to chromosome 12q13.11 (11), contains nine exons with at least six isoforms of exon 1, encodes a 427 amino acid protein.
Han et al. suggested that VDR polymorphisms, rather than vitamin D levels, could be developed as biomarkers for asthma susceptibility (12). The association between genetic variants of VDR and paediatric asthma has been studied in different ethnic groups (13-16). Over 900 genes may be transcribed by VDR (17,18). Jolliffe et al. suggested that variation in VDR might prove a more important determinant of the expression of diseases like asthma than circulating 25OHD (19).
Several single nucleotide polymorphisms (SNPs) in the VDR gene have been discovered, including ApaI and TaqI, which are named after the corresponding restriction enzymes used in restriction fragment length polymorphism (RFLP) analysis.
In this pilot study we aimed, first, to determine the VDR gene variants TaqI in exon 9 (T/C) and ApaI in intron 8 (C/T) in symptomatic paediatric asthmatics, and in healthy volunteers in Ireland; secondly, to investigate the impact of these polymorphisms in asthma susceptibility in relation to lung function, 25OHD, and other indices; and finally, to examine the possibility of using these polymorphisms as potential biomarkers for asthma.

Methods

The study was carried out at the National Children's Hospital, Tallaght, James Connolly Memorial Hospital Dublin, and Biomnis Ireland (Dublin, latitude, 53°N) after receiving institutional review board approval from both hospitals, and having obtained consent from parents, guardians and the healthy adults who were involved.

Subjects and study design

Asthmatic children were recruited from paediatric respiratory out-patient clinics for a vitamin D supplementation study (the trial was registered at ClinicalTrials.gov. Identifier: NCT02428322) (10). Our 44 subjects were Caucasian, aged 6-16, and established on anti-asthmatic medication with previous diagnosis of uncontrolled asthma according to the Global Initiative for Asthma 2011 guidelines (20). The healthy 57 subjects had no personal or family history of asthma or other respiratory illnesses, or bone, articular, renal or any other chronic diseases. Controls (healthy subjects) were recruited for this study of VDR. We examined two RFLPs in the VDR gene in both groups. We also studied the relationship between the polymorphisms and different biomarkers and subjective and objective asthma parameters in a cohort of asthmatic children. All these patients were known uncontrolled asthmatics on established anti-asthma therapy. A clinical nurse specialist assessed adherence to anti-asthma medication. Spirometry was carried out according to the American Thoracic Society / European Respiratory Society, with the spirometry module of the V-max Encore System (Carefusion). Results were presented as a percentage of predicted values (21). Subjective asthma control and quality of life scores were calculated. They combine the Global Initiative for Asthma score (GINA), the Childhood Asthma Control Test (C-ACT), and the Paediatric Asthma Quality of Life Questionnaire (PAQLQ).

Statistical analysis

Allele and genotype frequencies were calculated by direct counting. The χ^2 and (when the expected count was lower than 5) Fisher's exact tests were used to compare frequencies between cases and controls, and also for Hardy-Weinberg equilibrium determination. In investigating genotypic associations, odd ratios (OR) were reported for the allelic distribution. For group comparisons for biomarkers we used the t-test and Kruskal-Wallis test. Mean for biomarkers' values of genotypes in groups were compared with one-way ANOVA and Tukey's multiple comparisons test. P value < 0.05 counts as significant. We used GraphPad Prism 5, Version 5.01 software.

Laboratory Methods

Biochemistry and FBCs

Venous blood was collected into BD Vacutainer tubes® containing EDTA and no additive. Whole blood with EDTA was analysed for full blood count (FBC) using an automated analyser Sysmex XE-2100D (Sysmex America, Mundelein, IL 60060 USA) on the day of collection, and samples were kept for DNA extraction. Additional blood in non-gel serum tubes was centrifuged at 4000 RCF for 10 minutes, aliquoted, and frozen to -80 °C until further analysis.
Total serum 25OHD levels were analysed on Abbott Architect ci8200 (Abbott Laboratories, Abbott Park, Illinois, USA) using

chemiluminescent microparticle immunoassay (CMIA) method with between-run and within-run CVs < 6%. The assay is VDSP (Vitamin D Standardisation Programme) certified. It successfully passed the performance criterion of ± 5% mean bias of the Centres of Disease Control (CDC) and University of Ghent Vitamin D2 and D3 Reference Method with an overall imprecision of < 10% over the concentration range of 22-275 nmol/L for total 25OHD.

We divided 25OHD levels into two groups, based on the most up-to-date Institute of Medicine recommendations, according to which < 50 nmol/L indicates VDD and > 50 nmol/L indicates vitamin D sufficiency (VDS).

Serum concentrations of intact parathyroid hormone (PTH), albumin, total calcium, alkaline phosphatases, phosphate, total IgE, immunoglobulin A, and high sensitivity C reactive protein (hsCRP) were measured, using commercially available diagnostic kits on the automated analyser Abbott Architect ci8200. The between-run and within-run CVs for these assays ranged between 1% and 6%.

Eosinophil cationic protein (ECP) was analysed on the Phadia 250, using fluorescent enzymeimmunoassays (ImmunoCAP Technology) with a between-run CV < 7% and minimum detectable level of 2 ug/L (normal range: 11.1- 13.3 ug/L).

Serum levels of Interleukin-10 (IL-10) and CAMP (cathelicidin antimicrobial peptide) were determined by human enzyme-linked immunosorbent assay method (ELISA kit assays, Damastown, Dublin 15), with intra- and interassay CV < 8%. All assays were analysed with kits of the same lot number.

Genotyping of TaqI and ApaI polymorphisms

DNA isolation was performed on Maxwell 16 System (Promega Corporation, Madison, WI, USA). The outcome of this technique was high molecular weight DNA (> 20kb) that had no traces of RNA contamination and had a 260/280 absorbance ratio > 1.7. The isolated DNA was stored at -20 °C until required for analysis.

Based on a candidate gene approach, we selected two SNPs of the VDR gene, which have a functional impact on gene expression and function. Both polymorphisms have been widely studied in different populations in relation to various medical conditions, including asthma (1,2,13-15).

Genotyping of TaqI (rs731236, assay number C_2404008_10) and of ApaI (rs7975232, assay number C_28977635_10) was performed using TaqMan® SNP Genotyping Assay. Real Time PCR was carried out using 5 µl TaqMan® Genotyping Master Mix, 0.25 µl TaqMan® SNP Genotyping Assay (TaqMan probes) (40×), 3.75 µl Dnase Free Water and 1 µl DNA (1-10 ng). The final reaction volume was 10 µl. The thermal conditions of Real Time PCR were: initial denaturing at 95 °C for 10 min; 40 cycles of 95 °C for 15 sec (denaturing) and 60 °C for 1 min (annealing / extension). Approximately 20% of the samples from the first run were selected randomly for confirmation of the results, and 100% of them matched. The genotyping success rates for two SNPs were > 99%.

SDS 2.3 software was used for allelic discrimination (Applied Biosystems).

All the materials were used in the TaqMan® SNP Genotyping Assay (ABI), in compliance with the manufacturer's instructions and with information supplied on the Applied Biosystems website http://www.appliedbiosystems.com.

The laboratory where the analyses were performed is accredited against ISO 15189.

Results

44 uncontrolled paediatric asthmatics (23 male, mean 8.7 years, mean BMI 19.9 kg/m^2) completed all baseline measures. Their asthma was uncontrolled, based on poor symptom control, and was assessed by the Childhood Asthma Control Test (C-ACT). The median GINA score was 3 with a minimum of 1 and maximum of 5. 100 per cent of patients were on inhaled corticosteroids. More detailed clinical information for the patients has been provided in our previous paper (10). Mean 25OHD was 51 nmol/l (range: 24-80 nmol/l) and 22 children were VDD, while the other 22 were VDS. 25OHD levels and lung function parameters (FEV_1 and FVC%) were significantly higher in VDS vs. VDD patients ($p < 0.001$ and $p = 0.03$, respectively) (**table 1**). Subjective asthma parameters and biomarkers, such as C-ACT, PAQLQ, ECP and hsCRP did not show any significant difference. IgE was an exception (**figure 1**). Consistent with

Figure 1 *- Average of Total IgE level in VDS and VDD groups of 44 uncontrolled asthmatic children according to 25OHD levels of < 50 and > 50 nmol/L ($r^2 = 0.18$, $p = 0.0046$*).*

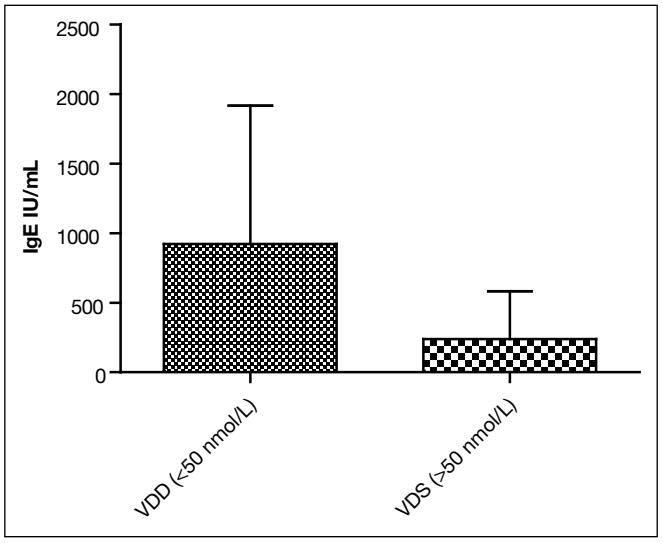
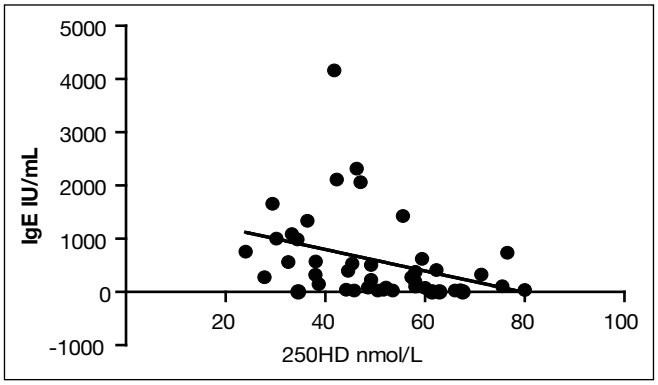


Figure 2 *- Relationship between serum 25OHD levels and serum total IgE (r^2 = 0.12, p = 0.023*) in 44 uncontrolled asthmatic children.*



previous reports, negative correlation was found between IgE and 25OHD levels (p = 0.023) in all patients studied (**figure 2**). We found that the distribution of T and C alleles and genotype frequencies varied significantly between asthmatics and controls for both polymorphisms (p < 0.05) (**table 2**, **3**). The alleles' frequencies were significantly different, as shown by 37% prevalence of C allele (TaqI) and 52% prevalence of T allele (ApaI) in asthmatic patients, versus 20% and 26% in the controls (p = 0.007, p = 0.0004). Children carrying the C allele for TaqI

Figure 3 *- Average WBC levels in uncontrolled asthmatic children according to TaqI genotypes. (Tukey's Multiple Comparison Test: TT vs TC and for TT vs CC, p < 0.05*).*

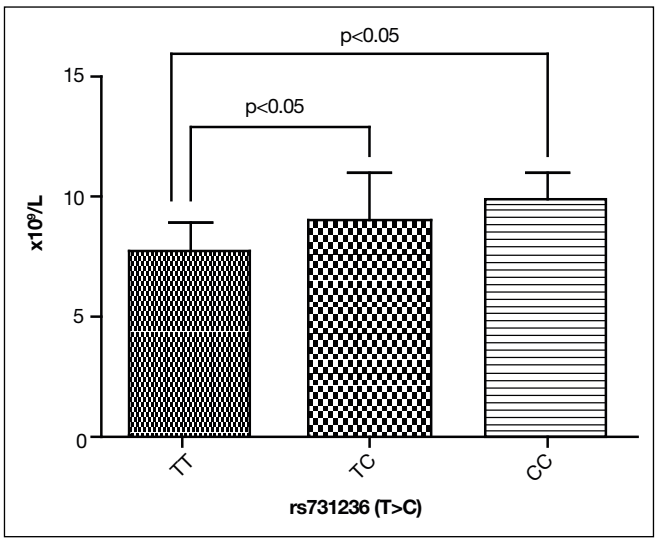
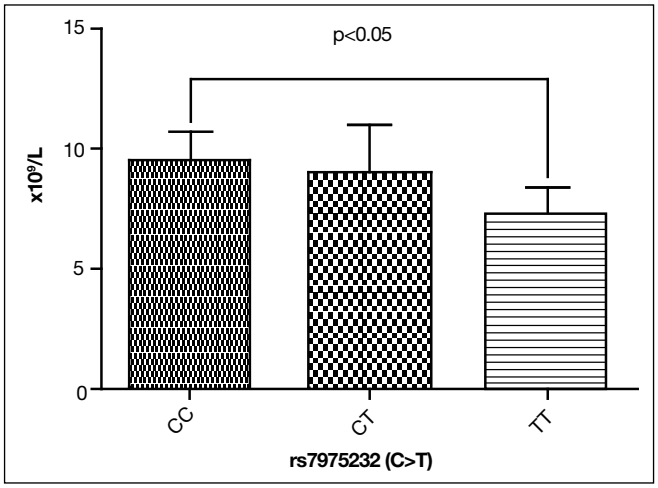


Figure 4 *- Average WBC levels in uncontrolled asthmatic children according to ApaI genotypes. (Tukey's Multiple Comparison Test: TT vs CC, p < 0.05*).*



are 2.37 times more likely to develop asthma (OR = 2.37, 95% CI (1.27-4.45)) and children carrying the risk T allele for ApaI are 2.93 times more susceptible for asthma development (OR = 2.93, 95% CI (1.62-5.3)) than healthy individuals.

