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Allergy immunotherapies for allergic rhinitis: systematic review and assessment of evolving quality

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Key words

allergic asthma; allergic rhinitis; allergy immunotherapy; grass allergy; quality assessment

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Summary

Background. *Heterogeneity in the design and quality of trials evaluating allergy immunotherapies (AITs) limits their comparability, making it difficult for physicians, patients, and payers to select the best treatment option.* ***Methods.*** *This systematic review evaluated the quality of randomised controlled trials (RCTs) of registered grass AITs using the National Institute of Health and Care Excellence checklist.* ***Results.*** *17 of 44 unique RCTs (38.6%) (sample size range: 18-1,501 subjects) were subcutaneous grass immunotherapy trials and 27 (61.4%) were sublingual grass immunotherapy trials (Allergovit, 5 trials; Alutard, 8; Grazax, 13; Oralair, 6; Staloral, 8; Pollinex, 2; Phostal and Purethal, 1 each). Three trials (6.8%; all Grazax) fulfilled every quality criterion. Quality assessments revealed inconsistencies in study quality and reporting. Study quality trended towards improvement over time, particularly after 2009.* ***Conclusions.*** *When assessing grass AIT, it is important to focus not only on endpoints but also on the quality of evidence.*

Introduction

Allergic rhinitis/allergic rhinitis with conjunctivitis (AR/ARC) and asthma are considered separate manifestations of the same disease: chronic airway inflammation, occurring in the upper airway in AR/ARC and in the lower airway in asthma (1,2). AR/ARC is one of the most important risk factors for asthma and typically precedes the development of asthma, contributing to unsatisfactory disease control (2-4). Early diagnosis and treatment of AR/ARC is crucial to halt the progression of the disease to asthma (3,5). Symptom-relieving pharmacotherapy for AR/ARC is not effective for all patients and does not prevent development of asthma symptoms because it does not treat the underlying disease (6). Allergy immunotherapy (AIT), or gradual exposure to an allergen to desensitise the immune response to trigger allergen, is a treatment option for patients with AR/ARC related to grass pollen and other allergens whose symptoms are inadequately controlled by pharmacotherapy. Allergy immunotherapy treats the underlying disease, thereby reducing symptoms (1,7,8).

Selection of the most appropriate AIT treatment for individuals with AR/ARC is complex, in part because of the historical background of the development of AIT. Historically, allergen extracts have not been seen as medical products in the European market, and named patient products, which may be distributed in European countries without a marketing authorisation (9), have been and continue to be widely used (10,11). Moreover, the first regulatory approvals were granted to AIT products with very limited or even no randomised controlled trials (RCTs) supporting a positive benefit-risk profile. For physicians and patients who decide to use a registered AIT product, the challenge is to find a product with well-documented evidence for the efficacy and safety.

Allergen-specific AITs for AR/ARC may be administered subcutaneously or sublingually. In European countries, subcutaneous immunotherapy (SCIT) has been available for more than a century (12). Sublingual immunotherapy (SLIT), which is available in drop formulation and, more recently, in tablet formulation, was first licensed in 2006 (13). There are limitations in the evidence base for SCIT and SLIT products. Early trials of AITs were often uncontrolled and included small sample sizes (10). Placebo-controlled clinical trials are now common, and the quality of these trials has improved in the past decade (14). Nevertheless, heterogeneity in trial design and population limits the comparability of trial results (10,11,14). The European Academy of Allergy and Clinical Immunology (EAACI) has published recommendations regarding clinical outcomes in AIT trials for ARC. These are likely to assist in standardising outcome measures to enable better analysis of clinical efficacy and improve the comparability of results (15).

Clinical guidelines recommend AIT for uncontrolled AR/ARC symptoms. The Allergic Rhinitis and Its Impact on Asthma (ARIA) clinical guidelines recommend AIT, including SLIT or intranasal allergen-specific immunotherapy, for the treatment of AR due to pollen (16). In 2017, the EAACI issued guidelines for AIT for the treatment of AR (17). These guidelines note that some AIT products do not provide sufficient data to support their efficacy in clinical practice and recommend that only standardised AIT products with documentation of efficacy should be prescribed. Specifically, the guidelines recommend preseasonal/co-seasonal SLIT for seasonal AR for short-term benefit and grass pollen SLIT tablets or solution with continuous therapy for AR for long-term benefit.

To support treatment decision making for AITs for AR/ARC, the objective of this study was to conduct a systematic literature review to identify placebo-controlled RCTs of grass AITs used for the treatment of patients with AR/ARC, with the aim of evaluating the quality of published evidence. The review was restricted to grass AIT products that are registered in Europe, including Allergovit, Alutard/ALK Depot, ALK start, Grazax, Oralair, Phostal, Pollinex, Polvac, Purethal, and Staloral.

Materials and methods

Searches were performed on the MEDLINE, Embase, Biosciences Information Service (BIOSIS), and Cochrane Library electronic literature databases on 25 January 2017, with no date, language, or geographical restrictions. Updated searches of the same databases were performed on 24 April 2018. In addition, conference abstracts (EAACI; American Academy of Allergy, Asthma and Immunology [AAAAI]; European Respiratory Society [ERS]; American Thoracic Society [ATS]) were searched from 1 January 2015 to 30 December 2016. Two study registries (ClinicalTrials.gov and the European Union Clinical Trials Register [https://www.clinicaltrialsregister.eu/]) were also searched for completed trials with results. Bibliographic lists of included recent relevant systematic literature reviews and meta-analyses were searched for further studies of interest.

Search terms included combinations of free text and Medical Subject Headings (MeSH). Specifically, the searches included terms for the population of interest (disease), including AR or ARC and grass or grass pollen (e.g., ("Rhinitis, Allergic" [MeSH] OR "allergic rhinitis"[Text Word] OR "allergic rhinoconjunctivitis"[Text Word]) AND grass[Text Word]); for the interventions or comparators of interest (e.g., "allergy immunotherapy"[Text Word] OR "sublingual immunotherapy-"[Text Word]); and for the study types of interest, including placebo-controlled, randomised, clinical trials (e.g., "Randomized Controlled Trials as Topic"[MeSH] OR "Randomized Controlled Trial"[Publication Type]). Animal studies, phase 1 trials, comments, and editorials were excluded.

The study selection process occurred in 2 phases, during which studies were screened for relevance based on study design, population, interventions included, and language of publication. **Table I** presents the inclusion and exclusion criteria that were used at the level 1 and level 2 screenings. Specifically, at level 1 screening, titles and abstracts of identified studies were reviewed independently by 2 researchers (double screening) for eligibility according to the inclusion and exclusion criteria. Any discrepancies were resolved; when a consensus was not reached, a third researcher was consulted. At level 2 screening, full texts of studies selected at level 1 were obtained and reviewed for eligibility, using the same inclusion and exclusion criteria. Single screening was performed for 52% of articles; double screening was performed for 48% of articles. The inclusion and exclusion processes were documented. Only articles published in the English language were reviewed.

Quality of the included studies then was assessed using a modified version of the checklist recommended by the National Institute of Health and Care Excellence (NICE) (18), which is a validated and accepted quality-assessment checklist and which has been used previously to assess study quality for AIT trials (19). **Table II** presents the items comprising the NICE checklist and the methods used to assess each item. Primary trial publications were the focus of this review; however, any previously published articles describing the study design or methodology of a trial that were cited in the primary publication for that trial also were consulted to identify additional details about the quality-assessment items. Although the quality-assessment items that constitute the NICE checklist are somewhat subjective, they were evaluated consistently across studies, supporting the comparability of the assessments.

Table I *- List of criteria for the inclusion and exclusion of articles.*

Criteria	Included	Excluded
Level 1		
Study design	Randomised, double-blind, placebo-controlled trials Long-term follow-up studies (e.g., open-label follow-up of randomised, double-blind, placebo-controlled trials) Systematic reviews and metaanalyses[a]	Nonrandomised studies Open-label randomised studies Phase 1 studies Proof-of-concept studies Prognostic studies Comments Editorials Letters Case reports Studies in animals but not humans
Population	Adults and children with grass pollen AR or ARC undergoing treatment with AIT	Patients without AR or ARC Patients with AR or ARC induced by allergens other than grass or grass pollen, e.g., house dust mites, animal dander/animal allergens, tree pollen or mould
Interventions	Trials that include AIT in at least 1 study arm. Terms for AIT may include: - allergen immunotherapy - specific immunotherapy (SIT) - allergen-specific immunotherapy - sublingual immunotherapy (SLIT) - subcutaneous immunotherapy (SCIT) - allergy vaccination	Articles that do not include AIT in at least 1 study arm
Outcomes	No limits	None
Language	English	Non-English
Level 2		
Study design	Same criteria as level 1	Same criteria as level 1
Population	Same criteria as level 1	Same criteria as level 1
Interventions	ALK start SQ/ALK 7 Allergovit Alustal Alutard/ALK Depot Grazax Oralair Phostal Pollinex Polvac Purethal Staloral	Treatments other than the treatments of interest
Outcomes	Efficacy (AR symptom reduction; AR medication use reduction; asthma symptom reduction; asthma medication use reduction) Safety and tolerability Quality of life Compliance Patient preference	Articles that do not report any of the outcomes of interest
Language	English	Non-English

Abbreviations: AIT, allergy immunotherapy; AR, allergic rhinitis; ARC, allergic rhinitis with conjunctivitis.
Note. Any issues with study design will be reported via the quality-assessment process.
[a]Systematic reviews and meta-analyses will be used for identification of primary articles.