Both cases and controls were in Hardy-Weinberg equilibrium for both ApaI and TaqI: p > 0.2 in three analyses, with the exception for TaqI in healthy individuals. The two SNPs were in linkage disequilibrium in cases (D = 1.000, r^2 = 0.633) but not in controls (D = 0.596, r^2 = 0.25). ApaI C allele was linked to TaqI C, and ApaI T to TaqI T in asthmatic children.

In relation to polymorphisms study in uncontrolled asthmatics, we found no association between genotypes and lung function, serum 25OHD levels and other biomarkers, including IgE, ECP, CAMP and hsCRP - except IL-10 and white blood cells count (WBC). IL-10 levels were significantly low in asthmatics with TC genotype for TaqI (p < 0.003) and significantly high in patients with TT genotype for ApaI polymorphism (p < 0.005) (**tables 4**, **5**). WBC was significantly high in patients with TC and CC genotypes for TaqI and significantly low in TT genotype for ApaI (**figure 3**, **4**). There was a trend toward greater Neutrophils count, respectively (p = 0.05) for TaqI, and (p = 0.08) for patients with CC genotype for ApaI. Only two of our children were obese (BMI > 30 kg/m^2). Both children were VDD at the baseline. After supplementation, an improvement in asthma condition was observed only in the patient with TT genotype for TaqI and ApaI polymorphisms. The other child, who had TC genotypes for both polymorphisms, registered no improvement.

Our haplotype analysis for two polymorphisms showed that TT and CC haplotypes were significantly associated with un-

Table 1- *Baseline data for VDD and VDS groups (44 asthmatics).*

	< 50 nmol/L (VDD)	**> 50 nmol/L (VDS)**	**p value**
n (patients)	22	22	
		Subjective parameters	
C-ACT (0-27)	17 ± 5	17 ± 5	0.42
PAQLQ (0-91)	64 ± 18	72 ± 17	0.07
VIDSun	3 ± 1	4 ± 1	0.012*
		Objective parameters	
FEV1%	93.6 ± 13.1	101.9 ± 15.1	0.03*
FVC%	88.9 ± 13.9	96.1 ± 9.2	0.03*
25(OH)D (nmol/L)	39.6 ± 7.4	62.2 ± 8.2	< 0.001*
IgE (IU/L)	960 ± 1000	232 ± 336	0.001*

Data shown as mean ± SD. PAQLQ = paediatric quality of life questionnaire; C-ACT = childhood asthma control test; VIDSun = Vitamin D and Sun questionnaire. *denotes statistically significant.

Table 2 - *Genotypic association analysis of VDR RFLPs (restriction fragment length polymorphisms) TaqI and ApaI between paediatric asthmatic patients and control individuals.*

Enzyme analysis	**Patients (44)**	**Controls (57)**	**Multiple comparison p value[1]**	**$\chi 2$**	**p value**
TaqI Genotypes					
TT	17 (38%)	34 (60%)			
CT	21 (48%)	23 (40%)		10.25	0.006*
CC	6 (14%)	0 (0%)			
TT/CT	17/21	34/23	0.2[1]		
TT/CC	17/6	34/0	0.003[1]		
CT/CC	21/6	23/0	0.025[1]		
ApaI Genotypes					
TT	11 (25%)	5 (9%)			
CT	23 (52%)	20 (35 %)		18.82	0.0001*
CC	10 (23 %)	32 (56 %)			
TT/CT	11/23	5/20	0.38[1]		
TT/CC	11/10	5/32	0.002[1]		
CT/CC	23/10	20/32	0.007[1]		

Data are: number (%); *$p < 0.05$ is considered significant; [1]Fisher's exact test.

Table 3 *- Allelic association analysis of VDR RFLPs TaqI (T > C) and ApaI (C > T) between uncontrolled paediatric asthmatic patients and control individuals.*

	Patients (44)	**Controls (57)**	**OR (95% CI)**	**p value**
TaqI allelic association				
T	55 (63%)	91 (80%)		
C	33 (37%)	23 (20%)	2.37(1.27 - 4.45)	0.007*
ApaI allelic association				
T	45 (52%)	30 (26%)	2.93 (1.62 - 5.3)	0.0004*
C	43 (48%)	84 (74%)		

Data are: number (%); OR = odds ratio; 95% CI (in parentheses); *denotes statistically significant.

Table 4 *- Relationship between serum IL-10 and TaqI (T > C) VDR RFLP (restriction fragment length polymorphism) genotypes in uncontrolled peadiatric asthmatics.*

	Serum IL-10 pg/mL			**Sig.**			
Taq-I RFLP	n	mean	SD	p value	CC vs.TT	CC vs.TC	TT vs.TC
TT	16	135	47.9	p = 0.046*	ns	ns	p = 0.0026*
TC	20	94	25.6				
CC	6	106	47.7				

n = number of subjects; SD = standard deviation; p < 0.05 is considered significant; Sig. refers to difference between means of homozygotes and heterozygotes (CC vs.TC), of the homozygotes and heterozygotes (TT vs. TC); ns = non-significant; *denotes statistically significant.

Table 5 *- Relationship between serum IL-10 and ApaI (C > T) VDR RFLP (restriction fragment length polymorphism) genotypes in uncontrolled peadiatric asthmatics.*

	Serum IL-10 pg/mL			**Sig.**			
Apa-I RFLP	n	mean	SD	p value	CC vs.TT	CC vs. CT	TT vs. CT
TT	10	154.8	45.5	p = 0.0053*	0.0163*	ns	p = 0.0015*
CT	22	95.3	25.4				
CC	10	103.0	41.8				

n = number of subjects; SD = standard deviation; p < 0.05 is considered significant; Sig. refers to difference between means of homozygotes and heterozygotes (CC vs. CT), of the homozygotes and heterozygotes (TT vs. CT); ns = non-significant; *denotes statistically significant.

controlled asthma (OR = 40.26, 95% CI (5.27 - 307.79), p < 0.001, and OR 43.74 (95% CI: (4.87 - 393.20), p < 0.001, respectively).

Discussion

In our pilot study we examined 25OHD levels and asthma symptom control in relation to TaqI and ApaI VDR polymorphisms in Irish children with uncontrolled asthma. We found a significant association between TaqI and ApaI SNPs and susceptibility to uncontrolled paediatric asthma.

The results of our work on the associations between the two polymorphisms examined and asthma agree with other studies (14,22) in which TaqI has been linked with asthma in paediatric patients. We also found an association between ApaI and asthma susceptibility. Children carrying the risk T allele for ApaI are nearly 3 times more susceptible to asthma. These findings

agree with studies by Saadi et al. (23) and Iordanidou et al. (2); the latter showed that ApaI "a" allele was also associated with improved asthma control in children.

The TaqI (rs731236, c.1056T > C, p.Ile352Ile) is a synonymous polymorphism at codon 352 (isoleucine) of the gene, and this T > C alteration does not result in amino acid sequence change (24,25). The ApaI (rs7975232, c.1025-49G > T) is located in the intron 8 of the VDR gene (17). The two tested polymorphisms do not cause any structural changes of the VDR protein, but they are linked with other functional SNPs and may take part in a complex gene network enhancing or inhibiting the expression of VDR target genes.

The ApaI and TaqI polymorphisms are located at the 3' end of the gene and are near the regulatory 3' untranslated region (3'-UTR) of mRNA. This indicates that they have the potential to alter splicing regulation. When the ApaI and TaqI SNPs are found in specific haplotypes, they affect VDR mRNA stability and the rate of transcription, and this may result in altered protein expression (24-28). For example, in our study both SNPs were in linkage disequilibrium in paediatric asthmatics, but not in healthy volunteers.

Alternatively, epigenetic modifications in the VDR gene can suppress VDR transcription. In a study on tuberculosis susceptibility in lymphoblastoid cell lines, Andraos et al. have demonstrated that the TaqI variant resides on a CpG island and the C allele is always methylated. They also showed that there are interactions between TaqI, methylation levels, ethnicity, and tuberculosis susceptibility (29). Consequently, we can hypothesise that this SNP may serve as a marker of methylation for other "functional" polymorphisms in the VDR gene or in nearby genes. TaqI SNP is located in the exon 9 which encodes the ligand-binding region of the VDR (30). The DNA methylation and histone modifications in these regions can change the chromatin state from an open to a closed conformation. It could lead to transcriptional repression of these genes. The expression of genes involved in Vitamin D metabolism are deregulated in various chronic diseases, and these changes may be partially accredited to epigenetic modifications (31).

Like some other researchers (32), we have found negative association in our paediatric patients between 25OHD level and total IgE. In comparison with other studies (22), we saw no significant association between TaqI and ApaI and IgE. But we should emphasise that 70% of our children were atopic with elevated IgE level.

In agreement with many other studies (1,13), we found no associations between genotypes and serum 25OHD. Interestingly, IL-10 levels were significantly low in asthmatics with TC and CT genotypes for TaqI and ApaI. WBC was significantly high with a trend toward a higher Neutrophils count in patients with TC and CC genotypes for both SNPs. IL-10 is widely expressed among innate and adaptive immune cells (33). It restricts the ability of antigen presenting cells to promote the differentiation and proliferation of $CD4^+$ T cells, and it influences the initiation and progress of adaptive T cell responses. IL-10 also inhibits the expression of numerous pro-inflammatory cytokines, thus further suppressing the ability of effector T cells to prolong inflammatory responses (34). Matilainen et al. showed that the effect of $1\alpha,25(OH)_2D_3$ on the expression of IL-10 is achieved through cyclic recruitment of VDR to Vitamin D response elements (VDREs) within a promoter region of the IL-10 gene (35). Up-regulation of IL-10 by vitamin D suppresses the innate immune response, in order to avoid the effects of long-lasting inflammation, such as tissue damage and development of chronic illnesses (36-39).

Based on our findings we can hypothesise that patients with specific genotypes for TaqI and ApaI have suppressed IL-10 production due to a decrease in expression of VDR. This can lead to the deregulation of innate immune responses and to the continuation of inflammatory processes. The increased levels of the neutrophils and WBC in patients with these SNPs support this interpretation. It may be possible to use these genotypes as predictive biomarkers of chronic asthma.

Over half of our patients were VDD, and low levels of 25OHD may be responsible for the suppression of their IL-10, due to insufficient production of the active form $1\alpha,25(OH)_2D_3$. Sufficient 25OHD levels may be vital, since they influence local tissue concentrations of the active metabolite (40,41). Ojaimi et al. have suggested that serum 25OHD levels as high as 120 nmol/L may be necessary for optimal immune function (42). This could explain why in our Vitamin D supplementation study (10) we did not observe any beneficial effects of vitamin D on asthma biomarkers in our patients. Only 20% of VDD subjects achieved 25OHD levels > 120 nmol/L after 15 weeks of supplementation with 2000 units each day. In the VDS group 63% of the patients achieved these levels. It can partially explain why after supplementation the VDS group had significantly fewer days of school missed and fewer steroid requirements than all other groups (10). We could not make any conclusions regarding the genotypes' effects on different asthma parameters and biomarkers after supplementation, probably due to the small number of children in both groups. We should point out the importance of phenotypical differences in our patients. Two of the children (both female) were obese and VDD. Only one of them improved significantly after supplementation without achieving 25OHD level over 120 nmol/L, and her genotype was TT for TaqI and ApaI. We hypothesise that in obesity-related asthma, genotypical investigation can be used to predict a beneficial response to vitamin D treatment. And we furthermore suggest that any benefit from vitamin D supplements can be achieved only in patients who have specific genotypes with particular asthma phenotypes.

Patient selection might help clarify whether vitamin D can be useful for enhancing asthma therapy.

We admit that the main limitation of our pilot study is our limited sample size, but in a small country it is difficult to recruit a sufficient number of paediatric patients with uncontrolled asthma. In this paper we explored only two VDR polymorphisms without including others ,such as FokI, BsmI and Tru9. We also limited our study to the IL-10 measurements, and we did not analyse other cytokines or VDR. But ours is a pioneering work in Irish paediatric asthmatic research, and we hope that it will open new horizons for future studies in this area.

In summary, we have revealed an association of TaqI and ApaI polymorphisms of the VDR gene with a susceptibility to uncontrolled asthma in a cohort of paediatric Irish patients. Also, we have shown that the patients with TC for TaqI, and CC and CT genotypes for ApaI have a significantly low level of IL-10 and increased WBC (neutrophils in particular). In our study we were the first to observe that TT and CC haplotypes were significantly associated with asthma in Irish children and could be potential biomarkers for paediatric asthma.

Further and more extensive functional studies will be necessary to confirm our findings in order to elucidate the underlying mechanisms in asthma that are related to vitamin D and VDR polymorphisms in specific asthma phenotypes.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Fecal microbiota profile in atopic asthmatic adult patients

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Key words

atopic asthma; density; diversity; gut microbiota; semi-quantitative stool culture

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Summary

Background. *Studies proposed a link between gut microbiota and airway tract.* ***Objective.*** *Study the diversity and density of gut microbiota in healthy and asthmatic patients.* ***Method.*** *Semi-quantitative stool cultures were performed from fecal samples collected from 80 adult asthmatic patients and 40 healthy individuals. Data on gender, age, dietetic history, clinical examination and investigations as skin prick test and pulmonary function testing were also collected.* ***Results.*** *Lactobacilli were found to be higher among patient group than control group.* E. coli *density was statistically higher in patient than control group. No significant difference was detected between male and female patients or controls.* Lactobacilli *were statistically more prevalent in stool culture of male cases than that of male controls. No difference was found between female cases and controls. There was no relationship between type of microbial growth and disease related parameters including age, duration of illness, number of allergens and pulmonary function test in cases.* ***Conclusion.*** *Atopic asthma is significantly associated with gut microbiota* Lactobacilli *and* E. coli. *It is important to determine the organism involved to focus on microbiome-driven disease and therapies.*

Introduction

Asthma is a complex disease that has influence on and is influenced by a colonization of the communities of microbes in the gut and the respiratory tract. These communities, in turn, are affected by environmental factors such as diet and antimicrobial administration in early life. Many of the animal studies proved the presence of a gut-airway axis. This opened up opportunities and ideas that aim at modifying the airway and/or gut microbiome in order to provide a novel strategy for prevention and treatment of allergic asthma (1).

Although more than 1000 different bacterial species represent human gut microbiota, only 150-170 predominate in any given subject (2,3). Infants during vaginal delivery are exposed to a population of microbes from the surrounding environment leading to formation of a colony of microbes in the human gut immediately at birth. As time passes, the gut flora of infants transforms into another form that is reported in young adults (4).

Fermentation of non-digestible dietary residue and control of intestinal epithelial cell growth maturation are some of the functions of the microbiota that are important for maintaining balance of the immune system (5,6). Yet, the human bacterial ecology has also been linked to pathogenesis and onset of many diseases, autoimmune disorders in particular, and of course allergy. These disorders, in return, cause further transformation and changes in the human microbiome (7-10). Therefore, biodiversity proves to be important in determining the wellbeing of human livelihood (11). This led to increasing interest in relationship between host and microbiota (12-14).

The relationship between allergic disease and gut microbiota was identified in several clinical studies. These studies reported a difference in the composition (diversity) of the bacterial mi-

crobiome in the feces (15-21). Differences in the gut microbiota diversity involve *Clostridium, Bacteroides, Bifidobacterium, Lactobacillus* and *Enterobacteriaceae* (22). One study reported that allergic children had fewer *Lactobacilli* and *Bacteroides* species, but higher aerobic bacteria, especially *Enterobacteriaceae* and *Staphylococci* (23). Collectively, studies on gut and environmental microbiota concluded that negative balance occurs due to decreased exposure to a certain diversity of microbes leading to increased risk of allergy in general and asthma in particular (24,25).
However, the scientific information about the relationship between allergy and gut microbiota dysbiosis is still controversial (26).
In asthma, no single straightforward mechanism can explain the gender differences amongst asthmatic subjects. Before puberty, it is more prevalent in boys than in girls; by adulthood, though, it is more prevalent in women than in men (27). Although several studies investigated microbiome in children (28-30), little is known about the complete microbiota profile of adult individuals suffering from asthma and relation to gender. Therefore, the aim of this work was to study the diversity of gut microbiota among atopic asthmatic adult males and females in comparison to matched controls, especially *Enterobacteriaceae, Staphylococci* and *Lactobacilli*.

Methods

This cross-sectional study included 120 adults; 80 of them were participants diagnosed with bronchial asthma according to Global Initiative for Asthma (GINA), 2015 (31). Cases were between the ages of 18 to 45 years, and were recruited from the Allergy Clinic of Ain Shams University over an eight-month period. The remaining forty adults were healthy individuals (controls). Exclusion criteria for standard analysis of gut flora (32) included adults < 18 years old, abnormal body mass index, unusual dietetic regimen, smokers, history of cancer colon, autoimmune diseases, presence of inflammatory bowel disease, an acute or chronic diarrhea in the previous 4 weeks before taking the stool sample, antibiotic, prebiotic and probiotic or vitamin administration of a period less than one month before fecal sampling. Participants who received immunosuppressive therapy and/or allergen specific immunotherapy in the last 5 years, and patients on oral glucocorticoid treatment during the 6 months prior to the sample collection date were also excluded from the study, as well as patients with any associated co-morbidities.
Cases and controls were evenly stratified by gender; into 40 atopic asthmatic females and 40 atopic asthmatic males, and controls into 20 females and 20 males. An informed consent was obtained from all participants, and the study was approved by the Research Ethics Committee of Ain Shams University.
Cases completed a questionnaire on asthma symptoms (33,34), triggering factors, and associated atopic diseases. Skin prick test (SPT) to allergens was used to determine atopic status. The most commonly inhaled allergens included mites, mixed pollen, mold, and animal epithelia. Positive (0.1% histamine in phosphate-buffered saline) and negative (physiologic saline) controls were used. A wheal size over 3 millimeters greater than the negative control after 15 minutes was considered a positive result (35). Spirometry was performed at the Pulmonary Functions Laboratory at Ain Shams University Hospital using the Flow Mate V Plus Spirometer (Spirometrics, ME, USA). Reference values used were adapted from the European Respiratory Society (36).

Stool sample collection

A single fresh stool sample was collected from each participant in sterile screw caped containers and rapidly delivered to the Central microbiology laboratory of Ain Shams University Hospitals for culture, according to Infectious Diseases Society of America (IDSA) guidelines, 2013 (37).

Semi quantitative stool culture

Fecal specimens were examined to demonstrate the fecal microbial diversity of normal intestinal flora among the cases and controls. Semi quantitative stool cultures were performed using the quadrant technique. The quadrant technique is recommended for samples that are rich in microflora and contain numerous colony-forming units, such as stool or sputum. Use of this technique allows for enhanced separation and enumeration of colonies. Part of the stool were cultured directly on MacConkey agar media plates (Oxoid, UK); to identify the microbial growth pattern of gram negative bacteria, on Xylose lysine deoxycholate (XLD) agar media plates (Oxoid, UK) to exclude *Shigella* and carrier state of *Salmonella*, on blood agar plates aerobic and anaerobic (Oxoid, UK) for isolation of gram positive and anaerobic bacteria respectively, and on Man-Rogoza Sharp agar media (MRs) (Oxoid, UK) for isolation of *Lactobacillus* species.
Feces were inoculated by dipping a swab into the specimen. After implantation of the inoculum, a wire loop was flamed and cooled. The loop was held between the thumb and index finger and passed at a 90 degree angle several times through the initial inoculum into the second quadrant of the plate (streak area 1). The plate was turned 90 degrees, and the process was repeated, streaking into the third quadrant (streak area 2), and finally, after another 90 degree turn, into the fourth quadrant (streak area 3). The loop was flamed between quadrants. When streak plates were used, the relative numbers of bacteria was reported. Several methods of semi-quantitation were used. Bacterial density was measured by use of (very few, few, moderate or many) growth with the use of (1+ to 4+) growth scoring criteria, heavy growth had a score 3 or 4 (38).
Man-Rogoza Sharp agar media (MRs) (Oxoid, UK) for isolation of *Lactobacillus species* was incubated anaerobically for 72-hours

in anaerobic conditions using (GasPak EZ Anaerobe, Becton Dickinson, Heidelberg, Germany) at 37 °C in jars (AnaeroPack, Mitsubishi Gas Chemical America Inc., NewYork, NY, USA), other plates were incubated aerobically for 48-hours at 37 °C. Colonies of different morphology grew and were identified according to standard phenotypic methods, as regards gram stain, colony morphology and biochemical reactions using Vitek 2 identification cards (39).

Fecal flora richness (alpha diversity)

We examined allergy associations with the number of observed species (richness) or alpha diversity of taxa with relative abundances.

Fecal flora Composition and density (beta diversity)

We studied the microbiome composition difference between patients and controls expressed by percentage of prevalence and density (colonization rate) which was expressed as the odds ratio (OR) and 95% confidence interval (CI). The associations were adjusted for sex, age, body mass index, time since last antibiotic use, probiotic and vitamin use.

Statistical methodology

Comparative analysis was performed using student t test, Chi-squared, and Fisher's exact test for continuous and categorical data, respectively. Continuous data was expressed as mean ± standard deviation, while categorical data was expressed as number and percent of total. An odds ratio (OR) is a measure of association between an exposure and an outcome. The OR represents the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in the absence of the exposure. The 95% confidence interval (CI) was used to estimate the precision of the OR. A p value of $p \leq 0.05$ was considered statistically significant. Data analysis was completed using PASW Statistics, version 18.