Table II *- Items assessed in the modified NICE RCT checklist.*

NICE RCT Checklist Item	Response
Was randomisation carried out appropriately?[a]	yes/no/not clear/NA
Was the concealment of treatment allocation adequate?[b]	yes/no/not clear/NA
Were the groups similar at the outset of the study in terms of prognostic factors [baseline characteristics]?[c]	yes/no/not clear/NA
Were the care providers, participants, and outcome assessors blind to treatment allocation?[d]	yes/no/not clear/NA
Were there any unexpected imbalances in dropouts between groups?[e]	yes/no/not clear/NA
Is there any evidence to suggest that the authors measured more outcomes than they reported?[f]	yes/no/not clear/NA
Were all randomised patients included in the analyses?[g]	yes/no/not clear/NA

NA, not applicable; NICE, National Institute of Health and Care Excellence; RCT, randomised controlled trial.
[a]The process of randomisation was found to be appropriate if the authors provided further elaboration on the methods used to generate the random allocation such as a table of random numbers or a computerised random number generator;
[b]Allocation concealment, which is the method used to implement the random allocation, was sufficient if participants had no prior knowledge of treatment assignment by using an external body, sequentially numbered containers, centralised assignments, or an automated system;
[c]Prognostic factors of treatment groups were classed as similar where authors reported that there were no significant differences in baseline characteristics or the reported baseline characteristics were similar across groups;
[d]Blinding was adequate if authors explicitly stated that participants were blinded and described the use of a placebo that was similar to the active drug;
[e]Dropout rates were considered to be balanced if the proportion of patients withdrawing from the trial were similar across the groups;
[f]Outcome reporting was considered adequate if authors reported all outcomes stated in the methods section or provided sufficient information about where the additional data could be located;
[g]The inclusion of all randomised patients in the analyses was considered adequate if all randomised patients were included in the efficacy and safety analyses.
Source: CRD (18).

Results

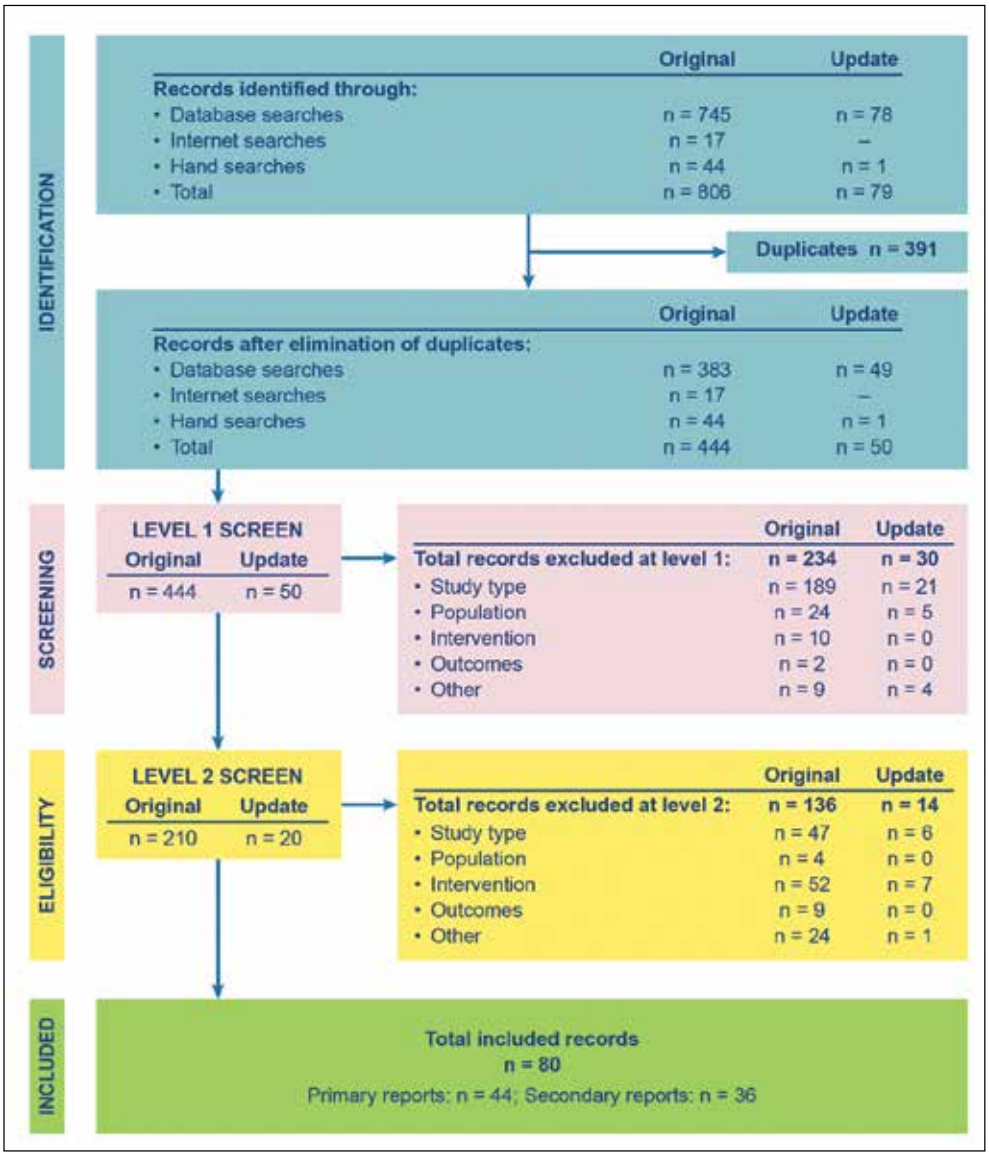
Search results

A total of 444 potentially relevant unique records from the January 2017 searches and 50 potentially relevant records from the April 2018 searches were identified for screening: 383 (from January 2017) and 49 (from April 2018) published studies from the database searches, 17 conference abstracts from the Internet searches (January 2017), and 44 (January 2017), and 1 (April 2018) published studies from hand searches of bibliographies. After level 1 screening, 210 (January 2017; databases 162; Internet searches 6; hand searches 42) and 20 (April 2018; databases 19; hand searches 1) studies were progressed for further screening. After level 2 screening, 80 articles were included (databases 61; Internet searches 1; hand searches 18); 44 were unique studies (primary reports) and 36 were secondary reports (1 of which was a randomised, double-blind, placebo-controlled study protocol). For the purposes of conducting the quality assessments, all conference abstracts then were excluded because they included insufficient detail about the assessment items (**figure 1**).
Among the 44 unique studies identified, 17 (38.6%) were SCIT trials and 27 (61.4%) were SLIT trials. Overall, 5 trials reported on Allergovit, 8 on Alutard, 2 on Pollinex, and 1 each on Phostal and Purethal (all SCITs); 13 trials reported on Grazax, 6 on Oralair, and 8 on Staloral (all SLITs). These treatments were compared with placebo in all trials. No studies reporting on ALK start or Polvac were identified.

Quality Assessment

Overall quality
The results of the quality assessment by year and by treatment type (SCIT vs. SLIT) are shown in **figures 2** and **3**, respectively. Overall, 3 of 44 trials (6.8%), all Grazax studies, fulfilled every quality criterion in the NICE checklist (**figure 3**).
The sample sizes of included trials ranged from 18 subjects (20) to 1,501 subjects (21). Trials of Grazax included the largest number of subjects across the included trials (5,832 subjects in total), followed by Oralair (2,227 subjects), Alutard (830 subjects), Staloral (789 subjects), Allergovit (281 subjects), Pollinex (258 subjects), Purethal (60 subjects), and Phostal (29 subjects). Nineteen trials included < 100 subjects: 4 of Allergovit, 5 of Alutard, 1 of Phostal, 1 of Purethal, 2 of Grazax, 1 of Oralair, and 5 of Staloral. Ten trials included 100 to 199 subjects: 1 of Allergovit, 2 of Alutard, 2 of Pollinex, 2 of Grazax, 1 of Oralair, and 2 of Staloral. Four trials included 200 to 299 subjects: 2 of Grazax, 1 of Oralair, and 1 of Staloral. Two trials, both of Grazax, included 300 to 399 subjects. Eight trials included 400 to 999 subjects: 1 of Alutard, 4 of Grazax, and 3 of Oralair. One Grazax trial included 1,501 subjects (21). Twenty-seven trials were conducted in adults (Allergovit, 3 trials; Alutard, 6 trials; Grazax, 8 trials; Oralair, 4 trials; Staloral, 2 trials; Phostal, 1 trial; Pollinex, 2 trials, Purethal, 1 trial), 9 included only children (Alutard, 1 trial; Grazax, 4 trials; Oralair, 1 trial; Staloral, 3 trials), and 8 included both children and adults (Allergovit, 2 trials, Alutard, 1 trial; Grazax, 1 trial; Oralair, 1 trial;

Figure 1 *- PRISMA Diagram.*



PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

Staloral, 3 trials). One trial conducted in children and 2 trials conducted in adults met all 7 of the quality criteria.