Results

Eighty participants diagnosed with bronchial asthma were included, together with forty healthy individuals as a control group. **Table 1** represents the demographic data between both studied groups, in which cases and controls were comparable as regards age and gender. There was no statistically significant difference between male and female atopic subjects as regards age, duration of illness, number of allergens and pulmonary function test.
As regards severity of bronchial asthma (interpretation of pulmonary function test), no statistically significant difference was found between male and female atopic subjects.
Regarding diversity and density of gut microbiota, *Lactobacilli* were found to be higher among patient group than control group with odds ratio 7.2 (1.6 - 32.4) CI (p value 0.01). At the same time, *Escherichia coli* density (heavy colonization) was statistically higher in patients than control group with Odds ratio 16.9 (5.75-50) CI (P value < 0.001). However, no significant difference was detected between male and female patients or controls (**table 2**).
On comparing male cases with male controls, and female cases with female controls, *Lactobacilli* were statistically more prevalent in stool culture of male cases than in male controls (p value = 0.045). On the other hand, no difference was found between female cases and controls (**table 3**).
Lastly, no relationship was detected between type of microbial growth and disease related parameters including age, duration

Table 1 *- Demographics.*

	Groups									
	cases					**control**				
	gender					**gender**				
	f (40)		**m (40)**		**p value**	**f (20)**		**m (20)**		**p value**
	mean	**SD**	**mean**	**SD**		**mean**	**SD**	**mean**	**SD**	
age	31.85	8.37	29.70	8.72	0.264	34.90	7.58	31.10	11.35	0.390
duration of illness	6.21	5.15	6.93	4.61	0.516					
number of allergens	3.55	1.34	3.95	1.45	0.204	-	-	-	-	-
FVC[1]	74.60	20.15	75.30	20.24	0.877	87.46	15.77	87.51	13.46	0.994
FEV1[2]	73.06	20.27	71.45	20.65	0.726	88.96	12.08	91.76	10.20	0.582
FEV1/FVC	90.90	11.58	92.02	14.14	0.70	97.55	11.45	99.27	12.99	0.757

Table 2 *- Distribution and density of isolated finding microbiota across the male and female in each group.*

	Cases (n = 80)			Control (n = 40)		
	female	**male**	**p value**	**female**	**male**	**p value**
Lactobacilli	8 (20.0%)	14 (35.0%)	0.133	2 (10.0%)	0 (0.0%)	0.468
density (heavy growth)	2 (25.0%)	2 (14.3%)	0.959	2 (100.0%)	0 (0.0%)	0.317
E. coli	35 (87.5%)	34 (85.0%)	0.745	18 (90.0%)	18 (90.0%)	1.000
density (heavy growth)	30 (85.7%)	22 (64.7%)	0.081	2 (11.1%)	3 (16.7%)	0.990
Klebsiella	8 (20.0%)	10 (25.0%)	0.592	2 (10.0%)	8 (40.0%)	0.068
density (heavy growth)	2 (25.0%)	1 (10.0%)	0.832	0 (0.0%)	1 (12.5%)	0.516
Proteus	5 (12.5%)	7 (17.5%)	0.755	0 (0.0%)	2 (10.0%)	0.123
density (heavy growth)	5 (100.0%)	7 (100.0%)	1.000	0 (0.0%)	2 (100.0%	0.545
Enterobacter	6 (15.0%)	1 (2.5%)	0.108	6 (30.0%)	0 (0.0%)	0.027
density (heavy growth)	3 (50.0%)	0 (0.0%)	0.876	3 (50.0%)	0 (0.0%)	0.608
Enterococci	3 (7.5%)	1 (2.5%)	0.615	0 (0.0%)	0 (0.0%)	-
density (heavy growth)	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-
Candida	1 (2.5%)	2 (5.0%)	0.677	2 (10.0%)	0 (0.0%)	0.468
density (heavy growth)	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-
Serratia	0 (0.0%)	1 (2.5%)	0.476	0 (0.0%)	0 (0.0%)	-
density (heavy growth)	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-
Citrobacter	2 (5.0%)	2 (5.0%)	1.000	0 (0.0%)	2 (10.0%)	0.468
density (heavy growth)	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-
Bacteroids	0 (0.0%)	2 (5.0%)	0.494	0 (0.0%)	0 (0.0%)	-
density (heavy growth)	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-
Provedencia	1 (2.5%)	0 (0.0%)	0.476	0 (0.0%)	0 (0.0%)	-
density (heavy growth)	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-
Morganella	00.0%)	1 (2.5%)	0.476	0 (0.0%)	0 (0.0%)	-
density (heavy growth)	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-
Pseudomonas	0 (0.0%)	4 (10.0%)	0.116	2 (10.0%)	0 (0.0%)	0.468
density (heavy growth)	0 (0.0%)	1 (25.0%)	0.329	0 (0.0%)	0 (0.0%)	-
Staph. aureus	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	2 (10.0%)	0.468
density (heavy growth)	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-

[1]FVC: forced vital capacity; [2]FEV1: forced expiratory volume during first second.

Table 3 *- Comparison of the distribution of isolated microbiota between cases and controls, stratified by gender.*

	Female			Male		
	cases	**control**	**p value**	**cases**	**control**	**p value**
Lactobacilli	8 (20%)	2 (10%)	0.665	14 (35%)	0	0.045
E. coli	35 (87.5%)	18 (90%)	1	34 (86%)	18 (90%)	1
Klebsiella	8 (20%)	2 (10%)	0.665	10 (25%)	8 (40%)	0.436
Proteus	5 (12.5%)	0	0.569	7 (17.5%)	2 (10%)	1
Enterobacter	6 (15%)	6 (30%)	0.358	1 (2.5%)	0	1
Enterococci	3 (7.5%)	0	1	1 (2.5%)	0	1
Candida	1 (2.5%)	2 (10%)	0.363	2 (2.5%)	0	1
Serratia	0	0	0	1 (2.5%)	0	1
Citrobacter	2 (5%)	0	1	2 (5%)	2 (10%)	0.496
Bacteroids	0	0	0	2 (5%)	0	1
Provedencia	1 (2.5%)	0	1	0	0	0
Morganella	0	0	0	1 (2.5%)	0	1
Pseudomonas	0	2 (10%)	0.2	4 (10%)	0	0.571
Staph. Aureus	0	0	0	0	2 (10%)	0.2

of illness, number of allergens and pulmonary function test in atopic patients.

Discussion

Competent immune system depends on T-helper (Th) CD4 lymphocyte population balance. The onset of allergic disease is due to an imbalance in this population and favoring immunity towards Th2 (40). Gut microbiota participates crucially in the evolution of the intestinal immune system as well as adjustment of the T helper cell balance. Moreover, the loss of biodiversity may have serious impact on human wellbeing (41). Studies showed that the risk of developing asthma and allergy happens due to change in diversity of the body's microbiota (42,43). This may explain why diseases such as asthma and allergy develop at any age. Also evidence showed that there is an increased incidence of asthma and allergies in industrialized countries during the last 50 years. Moreover, studies demonstrated correlation between allergic diseases and antibiotic use (44), altered fecal microbiota (45), and dietary changes (46). Some studies even suggest an association between growing up in a farm environment, early life consumption of unpasteurized milk, and decreased risk of developing allergy later in life (47-49). All the previous support the microbial hypothesis that proposed that microbiota is vital for immune hemostasis.

It is well known that the dendritic cells (DCs) are the antigen-presenting cells responsible for the activation of naive T cells. DC recognizes microbes in the intestine, samples them directly either from the lumen or through the gut-associated lymphoid tissue (GALT), then activates a cascade of events eventually ending in the differentiation of Th1, Th2, and Treg cells. (40). If dysbiosis prevails, increased susceptibility to airway colonization by certain bacteria or the microbiota may occur, resulting in switching immunity towards the inflammatory immune responses (50).

To the best of our knowledge, few studies investigated diversity of microbiota in adult atopic subjects. Most of the studies conducted before, focused on atopic children.

Several studies suggest a role for sex hormones in the pathogenesis of asthma. Asthma prevalence was found to be higher in women than men, as recorded by Rhodes and co-workers. On the other hand, other clinical studies pointed to distinctive changes in the prevalence and severity of asthma with age progression (51-55). The current study focused on adult asthmatic subjects and investigated the diversity of microbiota in males and females. The design of this study was done to eliminate the hormonal factor by dividing both subgroups equally into male and female group.

In our study, although diversity of bacteria was detected to be more towards *Klebsiella*, *Proteus*, *Enterobacter*, *Enterococci* in asthmatics, the results did not reach statistical significance dif-

ference except for predominance of *Lactobacilli* among patient group than in control group. At the same time, *E. coli* density (heavy colonization) was statistically higher in patients than in control group. However, no significant difference was detected between male and female patients. On comparing male cases with male controls and female cases with female controls, *Lactobacilli* were statistically more prevalent in stool culture of male cases than in male controls. On the other hand, no difference was found between female cases and controls.

Penders and co-workers reported that gut microbial differences contribute to allergy risk in humans (56). In two studies, infants who had a higher fecal abundance of *Clostridium difficile* had an increased risk of developing an allergy in the future (9,57). The current study showed low isolation rate of *Bacteroides* and no growth of *Bifidobacterium* in the conducted stool cultures as well as other species. This may return to the fact that there is pediatric age predominance of some strains as *Bifidobacterium*. The number of *Bifidobacteria* actually declines in the human body with age. In infants who are breast-fed, *Bifidobacteria* constitute about 90% of intestinal bacteria; however, this number is lower in bottle-fed infants and adults (58).

The diversity of the microbiota in healthy and allergic children was studied in two-year old children in Sweden and Estonia countries. Allergic children showed few colonies of *Lactobacilli* and *Bacteroids* and had higher counts of aerobic bacteria, especially *Enterobacteriaceae* and *Staphylococci*, irrespective of country of residence (23). Differences of the gut microbiota diversity between allergic and healthy children was also demonstrated by another case control study, but the differences identified concerned various particular genera and species, including *Bifidobacterium*, *Clostridium*, *Bacteroides*, *Lactobacillus* and *Enterobacteriaceae* (22). This is in accordance to our study, in which there was not only higher isolation of *Lactobacilli* in asthmatic patients, but also higher growth of *Lactobacillus* in male patients than male controls. *E. coli* density (heavy colonization) was statistically higher in patients than in control group. At the same time, the prevalence of *E. coli* as one of *Enterobacteriaceae* was more detected in asthmatic patients than control in a study by Bjorksten and co-workers (22). *Enterobacteriaceae* and *Staphylococci* were suggested by Penders et al. as potential candidates, as these have been associated with an increased risk of atopic diseases (10). This study detected *Staph. aureus* in 2 of control subjects only. Still, results were of no statistical significant difference.

Reduction of *Bacteroidetes, Lactobacilli* and *Bifidobacteria* were found in a study by Ouwehallnd et al. They demonstrated that this finding has been associated with an asthma phenotype (59). However, another study by Waligora-Dupriet and colleagues found that the prevalence of *Bifidobacterium* was similar in healthy and allergic subjects, regardless of the type of the allergic disease (58). On the other hand, Sjogren and colleagues recorded that *Bacteroides* colonization of the gut was not found to be related to allergy (17). Similarly, the present study showed growth of *Bacteroids* only in two male cases, yet no statistical significant value related was found.

Rook and co-workers established that *Lactobacilli* are microorganisms that stimulate immune regulation by triggering regulatory T-cell responses (60). However, He et al. suggested that *Bifidobacteria* was similar to *Lactobacilli* in their effects, and that the immune system may be species dependent because intestinal *Bifidobacterium* species showed to induce varying cytokine production by cells of the innate immune system (61). On the other hand, another study by Stsepetova et al. showed that allergy is related to restricted *Bifidobacterium* diversity (62). Bottcher and co-workers suggested an association between allergy and *Clostridium difficile* as they found that allergic infants had higher fecal concentrations of the rarely detected icaproic acid, which has been associated with the presence of *Clostridium difficile* (45), and Woodcock et al. detected higher *C. difficile* IgG antibody levels in allergic than non-allergic subjects (63). The present study could not prove presence or detect *Clostridium difficile* in neither case or control subject, because *Clostridium* is one of fecal flora characterized by attachment to mucosal surface of intestine rendering the culture and isolation very difficult (64). Furthermore, Kalliomaki et al. detected a reduced ratio of *Bifidobacteria* to *Clostridia* in the stools of atopic subjects in comparison to those of non-atopic subjects (57).

In contrast to the current study, Hevia and colleagues showed that the microbial alpha-diversity was not significantly different between healthy and allergic adult individuals. However, the analysis of specific bacterial groups detected significantly lower levels of *Bifidobacteria* in patients with long-term asthma. Also, in allergic individuals the *Bifidobacterium adolescentis* species prevailed within the bifidobacterial population (65). This in line with Hua and co-workers, who found statistically significant fecal dysbiosis across multiple allergies in adult allergic patients. Specifically, reduced richness and altered composition was found with all allergies except asthma, bee sting, and eczema. The dysbiosis was most marked with allergies to nuts and seasonal pollen, and it was driven by higher abundance of *Bacteroidales* and reduced abundance of *Clostridiales* (66).

Conclusions

The current challenge aimed to identify the role of gut microbiota in allergic disease, specifically atopic asthma, in adults. In addition, the study investigated the relationship between gender and gut microbiota profile. This study demonstrated predominance of *Lactobacilli* and *E. coli* in patients group. Interestingly, the study demonstrated higher levels of *Lactobacillus* in male patients. Yet, there was no relationship between type of microbial growth in stool culture of cases and parameters of the disease including age, duration of illness, number of allergens and pulmonary function test.

Further investigations are required, especially new methods like quantitative polymerase chain reaction, to investigate a relationship and detect whether microbiota could be considered an additional factor in pathogenesis of asthma. Proving this link may lead to new opportunities in diagnostic and therapeutic modalities. Further studies may be needed on large scale for evaluation of gut microbiota in asthmatic subjects using molecular methods to identify strains at the species level, evaluation of significance of certain strains, and probiotics as an adjuvant treatment of bronchial asthma. Evaluation of hormonal and genetic factors in comparison with microbiota would be preferable. Future research should include patients from other hospitals, from other areas, other countries and other races.

Conflict of interest

The authors declare that they have no conflict of interest.

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Role of nasal challenge and local eosinophilia in indirect exposure to cat in allergic rhinitis patients

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Summary

Introduction. *Sensitization to cat allergens is common worldwide. Currently, there is a trend towards costly and often unavailable diagnostic analysis.* ***Objectives.*** *The aim is to assess the reliability of skin prick test (SPT) and serum specific IgE (ssIgE) to cat sensitization, by performing nasal challenge test (NCT) in a community with low cat ownership but common presence of stray cats.* ***Patients and methods.*** *Forty-one patients with perennial allergic rhinitis (AR) who were mono or polysensitized (including cat) were included. We had 31 cat non-owners and 10 present cat owners. SPT (> 5 mm / diameter), ssIgE (≥ 0.70 IU/ml), nasal smear for eosinophil (Eo) and NCT were compared between groups. Outcomes included nasal challenge score, nasal Eo positivity, peak inspiratory and expiratory flow (PIF and PEF) 2 and 8 hours after the NCT, and were compared to baseline.* ***Results.*** *Baseline SPT wheal size and ssIgE level were similar in both groups. NCT positivity was more frequent in cat owners. The strongest nasal reaction was on the top concentration in both groups. Nasal Eo positivity in cat owners was higher before and 2 hours after NCT, but similar to non-owners at last measurement. NCT positive cat non-owners had bigger SPT wheal size than NCT negative non-owners, but smaller than NCT positive cat owners. In contrast to PEF, a significant fall in PIF was noticed in both groups. Mono and polysensitised patients showed similar NCT positivity.* **Conclusion.** *Stray cats may pose a relevant risk of developing perennial AR. Regardless of cat ownership status, SPT and ssIgE should be the first diagnostic tool. Nasal Eo and NCT seem to be good diagnostic tools in cat non-owners if diagnosis is elusive.*

Introduction

Studies have shown that the prevalence of pet sensitization is increasing over the past decades not only in western countries, but also all over the World (1). Together with house dust mites, cat allergens represent the major indoor allergens, and are known risk factors for rhinitis and asthma (2,3). Exposure and sensitization primarily depend on the prevalence of cat ownership, but is still considered ubiquitous (4). The sensitization prevalence varies among countries according to different cultures, climate, environmental factors, traditional and religious believes (5-10). Distribution of the main cat allergens indoors depends on their aero-dynamic diameter and on passive transport by clothes, shoes, or by human hair (11-17) from cat owning home to the environment that has never been occupied by the cat. A study by Woodfolk et al. (18) stressed out the importance of type of vacuum cleaner, which emits cat allergens with a mean of 90% on particles < 2.5μm/diameter. Similarly, Chapman et al. (19) have documented a wide variation of pet indoor allergens, from less than 1 μg to greater than 3000 μg/g of dust, clearly being the highest in pet-owners homes than in non-owners homes.
However, cat ownership should not be considered as the only index of exposure to cat allergens (4). Liccardi had suggested several modalities of exposure to pet allergens and possible con-

sequences in a "real life" condition, which exclude that any indirect and no apparent contact (d and e modalities) should be considered at lower risk of exposure.

Furthermore, Chen et al. (20) documented that exposure to cat allergen concentration as low as 0.24 - 0.63 μg/g could be positively associated with reported asthmatic respiratory symptoms in subjects who have experienced allergic symptoms when near animals (20). It means that in a community with low cat ownership and common presence of stray cats, the low concentration of cat allergens may be of sufficient magnitude to induce sensitization in susceptible people (10,19,21), and to develop respiratory symptoms after occasional animal contacts (5).

Although cat allergen is the third common allergen in the Middle East countries (22), exposure to cat allergen, sensitization, and its impact on developing allergic rhinitis (AR) and asthma is significant (23). Furthermore, available literature regarding sensitization to stray cats is conflicting (24-27). A study from Kuwait showed that despite low rate of cat ownership (4.1%) (28), the presence of cat allergens in public buildings is high (29). In contrast to low rate of cat ownership, the sensitization to cat was relatively high (27%) (30). This was similar to reports from the region (8,10,22). This could be related to numerous stray cats in Kuwait streets and gardens. Therefore, the diagnosis of sensitization to cats is important, irrespective of cat ownership. In up to 95% cases, the sensitizing allergen to cat (31-33) is Fel d 1, a glycoprotein which is produced by the sebaceous and salivary glands and transferred to cat fur (34). So cat fur is considered the primary source of cat allergens (35,36). Exposure occurs in public places mostly in countries with high rate of ownership (32,37-39), but also in countries with common stray cats (10,22,29,40). Recently there is a trend toward costly component resolved analysis (41-43) instead of standard diagnostic approaches, such as the extract based SPT and serology against native extract (44).

Study design and objectives

A randomized, controlled, prospective, experimental study was done on allergic rhinitis adult patients with indirect exposure to cat allergen in Al Rashed Allergy Center in Kuwait. The primary objective was to determine diagnostic reliability of SPT wheal size (mm/diameter) and level of cat serum specific in cat non-owners by performing NCT with cat allergen fur extract.

The secondary objective was to determinate the role of NCT. Nasal smear for Eosinophil, PIFR, PEFR, were used as objective measurements of NCT outcome.

Materials and methods

Forty-one randomly selected adult patients with perennial AR as defined by ARIA guidelines (45) sensitized to cat only or poly-sensitized to cat and at least one more common inhalant allergen, were included and divided into cat owners (n = 10) and cat non-owners (n = 31). Cat owners required a confirmed current direct domestic contact (≥ 5 years). Non-owners never kept cat at home and denied any known direct or indirect exposure to cat. The inclusion criteria included the following: 1, a positive skin prick test with a wheal size of (SPT ≥ 5 mm) in diameter, and serum specific IgE ≥ 0.7 IU/ml, to cat only or to cat and at least one common inhalant allergens using a battery of local inhalant allergens (with a long, almost-perennial, pollination); 2, baseline nasal PIFR and PEFR (Clement-Clarke International Ltd, Harlow, Essex UK) within a normal range. The exclusion criteria included: patients with allergic rhinitis and associated asthma.

SPT was performed with a battery of inhalant allergens (Stallergenes, France), including local pollens and cat. Normal saline and histamine were used as negative and positive controls. Skin wheal size (diameter/mm) was recorded after 15 minutes as the mean of 2 perpendicular measurement and was considered as positive as wheal diameter was ≥ 3mm. SSigE was determined by CAP (Phadia, Pharmacia Sweden).

NCT with cut fur allergen extract (Stallergenes, France) was done at least 3 weeks after acute episode of rhinitis, 1 week after discontinuation of oral antihistamine, nasal cortisone and decongestant, and 2 weeks after antidepressant and oral cortisone (≥ 10 mg/day). The NCT was performed out of the main pollination peaks following manufacturer recommendation. Frozen dried cat allergen extract (100 IR/ml), as an active substance, was freshly prepared by reconstitution with 9% diluent in different concentration starting from 0.1 to maximum 10 IR/ml. After patient's acclimatization (≈ 10 min) to the physician office condition, the NCT was performed by placing progressive doses of allergen in contact with a patient's nasal mucous membrane, using nebulized cap to deliver 100 **μl/1 puff of allergen solution in each nostril. Nasal reaction was assessed 20 minutes after each dose of allergen, keeping 10 minutes pinched and 10 minutes non-pinched nose as follows: sneezing: 0** = 0 - 2; 1 = 3 - 4; 3 = ≥ 5 sneezes; rhinorrhoea and nasal obstruction: 0 = absence, 1 = mild, 2 = moderate; 3 = severe; nasal palate, eyes and/or ears pruritus: 0 = absence, 1 = presence. The test was considered as positive when the total score was ≥ 5.

Eosinophil nasal smear, as wells as PIFR and PEFR (the best of three measurements was recorded) and compared at three steps: baseline, 2 hours and 8 hours after the challenge.

Nasal samples for Eosinophil positivity were collected by passing a sterile swab, from each nasal cavity, along the medial surface of the inferior turbinate 2 to 3 times, and the specimen smeared on a clear glass slide. Nasal smears were examined by light microscopy after staining with haematoxylin and eosin stain. Eosinophil positivity in nasal smear were calculated in the same time measurement points and compared with a baseline value. Results were interpreted by scale: weak positive (4 - 10 Eo/hpf), moderate (11 - 30 Eo/hpf), strong positive (> 30 Eo/hpf).

Non-parametric and parametric methods are used to calculate

statistical significance. The distribution value is determined by D'Agostino and Pearson omnibus test normality. Student's t-test, Mann-Whitney test, Fisher's test and $\chi 2$ test were used for calculating the difference between the groups. ANOVA test was used to calculate the relative difference distribution variance between variables. The statistical hypotheses were tested at the level of $\alpha = 0.05$, and the difference between the groups in the sample was considered significant when $p < 0.05$ or less. Statistical significance was depicted as: $p < 0.05$, $p < 0.01$ and $p < 0.001$. All data were analysed using GraphPad Prism version 7 (San Diego, California, USA).

This study was approved by the Ministry of Health ethics approval committee, number 2/2016.

Results

Cat owners and non-owners showed similar age and gender distribution ($p > 0.05$ for both measurements). The youngest cat owner was 24 years old (female), and the oldest was 54 years old (male). The youngest cat non-owners were 16 years old (male), and the oldest was 57 years old (male).

Mean wheal / diameter (mm) SPT for cat or pollens, as well as ssIgE antibodies level showed similar distribution in cat owners and non-owners ($p > 0.05$ for both measurements).

NCT positivity was more common in cat owners than in cat non-owners ($p < 0.05$).

Cat owners with positive NCT showed bigger wheal compared to cat non-owners with positive NCT ($p < 0.05$). However, in cat non-owners, wheal on SPT was bigger in NCT positive in comparison to NCT negative patients ($p < 0.05$).

PIF showed similar distribution in both groups compared to baseline levels, as well as 2 and 8 hours after NCT ($p > 0.05$ for all measurements). In cat owners, PIF decreased significantly 2 hours after NCT ($p < 0.01$), but despite recovering 8 hours after NCT it was still lower in comparison to baseline ($p < 0.05$). However, similarly to cat owners in cat non-owners PIF decreased significantly 2 hours after NCT ($p < 0.0001$), but 8 hours after NCT PIF recovered completely showing no difference in comparison to baseline ($p > 0.05$). Meanwhile, PEF remained the same during the challenge in either group ($p > 0.05$) (**table 1**).

In both cat-owners and non-owners, most patients reacted on 3rd concentration ($p < 0.01$), and then on 2nd concentration ($p < 0.05$). However, more frequent reactions on top concentration was observed in cat owners compared to non-owners ($p < 0.01$). On the other side, similar frequencies were observed among cat-owners and non-owners, on 1st, as well as on 2nd

Table 1 *- Patients' baseline and follow up characteristics.*

Patients	Cat owners	Cat non-owners	p value
number of patients	10	31	
age (years)	37.90 ± 13.36	31.94 ± 12.18	0.1959
gender f/m (number)	8/2	12/19	0.0564
SPT positive (cat only)	4 (40.0%)	12 (38.71%)	0.7642
SPT cat (mean wheal size in mm)	9.4 ± 2.38	8.42 ± 2.38	0.2673
SPT pollens (mean wheal/mm)	6.90 ± 6.08	7.13 ± 5.55	0.9122
cat ssIgE (IU/ml: mean ± SD) level	3 [0, 5]	3 [0, 5]	0.9934
positivity of NCT	10 (100%)	19 (61.29)	0.0179[1]
SPT in NCT positive patients (mean wheal diameter/mm)	10.0 ± 2.75	8.37 ± 2.06	0.0411[1]
SPT in NCT negative patients (mean wheal diameter/mm)	-	7.083 ± 1.38	-
p value	-	0.0118[1]	
PIFR before NCT	67.50 ± 8.58	65.58 ± 9.3	0.5669
PIFR 2 hours after NCT	29.50 ± 7.98	40.48 ± 18.04	0.0712
PIFR 8 hours after NCT	64.00 ± 7.38	64.32 ± 7.45	0.9056
p value	< 0.0001[1]	< 0.0001[1]	
PEFR before NCT	469.0 ± 62.8	459.4 ± 55.91	0.6476
PEFR 2 hours after NCT	459.0 ± 60.64	457.7 ± 45.51	0.9445
PEFR 8 hours after NCT	483.0 ± 51.43	488.1 ± 51.41	0.7879
p value	0.6582	0.2143	

[1]Difference is statistically significant.