In general, there was a trend towards improved study quality over time: more recent studies, particularly those published after 2009, appropriately addressed more of the quality-assessment items relative to older studies (**figure 2**). Furthermore, for each product, study sizes tended to increase over time (**figure 3**).

Randomisation and concealment of treatment allocation

The process of randomisation was found to be appropriate if the authors provided further elaboration on the methods used to generate the random allocation (e.g., a table of random numbers or a computerised random number generator). Allocation concealment was considered sufficient if participants had no prior knowledge of treatment assignment by using an external body, sequentially numbered containers, centralised assignments, or an automated system.

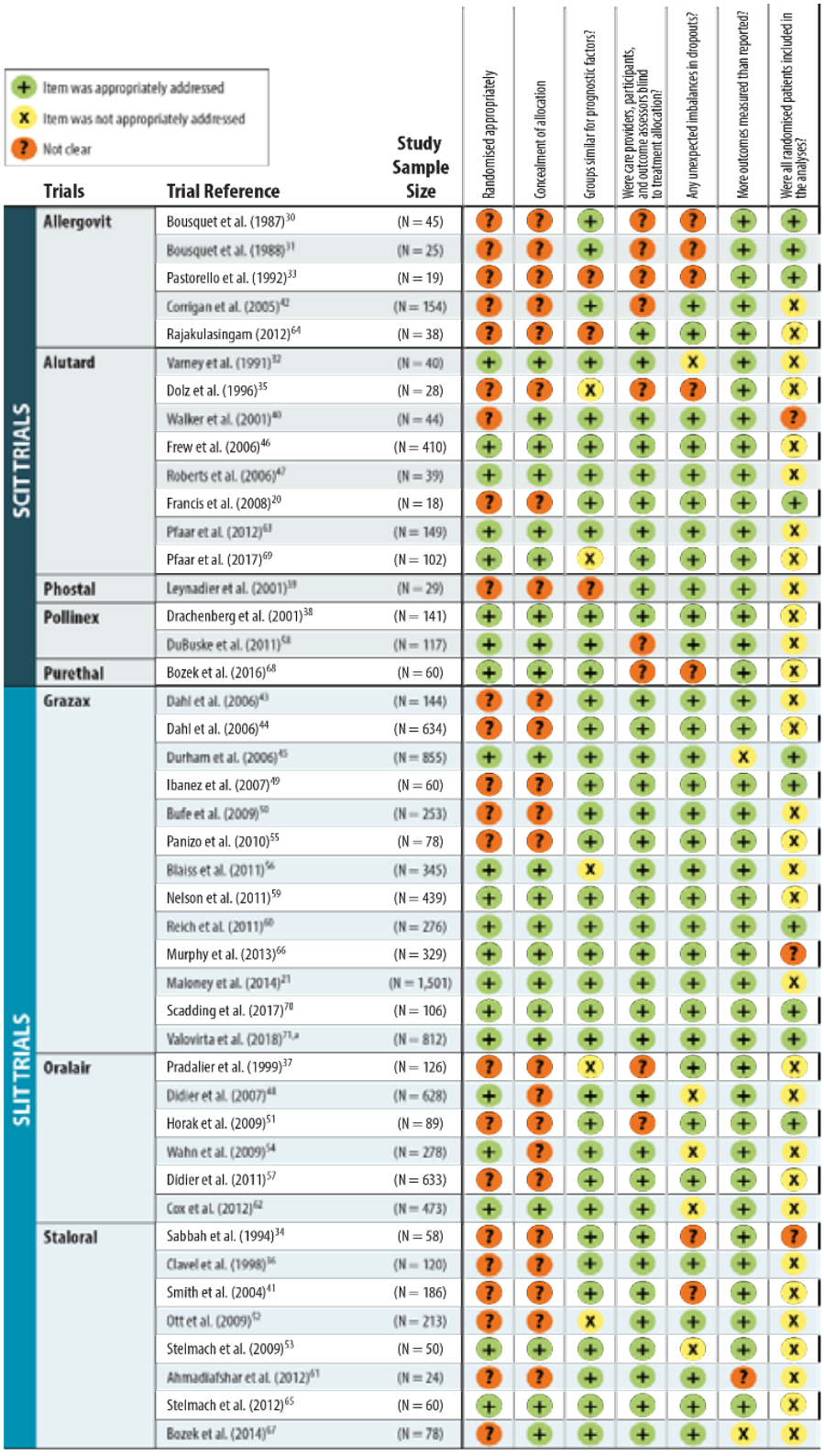
Of the 44 included trials, 21 (47.7%) reported methods of randomisation in detail and 21 (47.7%) adequately described concealment of treatment allocation. By treatment, appropriate randomisation methods were reported for 100% of Pollinex trials (2/2), 62.5% of Alutard trials (5/8), 61.5% of Grazax trials

Figure 2 *- Summary of quality assessment of included randomised controlled trials, by year.*

[a]Information about this trial's inclusion of all randomised patients in the analyses was presented in Valovirta et al. (72), which was cited as a methods paper in the primary trial publication.

Note. The process of randomisation was found to be appropriate if the authors provided further elaboration on the methods used to generate the random allocation such as a table of random numbers or a computerised random number generator. Allocation concealment, which is the method used to implement the random allocation, was sufficient if participants had no prior knowledge of treatment assignment by using an external body, sequentially numbered contain-ers, centralised assignments, or an automated system. Prognostic factors (or baseline characteris-tics) of treatment groups were classed as similar where authors reported that there were no sig-nificant differences in baseline characteristics or the reported baseline characteristics were simi-lar across groups. Blinding was adequate if authors explicitly stated that participants were blinded and described the use of a placebo that was similar to the active drug. Dropout rates were considered to be balanced if the proportion of patients withdrawing from the trial was sim-ilar across the groups. Outcome reporting was considered adequate if authors reported all out-comes stated in the methods section or provided sufficient information about where the addi-tional data could be located. The inclusion of all randomised patients in the analyses was consid-ered adequate if all randomised patients were included in the efficacy and safety analyses.

Figure 3 *- Summary of quality assessment of included randomised controlled trials, by SCITs vs. SLITs.*



SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy.

[a]Information about this trial's inclusion of all randomised patients in the analyses was presented in Valovirta et al. (72), which was cited as a methods paper in the primary trial publication.

Note. The process of randomisation was found to be appropriate if the authors provided further elaboration on the methods used to generate the random allocation such as a table of random numbers or a computerised random number generator. Allocation concealment, which is the method used to implement the random allocation, was sufficient if participants had no prior knowledge of treatment assignment by using an external body, sequentially numbered contain-ers, centralised assignments, or an automated system. Prognostic factors (or baseline characteris-tics) of treatment groups were classed as similar where authors reported that there were no sig-nificant differences in baseline characteristics or the reported baseline characteristics were simi-lar across groups. Blinding was adequate if authors explicitly stated that participants were blinded and described the use of a placebo that was similar to the active drug. Dropout rates were considered to be balanced if the proportion of patients withdrawing from the trial was sim-ilar across the groups. Outcome reporting was considered adequate if authors reported all out-comes stated in the methods section or provided sufficient information about where the addi-tional data could be located. The inclusion of all randomised patients in the analyses was consid-ered adequate if all randomised patients were included in the efficacy and safety analyses.

(8/13), 50.0% of Oralair trials (3/6), 25.0% of Staloral trials (2/8), and no trials of Allergovit or Phostal (**figure 3**). Adequate concealment of treatment allocation was reported for 100% of Pollinex trials (2/2), 75.0% of Alutard trials (6/8), 61.5% of Grazax trials (8/13), 37.5% of Staloral trials (3/8), 16.7% of Oralair trials (1/6), and no trials of Allergovit or Phostal. Among SCIT studies, 47.1% (8/17) reported methods of randomisation in detail and 52.9% (9/17) adequately described concealment of treatment allocation; among SLIT trials, 48.1% (13/27) and 44.4% (12/27), respectively, appropriately addressed these measures. In general, studies published after 2009 more consistently used and/or reported methods of randomisation and allocation concealment than older studies (**figure 2**).