Figure 1 *- Frequency of reactions on different concentration of allergen in NCT in cat owner and cat non-owners.*

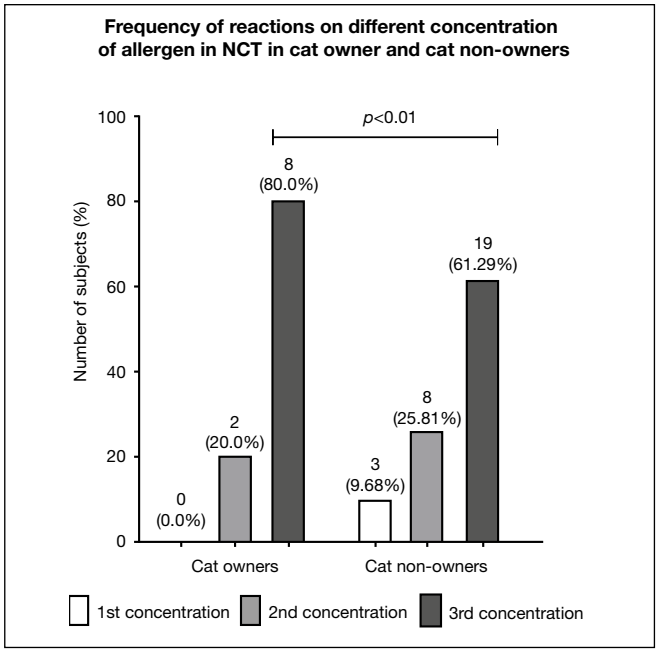
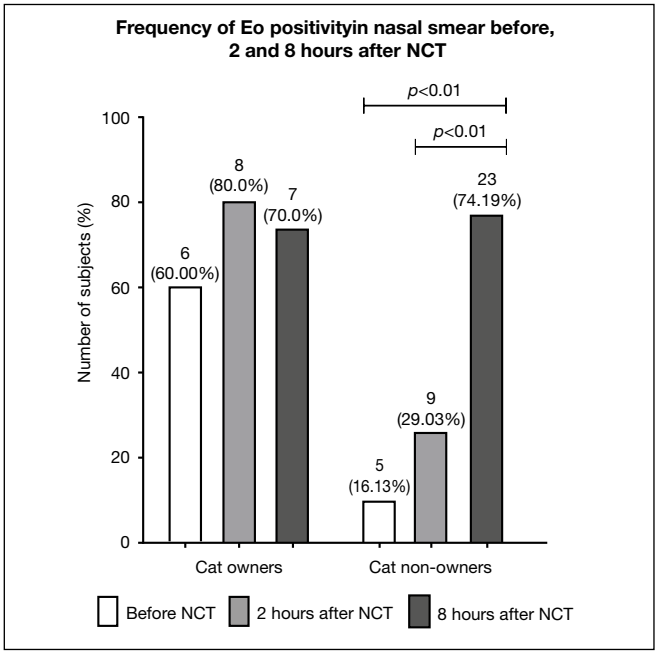


Figure 2 *- Frequency of Eo positivity of nasal smear before NCT, after NCT and 8 hours after NCT in cat owners and non-owners.*



concentration (p > 0.05 for both measurements) (**figure 1**).
Cat owners showed similar Eo positivity in nasal smear before NCT, 2 and 8 hours after NCT (p > 0.05). On the other side, cat non-owners showed more significant Eo positivity in nasal smear 8 hours after NCT compared to positivity before and 2 hours after NCT (p < 0.01 for both measurements). Eo positivity in cat non-owners was similar before and 2 hours after NCT (p > 0.05). However, in comparison to cat non-owners, Eo positivity in cat owners was more pronounced before NCT and 2 hours after NCT (p < 0.05 for both measurements), but 8 hours after NCT no significant difference was observed (p > 0.05) between cat owners and cat non-owners (**figure 2**).
NCT showed similar positivity between patients sensitised on cat only and patients sensitised on cat and pollen (p > 0.05).

Discussion

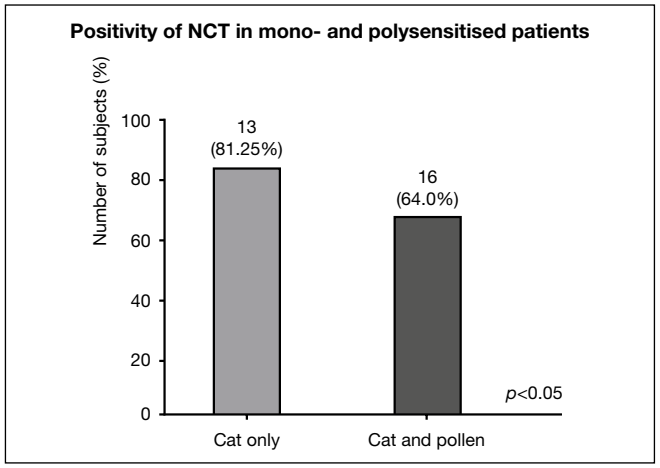
In contrast of dog allergy, the role of cat allergens delivered from stray cats in the development of perennial AR is less investigated, both in children and adults. However, the dose of allergen exposure is not always linear (15). So, the relationship between exposure and sensitization to cat allergen is complex and still inconsistent, based on difficulties to classify subjects to direct or indirect allergen exposure (5).
In a study performed in Turkey (10), where cat ownership is low but street cats are common, sensitivity to cat allergen by SPT in adults ranged from 11% to 44.7%. Our results have shown SPT positivity in 40% of cat owners and 38.71% of cat non-owners with no significant difference (**table 1**), which was higher than reported results by others (40).
Furthermore, in 2003 Linneberg A et al. (46) have shown that exposed to cat at home, in adult increased the risk of developing IgE sensitization to cat.
However, our results have shown similar distribution of ssIgE concentration in cat owners and non-owners (**table 1**). These findings may point out stray cats as a possible risk factor in the development of perennial AR in adults, regardless on cat owning. On the other side, advanced component based diagnostic testing could not replace SPT and native cat extract serology in the detection of sensitization to cats and differentiation between allergy and sensitization without clinical relevance (47).
Complementary with positive ssIgE antibodies, SPT to natural allergen extracts is highly predictive of symptoms development upon allergen exposure (48,49). However, atopy quantification using specific SPT wheal diameter (50) and IgE level may better predict the expression of rhinitis than using atopy as a dichotomous variable (presence / absence of sensitization) (51-53).
NCT may be helpful as additional measurements when the SPT results are not clinically informative enough regarding exposure. However, NCT has not yet been widely accepted in clinical practice (54) . Despite of significant difference in NCT positivity in cat owners and non-owners (100% vs. 61.29%, p < 0.05) in our study, high percentage of NCT positivity in cat

non-owners could be related to the exposure from stray cuts.
We observed bigger SPT wheal size in NCT positive cat owners in comparison to NCT negative cat non-owners ($p < 0.05$) (**table 1**), which could be explained by permanent exposure to higher doses of cat allergens in cat owners than in cat non-owners.
Performing a conjunctival challenge with cat allergen extract to determine importance of unnoticed exposure, Braso et al. (55) found positive challenge outcome in 15/20 SPT positive non-cat owners with a history of respiratory allergy and exposed to low level (mean of 0.4 microgram/g of dust) of cat allergen. Our results have also shown the bigger SPT wheal size in NCT positive cat non-owners than in NCT negative cat non-owners ($p < 0.05$) (**table 1**). All of these subjects had markedly positive SPT (> 5 mm/diameter) and ssIgE ≥ 0.70 IU/ml. So, being consistent with literature (55), results from our study support diagnostic importance of wheal SPT size and ssIgE antibodies level.
Although (56) Scadding et al. consider NCT as a recognized model that can help to understand the effect of challenging the upper airways on systemic or lower airway inflammation, these authors observed no significant change of PEF during up-dosing in NCT. Also in our study, similar PEF was noticed before, 2 and 8 hours after NCT (**table 1**) (56). As objective measurement of upper airway obstruction using PIF similar distribution in both group is shown when compared to baseline level, as well as 2 and 8 hours later ($p > 0.05$). We observed a significant fall in PIF value in the first measurement (2 hours) in both groups (cat owners: $p < 0.001$; cat non-owners: 0.0001) if compared with baseline value. We found recovering after 8 hours, seen by others (56), in non-owner group only ($p < 0.05$) (**table 1**). Our results might be explained with higher sensitivity, but lower specificity of PIF over PEF in detecting of obstruction (57).
In the Scadding et al. (56) study conducted on cat owners, significant increase in nasal response between second and highest concentration was absent during NCT. On the contrary, our results have shown that the total nasal score had increased with increasing concentration of cat allergen in both groups ($p < 0.05$) (**figure 1**). We speculate that other factors such as life style and climate may influence this phenomenon. The Kuwaiti dwellings, as well as all public buildings, are well ventilated by air conditioning system, which transfer cat allergens indoor. The harsh climate (high temperature, low humidity, and frequent presence of dry dusty wind (58), may increase dispersion and sedimentation of airborne allergens including cat allergen indoors without cats.
Eo in the nasal smear have been reported to display the best correlation with all the clinical and immunological parameters in allergic rhinitis (59). The sensitivity for nasal smear eosinophilia in the diagnosis of allergic rhinitis varies in different studies from 51.3% to 74%, with a specificity of 88.5% to 90% (60,61). However, Eo nasal smears are not necessary for routine use in diagnosing of AR, when the diagnosis is clearly supported by the history, physical examination, SPT and specific IgE diagnostic findings, but may be a useful adjunct when the diagnosis is questionable (62). In our study, Eo positivity in nasal smear was used to evaluate its importance in overall AR diagnostic approach, to evaluate difference between cat owners and non-owners and to estimate local reactivity after NCT as well in both groups. We have shown that cat owners have had significantly higher frequency of Eo positive in nasal smear before NCT, comparing to cat non-owners ($p < 0.05$). However, cat owners showed similar Eo positivity in nasal smear before NCT and 2 and 8 hours after NCT ($p > 0.05$). On the other side, cat non-owners showed more significant Eo positivity in nasal smear 8 hours after NCT compared to positivity before and 2 hours after NCT ($p < 0.01$), but in those patients Eo positivity was similar before and 2 hours after NCT ($p > 0.05$). However, Eo positivity in cat owners was more pronounced 2 hours after NCT ($p < 0.05$) than in cat non-owners, but 8 hours after NCT no significant difference was observed ($p > 0.05$) between owners and non-owners (**figure 2**). Regarding results on increased of Eo in nasal smear in cat owners, it seems that Eo nasal smear could be a helping tool in making a diagnosis of AR. On the other side, in cat non owners, elevated Eo in nasal smear 8 hours after NCT show on possibility of NCT using as decisive tool in making diagnostic of AR when the diagnose is indeterminate.
The evidence suggests that simultaneous exposure to more than one allergen might modify the effect of individual allergen (15). The same author (24) reported increased prevalence of sensitization to dust mites and pollens in adult pet owners in case of combined exposure. On the contrary, in our study, NCT showed similar positivity between mono (cat) and poly sensitised (cat and pollen) ($p > 0.05$) (**figure 3**). Such results suggest that cat allergy could be an independent risk factor for respiratory symptoms in our environment, where prevalence of sensitization to HDM in general is not high (30).
NCT is a safe and helpful procedure in allergy diagnostic. None of patients in either group during up dosing challenge withdrew from further procedure due to clinically significant lower airways symptoms or any other adverse reaction. Similar results are documented by other authors (54,56,63).
In conclusion, in an environment with common presence of stray cats, allergic sensitization to cat without direct exposure may be a relevant risk for developing perennial AR. Regardless of cat owning, SPT wheal size and level of ssIgE ≥ 0.70 IU/ml should be the first diagnostic tool. NCT and Eo nasal smear seem to be good further method in diagnostic of cat sensitization, especially in cat non-owners.

Patients consent

All patients were informed about the risk and outcomes of the procedure and provided informed consent.

***Figure 3** - shows positivity of NCT in mono- and poly-sensitised patients.*



Conflict of interest

The authors declare that they have no conflict of interest.

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Latex sensitization in patients with myelomeningocele: contribution of microarray technique

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Key words

latex; myelomeningocele; recombinant allergens; latex-fruit syndrome; allergy

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Summary

Background. *Microarray technique employing molecular allergens is pointed out as a new method to evaluate allergic patients.* ***Objective.*** *To evaluate if microarray technique (ImmunoCAP-ISAC®; I-ISAC®) is similar to fluorescence enzyme immunoassay (FEIA; ImmunoCAP®) in the diagnosis of latex allergy (specific IgE to latex plus symptoms) and latex sensitization (only antibody) in pediatric (9-mo to 14-yrs) patients with myelomeningocele undergone to surgical repair.* ***Methods.*** *Patients underwent skin prick testing (SPT) to latex and food (prick to prick), and dosage of serum specific IgE to latex and recombinant latex allergens (rHev b 1, rHev b 3, rHev b 5, rHev b 6.01, rHev b 6.02, rHev b 8, rHev b 9, and rHev b 11) by ImmunoCAP® and I-ISAC®.* ***Results.*** *The comparison between the two methods showed high level of concordance considering positive and negative results. A statistically significant correlation for rHev b 3 and rHev b 6.01 for the allergic patients, and for rHev b5 and rHev b6.01 for those sensitized to latex, was observed. I-ISAC® is limited to 5 recombinant latex allergens (rHev b 1, 3, 5, 6.01 and 8). Despite the presence of antibodies against pollens, LTP and profilins (I-ISAC®) in some patients, none of them reported symptoms related with intake of fruits and/or vegetables.* ***Conclusion.*** *Both methods are effective in assisting the diagnosis of latex allergy, but differ in the assessment of sensitized pediatric patients with myelomeningocele. The assessment of latex sensitized patients is more complete using the 8 recombinant latex fractions available for ImmunoCAP®, instead of I-ISAC®.*

Introduction

The recent advent of microarray technique employing molecular allergens represents a breakthrough for better evaluation of allergic patients, especially those with food allergy. This technique has allowed more extensive studies in allergic patients and provided a better understanding of cross-reactivity among different allergens, especially those derived from plants (1,2).

Patients allergic to latex may experience symptoms after eating certain foods, like fruits, vegetables and seeds. This characterizes the latex-pollen-fruit syndrome and is due to cross-reactivity of allergens from different sources called pan-allergens, such as lipid transfer proteins (LTP), storage proteins and profilins (3,4).

In a previous study, we evaluated the profile of latex sensitization/allergy in children and adolescents with myelomeningocele, followed up at a specialized center (5). We observed prevalence of sensitization and of allergy to latex among these children as 25% and 20%, respectively (5). Specific IgE to rHev b 1, rHev b 3, rHev b 5, rHev b 6.01, and rHev b 6.02 were detected in over 50% of children allergic to latex (presence of latex-specific IgE antibody plus clinical symptoms when exposed) (5). The higher frequency of specific IgE to rHev b 6.01, rHev b 6.02, and rHev b 5 than to rHev b 3 in our study was surprising, since the reports for this population indicate the opposite (5).

Determination of specific serum IgE against 8 recombinant *Hevea brasiliensis* allergen components was performed using a customized allergen microarray and a conventional fluorescence

enzyme immunoassay (FEIA) in 52 adults with immediate-type natural rubber latex allergy (6). These authors concluded that natural rubber latex-specific IgE recognition patterns and sensitization rates determined by microarray analysis were similar to those obtained by FEIA (6).

Do the patients with myelomeningocele also have this concordance between these diagnostic methods? Furthermore, would the microarray be able to detect sensitization to other allergens related to latex? To clarify these questions, we studied whether the microarray technique was able to detect sensitization to latex in the same way as FEIA in pediatric patients with myelomeningocele. Additionally, we tried to find out whether there are advantages in performing the microarray technique instead of FEIA in these patients.

Patients and methods

Twenty-five children and adolescents (9 months to 14 years, mean = 7.3 years) known to be sensitized to latex that had undergone surgical repair of myelomeningocele, followed at the Department of Neurosurgery of the Federal University of São Paulo, Brazil, were studied, as partially reported above (5).

Patients' parents or guardians were asked if their children had allergic diseases or symptoms, previous personal or family conditions related to corrective surgery of the myelomeningocele, or a current history of reaction to latex or foods. Patients underwent skin prick testing (SPT) to latex and food (prick to prick - avocado, apple, celery, chestnut, fig, jackfruit, kiwi, manioc, mango, melon, papaya, peach, pear, pineapple, potato and tomato) (7) and blood samples were obtained for quantification of specific IgE to latex and recombinant latex allergens (rHev b 1, rHev b 3, rHev b 5, rHev b 6.01, rHev b 6.02, rHev b 8, rHev b 9, and rHev b 11) by FEIA and microarray technique.

Specific IgE antibody levels in FEIA technique (ImmunoCAP®, Thermo Scientific®) were expressed in kU/L, and values equal to or greater than 0.35 kU/L were considered positive. Specific IgE antibody levels in microarray technique (I-ISAC®; Thermo Scientific®) were expressed in ISU (ISAC Standardized Units), and values equal to or greater than 0.3 were considered positive (8,9). The study was approved by the Ethic Committee of Federal University of São Paulo, and all parents / guardians have signed an informed consent.

Results

Table 1 summarizes the concordance of results of specific IgE to recombinant latex allergens obtained by both methods and shows high concordance for both groups of patients (allergic and sensitized).

Table 2 presents the results of the SPT to food and the presence of specific IgE to several allergens. In addition to specific antibodies to latex fractions, we detected sensitization to pollens, without a distinct pattern between allergic or sensitized to latex patients, in 5 subjects, as follows: a) Timothy grass: Grass group 1, rPhl p 1, n = 1; b) Berberine bridge enzyme, rPhl p 4, n = 3; c) Tree pollen: Olive pollen, nOle e 1 (common olive group 5), n = 1 and d) Plane tree, putative invertase inhibitor (rPla a 2), n = 1. Two other patients were sensitized to: a) Lipid transfer protein (nsLTP): peanut (rAra h 9), n = 1; walnut (nJug r 3), n = 2; b) peach (rPru p 3), n = 2; wheat (rTri a 14), n = 1; c) mugwort (nArt v 3), n = 1; d) olive pollen (nOle e 7), n = 1 and e) Plane tree (rPla a 3), n = 1. Sensitization to profilins, annual mercury (rMer a 1), n = 1, and to cross-reactive carbohydrate determinants (CCD, nMUFX3), n = 1, were also observed (**table 2**).

Discussion

According to extensive reports, the profile of specific IgE to latex fractions varies according to the subject evaluated. Healthcare

Table 1 *- Concordance (n / total, %) between presence (positive), absence (negative) or both (positive + negative) of specific IgE to recombinant latex allergens obtained by ImmunoCap® and ImmunoCap-ISAC® in patients allergic or sensitized to latex.*

Allergen	Allergic (n = 11)			Sensitized (n = 14)		
	positive	negative	total	positive	negative	total
rHev b 1	8/10 80.0%	1/1 100%	9/11 81.8%	4/6 75.0%	8/8 100%	12/14 86.0%
rHev b 3	4/6 75.0%	5/5 100%	9/11 81.8%	3/5 60.0%	8/9 88.9%	11/14 79.0%
rHev b 5	6/7 85.7%	4/4 100%	10/11 91.0%	2/3 66.7%	10/11 90.9%	12/14 86.0%
rHev b 6.01	5/8 62.5%	3/3 100%	8/11 73.0%	2/4 50.0%	10/10 100%	12/14 86.0%
rHev b 8	0/1 0.0%	9/10 90.0%	9/11 81.8%	1/1 100%	13/13 100%	14/14 100%

Table 2 - *Patients allergic or sensitized to latex according to skin prick test (prick to prick) with fruits and vegetables and presence of specific IgE identified by ImmunoCAP-ISAC (sIgE > 0.3 ISU-E).*

		Storage proteins	LTP	Profilins	Pollens	Gliadin	CCD	Fungi	Latex (ISU)				
Allergic													
Pt#									rHev b 1	rHev b 3	rHev b 5	rHev b 6.01	rHev b 8
9	potato avocado pineapple	nJug r 2 rAra h 6	nJu g r 3; rAra h9; rPru p 3; nArt v 3; nOle e 7 ;rTri a 14; rPla a 3	-	rPhl p 4; nOle e1; rPla a 2	-	-	-	2.2	1.5	48.0	4.5	2.2
12	manioc	-	-	-	-	-	-	-	-	-	7.4	1.4	-
24	tomato / potato manioc	nJug r 2	-	-	rPhl p 4; nCyn d 1; nCyn d 3; rPla a 2	-	nMUFX3	-	2.3	9.8	> 100	-	2.3
28	mango / jackfuit	-	-	-	rPhl p 7	-	-	-	12.0	-		-	12.0
37	chestnut	-	-	-		-	-	-	5.6	-	19.0	9.3	5.6
45	papaya	-	-	-		-	-	-	0.6	0.8	1.3	-	0.6
7	negative	-	-	-	rPhl p 1	-	-	-	0.5	-	-	0.8	-
55	negative	-	nArt v 3	-	rPhl p 4	rTri a 19	-	rCla h 8	-	2.2	5.3	-	-
Sensitized													
42	papaya/ pearl	-	-	-	-	-	-	-	1.2	0.3	-	8.8	-
13	negative	-	nJug r 3; nArt v 3	-	-	rTri a 19	-	-	-	0.4	-	-	-
41	negative	nJug r 2	-	-	-	-	-	-	3.0	22.0	7.2	1.5	-
50	negative	-	-	rMer a 1	-	-	-	-	-	-	-	-	0.5

LTP, Lipid transfer protein; peanut (rAra h 6, rAra h 9); walnut (nJug r 2, nJug r 3); peach (rPru p 3); wheat (rTri a 14, rTri a 19); mugwort (nArt v 3); Timothy grass (rPhl p 1, rPhl p 4, rPhl p 7); olive pollen (nOle e 1, nOle e 7); plane tree (rPla a 2, rPla a 3); annual mercury (rMer a 1); cross-reactive carbohydrate determinants (CCD, nMUFX3).

professionals are generally sensitized to Hev b 2, Hev b 5, Hev b 6.02, and Hev b 13, while patients with spina bifida are sensitized to Hev b 1, Hev b 3, and Hev b 7 (3,4). Recently, allergy to latex in patients undergoing multiple surgeries has been associated with specific IgE to rHeb v 5 and rHeb v 6.01 (4). Among our patients with myelomeningocele and latex allergy, 90.9% had specific IgE to Hev b 1 (5).

Although the levels of specific IgE to latex and fractions obtained by Immuno-CAP® were higher among latex allergic patients, they were not significantly different from those found in individuals sensitized to latex. Comparing these two methods (ImmunoCAP® vs. I-ISAC®) we observed a high level of concordance considering both positive and negative results. Since these methods are different, the quantitative comparison of their results is not possible (**table 1**).

The I-ISAC® available commercially contains 5 recombinant latex allergens (rHev b 1, 3, 5, 6.01, and 8), and using ImmunoCAP® we can quantify 8 fractions (rHev b 1, 3, 5, 6:01, 6:02, 8, 9, and 11). So, using ImmunoCAP® alone we would classify 11 patients as allergic and 14 patients as sensitized, whereas using I-ISAC® the results were 11 allergic and 9 sensitized. Five patients were not identified as latex-sensitized because I-ISAC® test does not contain the latex fractions to which they were sensitized (rHev b 6.02, 9, and 11).

Despite the presence of antibodies against pollens, LTP and profilins in some patients, none of them reported symptoms related with intake of fruits and/or vegetables. However, they were submitted to SPT (prick to prick) for foods and some of them were positive for: papaya (n = 2), potato (n = 2), avocado (n = 1), chestnut (n = 1), jackfruit (n = 1), mango (n = 1), manioc (n = 1), tomato (n = 1), pear (n = 1), and pineapple (n = 1), and were negative for apple, banana, celery, fig, kiwi, melon and peach (**table 2**). Another point to be emphasized was the presence of specific IgE to pollens, grasses and trees to which our patients probably were not exposed.

Comparing results of SPT with specific IgE to all different components tested in I-ISAC®, we did not observe a suggestive pattern to differentiate allergic and sensitized patients to latex, although we have found some patients with negative SPT and positive specific IgE to storage proteins, profilins and LTPs (**table 2**).