Similarity of baseline characteristics

Baseline characteristics of treatment groups were classed as similar where authors reported that there were no significant differences in baseline characteristics or the reported baseline characteristics were similar across groups.

Overall, in 37 of 44 trials (84.1%), treatment and placebo groups had similar baseline characteristics. Baseline characteristics were similar between groups in a majority of trials for each treatment: Pollinex, 100% of trials (2/2); Purethal, 100% (1/1); Grazax, 92.3% (12/13); Staloral, 87.5% (7/8); Oralair, 83.3% (5/6); Alutard, 75.0% (6/8); Allergovit, 60% (3/5); and Phostal, 0. Among SCIT trials, 70.6% (12/17) had treatment groups with similar baseline characteristics, whereas 88.9% of SLIT trials (24/27) had treatment groups with similar baseline characteristics.

Blinding of treatment allocation

Blinding of treatment allocation was considered adequate if authors explicitly stated that participants were blinded and described the use of a placebo that was similar to the active drug. Although all trials were reported to be double blind, it was unclear whether subjects were blinded appropriately in 9 of 44 trials overall (20.5%). By treatment, 100% of Grazax trials (13/13), 100% of Staloral trials (8/8), 87.5% of Alutard trials (7/8), 66.7% of Oralair trials (4/6), 50% of the Pollinex trials (1/2), 20% of Allergovit trials (1/5), and the Phostal trial clearly reported on blinding procedures, whereas the Purethal trial did not. More SLIT trials (92.6%, 25/27) than SCIT trials (58.8%, 10/17) clearly reported on blinding procedures.

Unexpected imbalances in dropouts

Dropout rates were considered to be balanced if the proportion of patients withdrawing from the trial was similar across treatment groups.

In 5 of 44 trials (11.4%), there were unexpected imbalances in dropouts between treatment groups, and this item was not clearly reported in 7 trials (15.9%). By treatment, 60.0% of Allergovit trials (3/5), 37.5% of Staloral trials (3/8), 50.0% of Oralair trials (3/6), 25.0% of Alutard trials (2/8), and the Purethal trial either included or did not clearly report on imbalances in dropouts. No such imbalances were included in any of the 13 Grazax trials, the 2 Pollinex trials, or the Phostal trial. Proportionally more SCIT trials (35.3%, 6/17) than SLIT trials (22.2%, 6/27) included or did not clearly report on imbalances in dropouts.

Evidence of outcomes assessed and not reported

Outcome reporting was considered adequate if authors reported all outcomes stated in the methods section or provided sufficient information about where the additional data could be located.

Overall, 2 of 44 trials (4.5%) did not report all outcomes assessed, and this was unclear in 1 trial (2.3%). All trials of Allergovit (5/5), Alutard (8/8), Pollinex (2/2), Oralair (6/6), and Phostal and Purethal (1 each) reported on all outcomes assessed, whereas 92.3% of Grazax trials (12/13) and 75.0% of Staloral trials (6/8) reported on all outcomes assessed. All 17 SCIT trials and 88.9% of SLIT trials (24/27) reported on all outcomes assessed.

Inclusion of all randomised patients in the analyses

The inclusion of all randomised patients in the analyses was considered adequate if all randomised patients were included in the efficacy and safety analyses.

Overall, 10 of 44 trials (20.7%) included all randomised patients in the analyses. By treatment, 60% of Allergovit trials (3/5), 38.5% of Grazax trials (5/13), 16.7% of Oralair trials (1/6), and 12.5% of Alutard trials (1/8) included an all randomised patients in the analyses; none of the Staloral, Pollinex, Phostal, or Purethal trials included all randomised patitents in the analyses. Such analyses were included in 23.5% of SCIT trials (4/17) and 22.2% of SLIT trials (6/27).

Discussion

In this systematic literature review to assess the quality of 44 placebo-controlled trials of grass allergy AITs, only 3 trials, all Grazax studies, fulfilled all quality criteria in the modified NICE checklist. Consistent with previous findings that more recently conducted AIT trials are of better quality than older trials (22), our review found that more recent trials (published after 2009) were generally of better quality and reporting than older trials, both overall and for individual products. The trend towards improved quality over time is potentially a reflection of evolving standards for both trial design and reporting. More recent studies also tended to include larger sample sizes relative to older studies, both overall and for the individual products. Notably, 5 Grazax trials, 3 Oralair trials, and 1 Alutard trial each enrolled more than 400 subjects, and 1 of these Grazax trials included 1,501 subjects. Trials of SLIT products generally included larger sample sizes than SCIT trials.

Inconsistencies in the quality and reporting of trial methods were revealed when quality assessments were performed. Several trials reported that they were randomised, but only a few reported the methods used for randomisation, concealment of treatment allocation, inclusion of all randomised patients in the analyses, or handling of missing data. In particular, studies published after 2009 more consistently addressed randomisation and concealment of treatment allocation compared with older trials. In most trials, treatment groups had similar baseline characteristics. Although all trials were double blind, it was unclear whether blinding procedures were appropriate in approximately 20% of trials overall; more SLIT trials than SCIT trials clearly reported on blinding methods. Few trials overall, and proportionally more SCIT trials than SLIT trials, reported imbalances in dropouts between treatment groups. All SCIT trials and all but 3 SLIT trials reported on all outcomes assessed. Only a quarter of trials overall, and similar proportions of SCIT trials (approximately 24%) and SLIT trials (approximately 22%), included all randomised patients in the analyses. When studies were compared across the treatments reviewed, Grazax studies were of high quality relative to trials of other treatments, according to the quality assessments. Specifically, all Grazax trials included appropriate blinding methods and avoided unexpected imbalances in dropouts. In addition, 92% of Grazax trials reported on all outcomes assessed. Three Grazax trials appropriately addressed all 7 quality criteria. Although fewer trials of Alutard than of Grazax were identified (8 vs. 13), Alutard trials were also of good quality overall, with 87.5% of trials appropriately addressing 5 or more quality criteria.

The quality-assessment results from this study provide important context for the assessment of clinical endpoints and other outcomes in AIT. For example, previous research has explored the effects of SCIT versus SLIT for respiratory allergy. Although both SCIT and SLIT have been shown to be effective, SCIT is associated with a higher risk of life-threatening systemic reactions than SLIT (23). In contrast with SCIT, SLIT is suitable for at-home administration, is less painful and more convenient owing to a lack of injection, has a lower risk of anaphylaxis, has lower indirect costs, and has been shown to be cost saving relative to pharmacotherapy (15,24-26).

Physicians, patients, and payers considering AIT options for respiratory allergy should consider not only the attributes and outcomes of available treatments but also the robustness of the underlying evidence. Given the unique regulatory history of AIT products in Europe, some products have been registered or are in use that lack a solid evidence base. Nevertheless, evidentiary standards for AIT products are evolving, as can be observed from the AIT landscape in Germany. Guidelines on the use of AIT issued jointly by German, Austrian, and Swiss professional organizations in 2014 acknowledge that data from SCIT and SLIT trials differ in quality and scope and recommend product-specific evaluations to inform treatment decisions (27). In conjunction with these guidelines, a summary of the currently available AIT products (including registration dates) and a separate summary of the evidence fulfilling defined quality criteria, including study quality, supporting the available products are issued every 6 months (28,29). The intent of these resources is to enhance transparency for AIT products to support the physicians in their guidelines-based therapy decisions. Whether other health care systems will adopt a similar focus on quality of evidence for AIT products remains to be seen.

Some strengths and limitations of this study must be considered when the results are interpreted. Studies were identified systematically using a comprehensive search strategy with no date limitations and were screened according to predefined inclusion and exclusion criteria. Study quality was assessed using an accepted, validated measure that has been used previously to assess the quality of AIT evidence (19). Nevertheless, there are some limitations associated with the quality-assessment method in that studies indicating that they fulfilled a particular quality-assessment item (e.g., randomisation) but did not clearly describe the methods used for that item were classified as "not clear." Such classifications reflect incomplete reporting of the trial, potentially in line with reporting standards that were in place when the trial was published, and not necessarily poor quality. In particular, 5 trials (3 of Allergovit, 1 of Alutard, and 1 of Staloral) were published before the first CONSORT statement was issued in 1996. Finally, only articles published in the English language were reviewed, and thus trials published in other languages are not reflected in the results.