Some conditions may explain these findings. The food profile on I-ISAC® (walnuts, peanuts, peach, wheat) does not seem to be the most appropriate to investigate the latex-fruit syndrome, because it does not contain many foods known to cause of the syndrome (e.g. avocado, apple, celery, chestnut, fig, jackfruit, kiwi, manioc, mango, melon, papaya, peach, pear, pineapple, potato and tomato) (3,4,5). In addition, latex-fruit syndrome is reported to be more frequent among healthcare professionals than in patients with myelomeningocele. These patients usually show asymptomatic sensitization, while healthcare workers may present clinical manifestations as well as anaphylaxis (10).

In conclusion, we observed that both methods are effective in assisting the diagnosis of latex allergy (sensitization + symptoms), but they differ in the assessment of sensitized patients (without symptoms) in the pediatric patients with myelomeningocele. The assessment of patients sensitized to latex is more complete when using the 8 recombinant latex fractions available in ImmunoCAP® compared with I-ISAC®.

The negative predictive value of SPT for diagnosing food allergy in pediatric patients with myelomeningocele is high. In this population, the latex-fruit syndrome is better evaluated using fresh suspected food (prick to prick) than when using I-ISAC®. In pediatric patients with myelomeningocele, the I-ISAC® test has proven not to be the best method for investigation of latex-pollen-fruit syndrome.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Localized salt-dependent aquagenic urticaria, a rare subtype of urticaria: a case report

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Key words

aquagenic urticaria; localized; hypertonic saline; sea water; salinity

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Summary

Aquagenic urticaria (AU) is a rare form of chronic inducible urticaria elicited by water at any temperature. We describe the case of a 25-year-old atopic woman who presented to our unit with a 4-year history of recurrent urticarial rashes, highly pruritic, confined to the neck and lower part of the face, occurring solely on contact with sea water. The lesions were reproduced by challenge tests with aqueous 3.5% NaCl and other hypertonic aqueous solutions but not with 20% glucose neither tap water.

Our case supports the existence of a distinct salt-dependent subtype of aquagenic urticaria (SDAU), which seems to be triggered mostly by sea bathing, affects young women and has a characteristic localization on the inferior facial contours and neck. To the best of our knowledge, only eight cases of SDAU have been reported in the literature.

Introduction

Aquagenic urticaria (AU) is a rare form of chronic inducible urticaria elicited by water at any temperature. Pruritic wheals develop immediately or within minutes at the sites of contact of the skin with water, irrespective of temperature or source, and clear within 30-60 min. Sweat, saliva, and even tears can precipitate a reaction. Symptoms often start in puberty, but cases presenting in childhood are also reported (1-3). It affects both genders, but has a female predominance. Most cases are sporadic but familial cases are also reported (4-10), and it was recently described in monozygotic twins (11), although no specific genetic mutation has been found yet.

Systemic symptoms are rare but have been reported, including wheezing, dysphagia, and respiratory distress (12,13).

Water challenge test performed at body temperature for 20 min is recommended for diagnosis of AU. Differential diagnosis includes aquagenic pruritus, in which intense itching occurs after contact with water, but without visible skin lesions; cholinergic urticaria, where wheals develop in response to heat, exertion, sweating or emotional stress, and cold urticaria, characterized by rapid appearance of itching wheals and occasionally angioedema after cold stimulation (14,15).

AU was first described by Shelley and Rawnsley, who reported three cases in 1964 (10) and fewer than 100 cases have since been published in the literature (13-19).

A distinct salt-dependent subtype of aquagenic urticaria (SDAU) seems to be triggered mostly by sea bathing, and affects young women, with a characteristic localization on the inferior facial

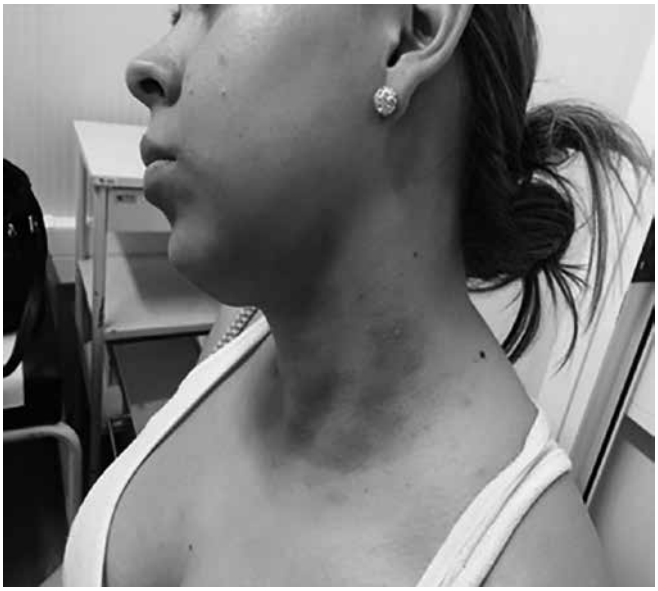
contours and neck. To the best of our knowledge, only eight cases of SDAU have been reported in the literature (20-22).

Case report

A 25-year-old atopic woman, with a history of mild persistent allergic rhinitis for several years induced by both house dust mites and grass pollens, was referred to our department due to recurrent episodes of urticaria. She reported a 4-year history of recurrent urticarial rashes, highly pruritic, confined to the neck and lower part of the face, occurring solely on contact with sea water. The lesions appeared within about 10 min after the start of bathing in sea water and cleared spontaneously within 30 min after showering with tap water, leaving no residual mark. Her symptoms had occurred several successive summers while bathing in the Atlantic sea. The water temperature and the time of exposure were irrelevant. She did not report angioedema, wheezing or dyspnea during these episodes. Water ingestion and contact with swimming pool, river and tap water had no effect. No other types of inducible urticaria were present. There was no family history of atopy, and none of the girl's relatives reported similar skin reactions related to water exposure. Her symptoms were not associated with food ingestion or drug intake before water contact. She denied insect stings, minor trauma, infection, application of retinoids or any other irritant topical drug. Apart from oral contraceptives, she denied the use of other medications. Antihistamines taken before sea bathing were not effective.

A general physical examination was unrevealing, and there was a notable absence of dermographism. Laboratory evaluation, which included complete blood count, erythrocyte sedimentation rate, C-reactive protein, urine analysis, serum electrophoresis, immunoglobulins (G, A, M), thyroid function tests, anti-thyroid antibodies, antinuclear antibodies, rheumatoid factor and complement fractions (C3, C4, and C1q) revealed no abnormalities. Cryoglobulins were negative. There were no parasites or eggs in the stool sample. Level of total immunoglobulin E was 329 IU/ml. Her skin tests were positive for house dust mites and grass pollens. An ice cube test elicited no reaction. Water challenge tests were performed with fresh sea water, tap water, aqueous hypertonic saline (3.5% NaCL, iso-osmolar with sea water), and 20% glucose solutions and also with different hypertonic aqueous solutions (3.5% KCL, 5.3% NaHCO3) at 35 °C, by means of soaked compresses applied to the patient's submandibular area and neck and, as controls, to the antecubital flexures. The compresses were left in place for 20 min. The patient reacted intensely with pruritus, micropapular eruption and erythema in the contact area (**figure 1**), to fresh sea water and to all solutions containing salts, but she did not react to a similar osmotic load with 20% glucose neither to tap water. Control tests in the antecubital flexures were all negative. We also performed a chemical analysis of a sea water sample (Praia de Salgueiros - Atlantic sea, Vila Nova de Gaia, Portugal), where she used to take **baths**, and we found a salinity of about 3.5%. A diagnosis of SDAU was made. Short baths, immediate washing of the body surface with fresh water and application of protective hydrophobic barrier creams before sea bathing were recommended. We also prescribed medical treatment with oral levocetirizine 5 mg.

Figure 1 *- Localized erythema, micropapular eruption and scattered urticarial lesions 20 min after applying a compress soaked in fresh sea water and in different aqueous hypertonic solutions (3.5% NaCL, 3.5% KCL, 5.3% NaHCO3) at 35 °C to the neck.*



Discussion

Only a few cases of SDAU have been reported in the literature. The first one was described by Gallo et al. in 2001 (20): the patient was a young woman whose urticarial rashes were restricted to the lower facial contours, and were more intense on contact with sea water and hypertonic saline than with tap water. The same authors reported six further cases with similar characteristics in 2013 (21). The lesions appeared a few minutes after sea bathing, and had a selective localization on the inferior facial contours and neck, as in our patient. The challenge tests with hypertonic saline (3.5% NaCl) were positive in all six patients. Two patients reacted also to tap water or to normal saline, but less intensely. One patient had also been tested with different water solutions that were iso-osmotic with sea water (3.5% KCl, 5.3% NaHCO3 and 20% glucose), and also with 3.5% NaCL, distilled and tap water. She only reacted to solutions containing

salts (3.5% NaCL, 3.5% KCl, and 5.3% NaHCO3), as we stated in our patient.

The pathogenesis of AU is not well established, however, several hypotheses have been proposed (22). The authors who first described this condition proposed the formation of a toxic substance by the combination of water and sebum that causes mast cell degranulation (10). Further support for this hypothesis developed when Chalamidas and Charles (23) reported that patch testing with a patient's own sweat did not produce urticaria while patch testing with the patient's own sweat and sebum produced marked perifollicular urticaria. Czarnetzki et al. (8) hypothesized the existence of a water soluble antigen at the epidermal layer. The antigen diffuses into the dermis by water and then causes release of histamine from mast cells. Tkach (24) hypothesized that hypotonic water sources could lead to osmotic pressure changes, resulting in indirect provocation of urticaria. Others have stated that 5% saline was more effective than distilled water for eliciting the wheal-and-flare reaction. They hypothesized that the salt concentration and/or water osmolarity may influence the pathogenic process of AU, possibly by enhancing solubilization and penetration of a hypothetical epidermal antigen, in the same way as has been postulated for enhancement of organic solvents (25). Another proposed chemical mediator in AU is acetylcholine, because of the ability of the acetylcholine antagonist scopolamine to suppress wheal formation when applied to the skin before water contact (26). However, another study failed to reproduce this finding when pretreatment with atropine did not result in suppression of subsequent wheal formation (8).

Gallo et al (21) have recently suggested the involvement of a transient receptor potential vanilloid subtype (TRPV1) channel in the salt-dependent aquagenic urticaria's pathophysiology, based on previous findings of Ständer et al. (27), which reports the wide distribution of the TRPV1 in human skin including sensory nerve fibers, mast cells, epidermal keratinocytes, blood vessels, epithelial cells of hair follicle, eccrine sweat glands, and sebaceous glands. This receptor is a non-selective cation channel that could be activated by capsaicin (a substance not synthesized in the human body) or by the cannabinoid anandamide (28-31), through an endogenous activation by an increase of temperature within a noxious range (above 42 °C), and by protons (pH below 5.9). This non selective sodium permeable channel can act as a "cellular sensor" of osmotic concentration and its presence on mast cells could lead to activation and therefore to the histamine release.

Because of its unclear pathogenesis, treatment options are limited and produced varied responses at best. Antihistamines are usually used as first line treatment, up to four times the standard single daily dose to treat aquagenic urticaria, although response varies from patient to patient.

In refractory cases ultraviolet monotherapy alone or in combination with antihistamines have been used with some efficacy (32). It has been hypothesized that the ultraviolet therapy induces thickening of the epidermis, which may prevent water penetration and further interaction in the epidermal milieu. Other therapeutic options have included topical barrier creams and acetylcholine antagonists (26) and it was recently described a case of aquagenic urticaria successfully treated with omalizumab (33).

Treatment of SDAU consists of reducing the time contact with sea water and the application of barrier creams before sea bathing. Oral antihistamines are also required, but response may vary.

Conclusions

We described a rare case of a young women with aquagenic urticaria triggered only by sea bathing and strictly localized on the inferior facial contours and neck.

Urticarial lesions were reproduced by challenges with aqueous 3.5% NaCl and other hypertonic aqueous solutions (3.5% KCL, 5.3% NaHCO3) but not with 20% glucose neither tap water.

Our case, as previous reports, suggests the existence of a SDAU and supports the hypothesis that the salt concentration and/or osmolarity of water may influence the pathogenic process of aquagenic urticaria. We think the reaction to be dependent on the salinity of the water rather than on its osmolality, because the patient did not react to hypertonic glucose.

Patient consent

Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

Conflict of interest

The authors declare that they have no conflicts of interest.

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