Conclusions

Considering the historical perspective on and the evolving evidentiary standards for AIT trials, it is important to understand the quality of the existing clinical evidence. Although the results here are only for grass AIT, it is likely that similar results would be found for other AIT products. The marketing and use of AIT products in Europe and worldwide are heterogeneous and historically have been guided by expert clinical opinion rather than close regulatory oversight (9). The standards for clinical evidence for AITs have evolved in recent decades, however; accordingly, the quality of AIT trials has tended to improve over time, with more recent trials generally including higher numbers of patients and appropriately addressing more quality-assessment items than older studies. This SLR focused on the published data for registered grass AIT. Published evidence is of better quality or is more extensive for some of the reviewed treatments than others. In particular, numerous trials have been published for Grazax and Alutard, which were of good quality on the whole. In comparison, evidence was limited for Phostal and Purethal. Our results support previous recommendations

that future trials in AIT should be robustly designed, in line with accepted quality metrics, and should consistently and completely report findings to aid their appraisal and interpretation (17,27). In particular, trials should use appropriate methods for randomisation, allocation concealment, blinding, inclusion of all randomised patients in the analyses, and accounting for missing data; should ensure balance between treatment groups in baseline characteristics and report on unexpected imbalances in dropouts; and should ensure reporting of all outcomes assessed. Evidence-based treatment decisions for AITs should rely on not only trial outcomes but also the quality of the evidence base.

Conflict of interest

Annete Njue, Weyinmi Nuabor, Matthew Lyall, and Anne Heyes are salaried employees of RTI Health Solutions. Lisa Elliott is a salaried employee of ALK-Abelló. Anne Domdey was a salaried employee of ALK-Abelló when this research was conducted.

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Author contributions

Anne Domdey initiated the study, secured funding, designed the study, interpreted the data, and participated in writing the manuscript. Annete Njue designed the study, screened the articles, led and conducted the analyses, interpreted the data, and participated in writing the manuscript. Weyinmi Nuabor and Matthew Lyall screened the articles, conducted the analyses, interpreted the data, and participated in writing the manuscript. Anne Heyes provided study oversight, contributed to the study design and analyses, interpreted the data, and participated in writing the manuscript. Lisa Elliott secured funding, contributed to the study design, interpreted the data, and participated in writing the manuscript.

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Circulating microRNAs as potential non-invasive biomarkers in pediatric patients with celiac disease

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Key words
celiac disease; microRNA; gluten free diet; biomarkers; auto-antibodies

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Summary
Celiac disease is an enteropathy induced by ingestion of gluten triggering an immune response in genetically predisposed individuals. MiRNAs are small non-coding RNAs that have a role as regulators of gene expression at the post transcriptional level. The aim of this study is to evaluate the possibility of using circulating miRNAs as non-invasive biomarkers in pediatric patients with celiac disease. In addition, we examine the effect of a gluten-free diet on the expression of these miRNAs in serum of CD patients. The expression pattern of miR-21 and miR-31 was estimated in serum of 25 untreated CD patients (recently diagnosed), 25 treated CD patients (on gluten-free diet) and 20 healthy controls using qRT-PCR. Our results demonstrated the significant up-regulation of microRNA-21 in the untreated celiac patients in comparison with the treated group and healthy controls. Moreover, miR-31 expression was significantly under-expressed in the untreated celiac patients in comparison with the treated group and healthy controls. Furthermore, the results showed that miR-21 expression level was significantly positively correlated with the tTG IgA auto-antibodies. In conclusion, circulating miRNA-21 and miRNA-31 could serve as potential non-invasive biomarkers for pediatric CD patients.

Introduction

Celiac disease (CD) is an autoimmune disease affecting the small intestine with a prevalence of 1 in 100 to 1 in 200 subjects, particularly children (1-3). This enteropathy is triggered by the interaction of environmental and genetic factors (3,4). CD is characterized by an immunological reaction against the TG2 (transglutaminase type 2) enzyme triggered by the ingestion of gluten peptides from wheat and related cereals in genetically predisposed individuals (2,5,6). This autoimmune reaction induces a duodenal damage characterized by villous atrophy, intraepithelial lymphocytosis, infiltration of inflammatory cells in the lamina propria and crypt hyperplasia (7,8). Defects in regulation of gene expression through microRNAs (miRNAs) could be responsible of the changes in intestinal permeability and intestinal immune system (2), suggesting their implication in the dysfunction of intestinal barrier and their association with certain clinical manifestation (9).
MiRNAs are small endogenous single-stranded non-coding RNAs that regulate gene expression through the control of stability and translation of the mRNA (10,11). MiRNAs have been associated with various pathological conditions of the immune system (12). Many studies have reported the aberrant expression of miRNAs in intestinal biopsies of celiac patients, while the role of circulating miRNAs and their expression levels are still undefined compared to that of tissue miRNAs (4). Capuano et al. (2011) evaluated the miRNA expression pattern in the small intestine of children with active CD, children with CD on GFD and control children without CD. Their results showed the overexpression of miR-449a and the decrease of miR-124a expression in CD patients and GFD treated CD patients than in controls (13).

In this study, we evaluate the possibility of using circulating miRNAs as non-invasive biomarkers in pediatric patients with celiac disease for diagnosis and prognosis. We investigate the deregulated expression pattern of miRNA-21 and miRNA-31 in serum of celiac disease patients. In addition, we examine the effect of a gluten-free diet on the expression of these miRNAs in serum of CD patients. We then analyze the correlation between these miRNA expression levels and the auto-antibodies of CD patients.

Subjects and methods

Ethics

This study was approved by the ethics committee of National Research Center, Giza, Egypt, and written informed consents were obtained from the parent/guardian of all children involved in our study before their enrollment.

Study subjects

This study included 70 subjects with age ranging from 2 to 14 years. They were divided into 3 groups: Group 1 consisted of 25 untreated CD patients (recently diagnosed); Group 2 consisted of 25 treated CD patients (on gluten-free diet) for about 5 years; Group 3 consisted of 20 healthy normal subjects matched for age and gender as a control group.
Patients were obtained from Cairo University Specialized Children Hospital, Cairo, Egypt. They were diagnosed according to the criteria of the European Society for Paediatric Gastroenterology Hepatology and Nutrition (analysis of autoantibodies [anti-tTG and anti-endomysium IgA] by ELISA with investigation of intestinal biopsy) (14). Clinical manifestations and treatments of CD patients are summarized in **table I**.

RNA extraction and quantitative real-time PCR

MicroRNA was extracted and isolated from plasma of all subjects of the study populations using miRNeasy Mini kit of Qiagen (Germany) according to the manufacturer's instructions. For miRNA-specific reverse transcription, microRNA was reverse-transcribed to cDNA using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) and using specific primers according to the manufacturer's instructions. Reverse transcription was performed under the following conditions: 30 min at 16 °C, 30 min at 42 °C, and followed by 5 min at 85 °C, and the resulting cDNA was kept at -80 °C until use.
A real-time quantitative PCR (qRT-PCR) was carried out to quantify the expression levels in triplicate of mature miR-31 and miR-21 using TaqMan® MicroRNA Assay kit and TaqMan® Universal Master Mix (Applied Biosystems) using step one real-time PCR system according to the manufacturer's instructions. RNU6B was used as endogenous control to normalize the expression levels of target miRs. Relative quantification (Rq) of miRNA expression was calculated using the $2^{-\Delta\Delta^{CT}}$ threshold cycle method. ΔCt was determined by subtracting the Ct values for RUN6B from the Ct values for the gene of interest. qRT-PCR was performed under the following conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 50 cycles at 95 °C for 15 s, and at 60 °C for 1 min.

Table I *- Clinical characteristics of the studied subjects.*

Characteristic	Treated patients with celiac disease	Untreated patients with celiac disease (recently diagnosed)	Normal healthy controls
no. of cases	25	25	20
sex, no. male/female	14 / 11	16 / 9	8 / 12
age, (range)	2 - 14	2 - 14	2 - 14
disease duration, mean ± sd (years)	4.88 ± 1.6	0	0
tTG IgA antibodies, no. positive/ negative	13 / 12	18 / 7	0 / 20
EMA IgA antibodies, no. positive/ negative	9 / 16	15 / 10	0 / 20
medications (GFD)	25 / 25	0 / 25	0 / 20
biopsy	villous shortening and relative increase in intraepithelial lymphocytes and mild chronic duodenitis	moderate enteritis with villous atrophy and moderate duodenitis	-

EMA IgA, auto-antibodies anti-endomysium IgA; GFD, gluten-free diet.

Statistical analysis

Data were statistically analyzed using SPSS version 16.0 software (SPSS Inc., Chicago, Illinois, USA). Nonparametric T test (Kruskal-Wallis test) was used to compare gene expression levels between groups, and Spearman's rank correlation to test association between gene expression levels and auto-antibodies of patients. Data were presented as the mean ± SEM. A p value of less than 0.05 was considered statistically significant. Receiver operating characteristic (ROC) curve was constructed for each miRNA to evaluate the efficiency of miRNAs as biomarkers for CD patients against controls. Area under curve (AUC) values and 95% confidence intervals for each miRNA were calculated.

Results

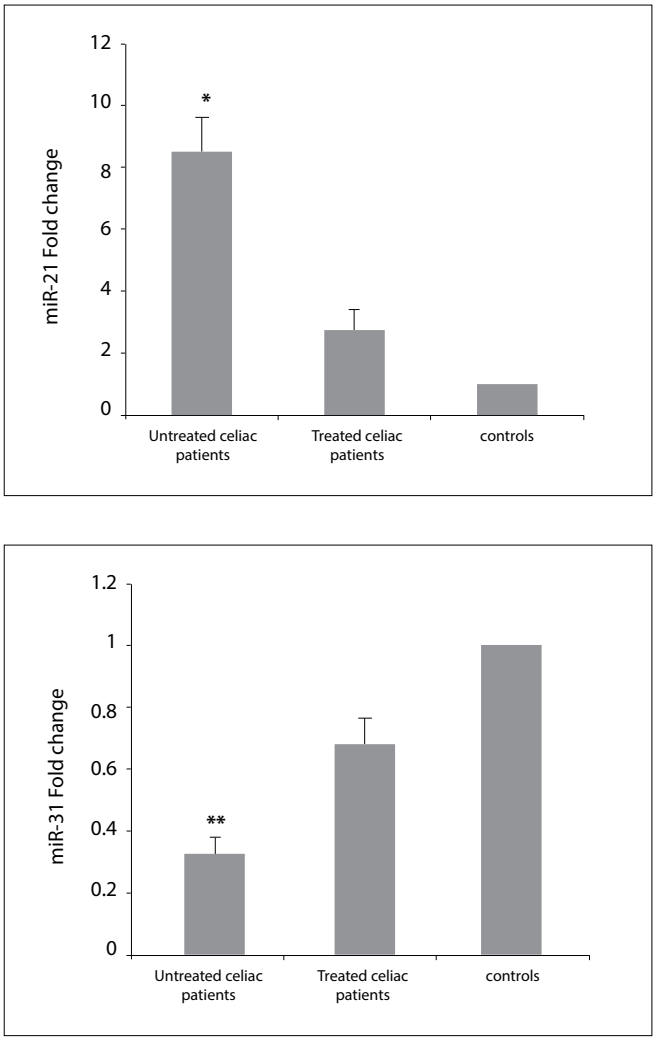
Deregulation of microRNA-21 expression pattern in plasma of CD patients

This study demonstrated the significantly increased expression levels of microRNA-21 in the untreated celiac patients in comparison with the treated group and healthy controls (**table II**). The miR-21 expression was over-expressed in the treated CD patients compared with the control group (**table II**). In the untreated group, the expression of miR-21 was 8.49-fold increased compared to the control group (**figure 1**). In the treated group, miR-21 expression was 2.8-fold higher compared to normal controls (**figure 1**).

Deregulation of microRNA-31 expression pattern in plasma of CD patients

Our results indicated that miR-31 expression was significantly down-regulated in the untreated celiac patients in comparison with the treated group and healthy controls and with insignificant down regulation in CD patients compared with the control group (**table II**). MiR-31 was 3-fold lower expressed in the untreated CD patients compared to the healthy controls, and 1.5-fold decreased compared to normal controls (**figure 1**).

Figure 1 *- Fold changes of miR-21 and miR-31 expressions of the untreated and treated CD patients were determined relative to normal controls. Bar graph represents the mean ± SEM fold change.*



[1]Statistically significant at p < 0.01 versus controls (by Kruskal-Wallis test); [2]Statistically significant at p < 0.001 versus controls (by Kruskal-Wallis test).

Table II *- Expression levels of miR-31, miR-21 in plasma of untreated CD patients, treated CD patients, and normal healthy controls. The results were expressed as mean ± SEM.*

Parameters	Untreated CD patients (mean ± SEM)	Treated CD patients (mean ± SEM)	Normal controls (mean ± SEM)
miR-21 expression level	135[1] ± 18	44.3 ± 10	16 ± 8
miR-31 expression level	0.45[2] ± 0.06	0.9 ± 0.13	1.3 ± 0.3

[1]Statistically significant at p < 0.001 versus normal controls (Kruskal-Wallis test); [2]Statistically significant at p < 0.01 versus normal controls (Kruskal-Wallis test).

Correlations between serum microRNAs and auto-antibodies in the untreated and treated CD patients

In the untreated and treated group, the results clarified that there is a significant positive correlation between miR-21 and tTG IgA auto-antibodies, while it has no correlation with EMA IgA auto-antibodies. Our data also showed no significant correlation between miR-31 expression levels with tTG IgA or EMA IgA auto-antibodies (**table III**).

ROC curve of microRNA-21 and microRNA-31

ROC curve showed that miR-21 has an AUC value of 0.847 (95% CI 0.704 - 0.991), while miR-31 has an AUC value of 0.801 (95% CI 0.658 - 0.944) at $p < 0.001$ versus normal controls. These findings revealed that both miR-21 and miR-31 could function as good biomarkers for CD patients against healthy controls (**figure 2, table IV**).

Discussion

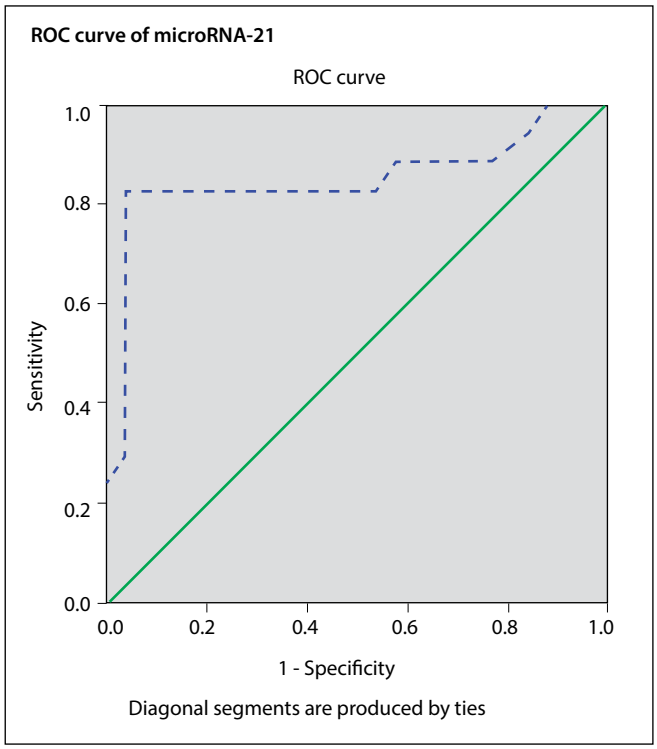
Specific miRNAs have been reported to be modulated in intestinal mucosa of CD patients, suggesting their involvement in the pathogenesis of CD and the probability of using thereof as diagnostic tools or as predictors of gluten free diet outcome in CD patients (2,4).

Table III *- Correlations between plasma miR-21 and miR-31 with auto-antibodies in the untreated and treated CD patients.*

Untreated CD patients	
parameters	R (Spearman correlation)
miR-21 expression ~ tTG IgA auto-antibodies	0.661[1]
miR-21 expression ~ EMA IgA auto-antibodies	-0.123
miR-31 expression ~ tTG IgA auto-antibodies	-0.257
miR-31 expression ~ EMA IgA auto-antibodies	0.112
Treated CD patients	
parameters	R (Spearman correlation)
miR-21 expression ~ tTG IgA auto-antibodies	0.775[2]
miR-21 expression ~ EMA IgA auto-antibodies	-0.160
miR-31 expression ~ tTG IgA auto-antibodies	0.291
miR-31 expression ~ EMA IgA auto-antibodies	-0.341

[1]Correlation is significant at the 0.01 level (2-tailed); [2]Correlation is significant at the 0.001 level (2-tailed).

Figure 2 *- ROC curve of miR-21 and miR-31 for patients with celiac disease versus healthy controls.*



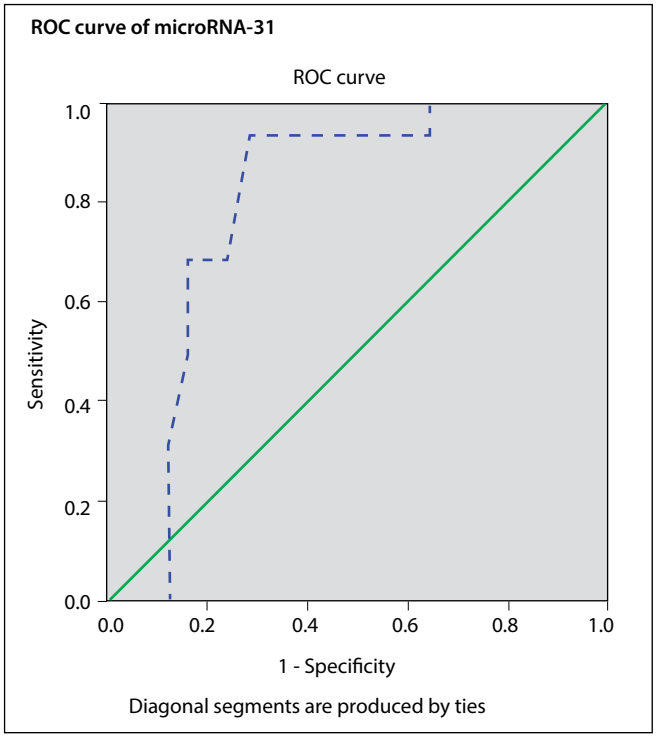


Table IV - *AUCs and 95% Confidence Intervals of miRNA-31 and miRNA-21 for CD patients versus normal controls.*

	CD patients against normal controls							
	AUC[1]	**standard error[2]**	**95% confidence intervals**	**sensitivity**	**specificity**	**LR+**	**LR-**	**cut-off value**
miRNA-21	0.847[1]	0.073	0.704 - 0.991	82.4%	80.8%	4.3	0.22	61.28
miRNA-31	0.801[1]	0.073	0.658 - 0.944	93.8%	72%	3.35	0.086	0.7

[1]Statistically significant at $p < 0.001$ versus controls; [2]Under the nonparametric assumption.

Our study demonstrated that miR-21 expression was significantly up-regulated in active CD patients compared to healthy controls and also children with CD on a gluten-free diet. In addition, the expression level of miR-21 was decreased in the treated CD patients compared to untreated patients. Moreover, in the treated and untreated CD patients, the results showed that miR-21 expression level was significantly positively correlated with the tTG IgA auto-antibodies, while there was no correlation between miR-21 and EMA IgA auto-antibodies. These findings are in line with results of Buoli Comani et al. (2015), who found a significant over-expression of miR-21-5p expression in the duodenal biopsies of active CD patients in comparison with controls. In addition, CD patients on a gluten-free diet showed a decrease in miR-21-5p compared to controls with a non-significant difference in expression patterns. MiR-21 is also widely reported as dysregulated in UC and CD (15). Ludwig *et al.* (2013) found that miR-21 was upregulated in IBD-associated dysplastic lesions compared with active patients with IBD (16). In a study of intestinal miRNA levels in CD, Wu et al (2010) identified several miRNAs that are upregulated (miR-16, -20a, -21, and -106a) (17). Let-7b, miR16, and miR-21 were greatly expressed in human dendritic cells, which likely contribute to the chronic inflammation of CD (18,19).

There were many studies reported that miR-21 had a proinflammatory role in IBD by impairing intestinal barrier function. Paraskevi *et al.* (2012) and Yang *et al.* (2013) found up-regulation of miR-21 in patients with UC in both the mucosal and blood samples (20,21). MiR-21 was found to affect the intestinal epithelial permeability by targeting RhoB, which was found significantly decreased in the patients with UC. In addition, intestinal integrity and morphology were declined in Caco-2 cells and in UC patients exhibiting overexpression of miR-21 through targeting RhoB (21).

Similarly, in IBD patients Shi et al. (2013) demonstrated the up-regulation of miR-21. Furthermore, their results showed the increase of intestinal permeability and epithelial cell apoptosis promoted by dextran sulphate sodium were attenuated in miR-21 knockout mice (22).

On the other hand, our study results showed that miR-31 expression was significantly under-expressed in the untreated celiac patients in comparison with the treated group and healthy controls. Moreover, its expression was restored in the treated CD patients compared to the untreated celiac patients. In addition, miR-31 expression level was not correlated with tTG IgA nor EMA IgA auto-antibodies in both the untreated CD group and treated CD group.

These results are similar to that of previous studies in which, Vaira et al. (2014) investigated the duodenal mucosa miRNA expression profile and confirmed significant deregulation of miR-31-5p and miR-551b-5p in classical CD patients, and five miRNAs in anaemic CD patients (miR-31-5p, miR-192-3p, miR-551b-5p, miR-638 and miR-1290) compared with non-CD controls (6). In addition, the duodenal fibroblasts obtained from patients are then incubated with gliadin peptides (13 and 33 mer) and measured for the miRNA expression. The deregulation of miRNA levels was observed in untreated CD patients for miR-192-3p, miR-31-5p and miR-1285-3p (6).

Buoli Comani et al. (2015) analyzed duodenal biopsies of pediatric celiac patients for examining a panel of miRNAs and their target genes compared to controls. After that, they evaluated the circulating miRNA patterns in untreated CD patients or on a gluten-free diet compared to controls. They found that miR-31-5p and miR-338-3p were underexpressed in the duodenum samples of CD patients (2). In addition, circulating miR-31-5p in untreated CD patients displayed a significantly decline compared to controls. Moreover, there is no statistically significant difference in miRNA levels between controls and CD patients on a gluten-free diet (2).

In a study performed by Magni et al. (2014) in the duodenum of adult CD patients, the significantly decreased expression of miR-192-5p, miR-31-5p, miR-338-3p, and miR-197 were demonstrated as compared with controls (5). FOXP3, the target of miR-31-5p, showed upregulation in CD patients. Furthermore, exposure of CD patients to gliadin led to changes in the expression of FOXP3, miR-192-5p, miR-31-5p, CXCL2 and NOD2 (5).

Olaru et al. (2011) found that miR-31 was increased successively at each stage of IBD progression from non-inflamed to inflamed non-neoplastic, dysplastic, and finally cancer (23). In addition, RT-PCR analysis revealed altered expression of miR-

31, -125a, -142-3p, and -146a discriminating between the inflamed mucosa of CD and UC (24).

In our previous study, we showed the aberrant expression of miR-31 and miR-21 in SLE patients compared to their first-degree relatives and controls. Moreover, our results indicated that both of miR-31 and miR-21 could serve as regulatory biomarkers in patients with SLE (25). This may indicate the altered expression of these circulating miRs in the autoimmune diseases.

Conclusion

Our study demonstrated the deregulation of circulating miRNA-21 and miRNA-31 expression levels in children with CD and showed that miR-21 expression level was positively correlated with the tTG IgA auto-antibodies. In addition, our findings indicated that a gluten free diet has influenced the expressions of miRNA-21 and miRNA-31 in serum of pediatric CD patients. Therefore, circulating miRNA-21 and miRNA-31 could be used as potential non-invasive diagnostic and prognostic biomarkers for pediatric CD patients.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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The incidence of immunofluorescence patterns and specific autoantibodies observed in autoimmune patients in a tertiary care centre

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Key words
anti (ANA); autoimmune diseases (ADs); indirect immunofluorescence (IIF); extractable nuclear antigens (ENA); autoantibodies

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Summary
Objectives. *Testing for antinuclear antibodies (ANA) facilitates the diagnosis of autoimmune diseases (ADs). Here, we report an incidence of ANA positivity and its patterns by indirect immunofluorescence (IIF) and specific autoantibodies through immunodot assay.* **Methods.** *Sera from 993 patients presenting with various ADs were tested by IIF and immunodot assay.* **Results.** *ANAs were detected in 39.7%, of which speckled pattern was predominantly observed (50.8%). 56.8% of samples were positive on the immunodot assay, with SSA Ro 60 antibody being the most prevalent (30.7%).* **Discussion**. *A significant correlation ($p < 0.0001$) was observed between patterns and auto-antibodies. Coarse speckled (CS) and homogeneous were overly represented by antibodies SSA Ro 60 (13%) and nucleosomes (5.8%), respectively. Mi-2, PL-7, PL-12, and SP-100 were the rarest autoantibodies specificities found.* **Conclusions.** *The presence of a particular IIF pattern is predictive of a specific autoantibody in the sample. Association of IIF patterns and specific autoantibody are relevant for a more accurate diagnosis of disease.*

Introduction

Autoimmune diseases (ADs) arise due to the pathologic activity of the immune system of an organism, directed against its own cells and tissues leading to the production of autoantibodies (1). However, the etiology of these conditions is unclear. Studies have suggested a controversial functional role of environmental factors, drugs, chemicals and toxins in triggering ADs (2).
The geo-epidemiology of ADs has grabbed attention recently (3-5). Studies have identified considerable variability in the epidemiology of different ADs, ranging from common, such as Hashimoto's thyroiditis and rheumatoid arthritis with a reported prevalence of approximately 1%, to others that are so rare that the only clues to their prevalence are a small number of case reports and small studies (6-10). Overall, the prevalence of a broad group of ADs is 12.5% (11). Studies in some centers in India reported the prevalence of presence of common ADs from 7.5 to 12%, which indicated a significant health care burden (12).
Serum antibodies against nucleus and anti-extractable nuclear antigens (ENA) are widely used in clinical practice and are included in the diagnosis of ADs (13,14). The most common autoantibodies are ANAs, which are conventionally assessed by IIF and include antibodies to both nuclear and cytoplasmic components (15). The immunofluorescence staining patterns and specific autoantibodies are clinically relevant as they are associated with particular ADs (16). Due to the ethnic variations, genetic and environmental factors, there is a significant variation in incidence and disease-specific autoantibodies (17,18). Nonetheless, ANA is frequently found in a considerable proportion of healthy subjects although studies are generally performed in selected populations, such as blood donors or employees, while data on ANA prevalence (19-21) and clinical significance over time (22) in an unselected general population, are limited.

In light of the above backdrop, this study hypothesized that a definite spectrum of autoimmune disorders might correlate with the clinicopathological endpoints of the disease, and therefore investigated the epidemiological profile of ANA staining patterns by IIF and specific autoantibodies through immunodot assay from patients presenting with autoimmune disorders in a tertiary care hospital. Taken together, the results of this study may throw intriguing insights on the detection of specific autoantibodies with a specific ANA staining pattern.

Materials and methods

Study population and specimens

A total of 993 consecutive patients clinically suspected of various autoimmune disorders were recruited with written informed consent from January 2016 to October 2016, from different medical specialties of the hospital. The study protocol conformed to the provisions of the 1975 Declaration of Helsinki (as revised in Seoul, Korea, October 2008).
Whole blood was collected from the patients by venipuncture into plain vacutainers, and separated sera were stored at -80 °C until assessed. However, to prevent repeated freezing and thawing, serum aliquots were immediately processed to prevent discrepancies in results. Based on standardized assays used in our laboratory, screening of ANA was achieved through indirect immunofluorescence assay using Hep-2 substrate, and specific autoantibodies were confirmed by immunodot assay using a panel of 25 nuclear and cytoplasmic antigens.

Indirect immunofluorescence (IIF)

Screening of ANAs was performed using the Bio-Rad Kallestad Hep-2 substrate (cat no. 30472). Screening dilution of 1:80 was used for adult patients and 1:40 was used for pediatric patients. Briefly, samples were processed using an automated processor (Bio-RAD, serial no. 2003-215). Positive samples were further diluted till 1:320 to determine the titer. Serum diluted in phosphate buffered saline (PBS) was incubated with fixed Hep-2 cell substrate for 30 minutes at room temperature. Slides were washed twice for five minutes with PBS, incubated for an additional 30 minutes with fluorescent-labeled conjugated anti-human IgG (cat no. 30446). Subsequently, a cover-slip was placed over the slide and analyzed using a fluorescence microscope at 40x magnification. The fluorescence of each sample was compared with the negative control, and the pattern of fluorescence was determined and recorded.

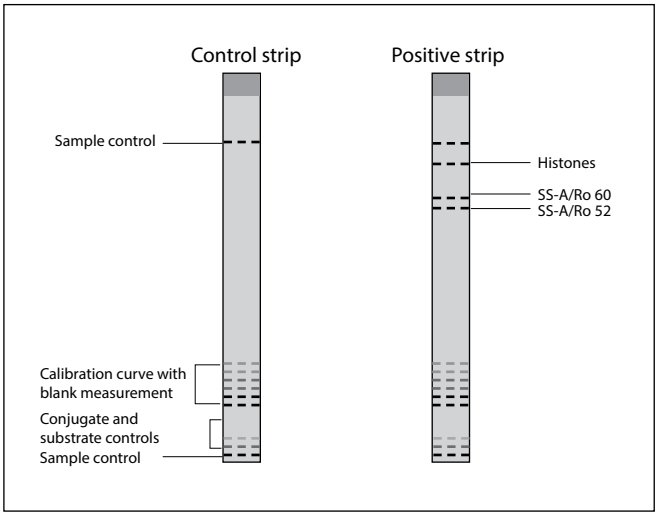
Immunodot assay

Immunodot assay is based on classical enzyme immuno assay (EIA) in which multiple parameters can be tested simultaneously. D-tek Blue Diver ANA Quantrix (cat no. ANA25Q-24) system was used for the quantitative detection of a 25-antigen panel according to manufacturer's instructions. Purified nucleosomes, ds-DNA, histones, Sm, RNP68kD/A/C, Sm/RNP, SSA/Ro 60kD, SSA/Ro 52kD, SSB, Scl-70, Ku, PM-Scl 100, Mi-2, Jo-1, PL-7, PL-12, SRP-54, Ribosomes P0, CENP-A/B, PCNA, sp100, gp210, M2 recombinant, M2 native and F-actin are bound in triplicate on the nitrocellulose membrane. Two sample controls, a calibration curve with blank measurement, conjugate controls, and substrate controls are also present in triplicate in the strip (**figure 1**). Post assay, strips were scanned and the intensities were evaluated using a computerized Dr. Dot software. Specifically, samples with values > 6 AU/ml were considered positive, samples < 6 AU/ml negative.

Statistical analysis

Categorical variables were expressed as a percentage, and a two-tailed probability level of $p < 0.05$ was considered statistically

Figure 1 *- Immunodot assay strips (D-Tek) showing control and positive sample strip.*



Nuc, nucleosomes; dsDNA, double stranded DNA; His, histones; Sm, Smith; RNP68kD, ribonucleoprotein 68; Sm/RNP, Smith antigen/ribonucleoprotein; SS-A /Ro60 kD, soluble substance A/ Robert antigen 60 kDa; SS-A/Ro52 kD, soluble substance A/Robert antigen 52kDa; SS-B, soluble substance B; Scl-70, scleroderma antigen 70 kDa; Ku, DNA helicase; PM-Scl 100, polymyositis scleroderma 100 kDa; Mi-2, chromodomain helicase DNA binding protein4; Jo-1, histidyl-tRNA synthetase/John-P; PL-7, threonyl-tRNA synthetase; PL-12, alanyl t-RNA synthetase; SRP54, signal recognition particle 54; CENP-A/B, centromere protein A/B; PCNA, proliferating cell nuclear antigen; sp100, sp100 nuclear antigen; gp210, nuclear pore glycoprotein-210; M2 rec, mitochondrial recombinant protein; M2 native, mitochondrial native protein; F-actin, filamentous actin.

significant. All computations were performed considering 80% power and 95% confidence interval using IBM SPSS, Ver20 (http://www-01.ibm.com/software/analytics/spss/) statistical software. Association of patterns with the antibodies were analyzed using a chi-square test.

Results

The incidence of serum ANA and specific autoantibodies

Upon IIF investigation, serum ANA was detected in 55.9% (555 out of 993) at 1:80 dilution, of which 70.9% (394/555) were positive at titers ≥ 1:160. Among the various patterns observed in ANA-positive cases, the most common pattern was homogeneous 26.4% (104/394), then fine speckled 25.9% (102/394) and coarse speckled 24.9% (98/394). 11.4% (45/394) serum samples had more than one immunofluorescence pattern. 56.8% (224/394) samples were positive by immunodot assay. Among these, SSA Ro 60 (30.7%) was found to be the most prevalent antibody along with SSA Ro 52 in 26.1% cases. Mi-2, PL-7, PL-12, and SP-100 were the rarest autoantibodies specificities found (**table I**).

Correlation of ANA patterns with specific autoantibodies

The presence of a specific autoantibody is associated with a specific immunofluorescence pattern. A significant association ($p < 0.0001$) was observed between patterns and an-

Table I *- Distribution of various ANA patterns and antibodies against specific antigens in "ANA positive (> = 1:160)" patients (n = 394).*

Immunofluorescence		**Immunodot assay**	
pattern	**number of positive samples (%)**	**antigen**	**number of positive samples (%)**
HOM	104 (26.4)	SSA Ro60	121 (30.7)
FS	102 (25.9)	SSA Ro52	103 (26.1)
CS	98 (24.9)	SSB	48 (12.2)
NUC	28 (7.1)	Sm/RNP	40 (10.2)
CENT	17 (4.3)	nucleosomes	39 (9.9)
HOM with NUC	17 (4.3)	RNP68	28 (7.1)
FS with NUC	11(2.8)	dsDNA	28 (7.1)
MP	7 (1.8)	histones	26 (6.6)
FS with MND	5 (1.3)	Sm	24 (6.1)
MND	3 (0.8)	Scl-70	24 (6.1)
CS with FND	2 (0.5)	CENP-A/B	21 (5.3)
		PCNA	13 (3.3)
		SRP-54	4 (1.0)
		M2-recombinant	3 (0.8)
		M2-native	3 (0.8)
		F-actin	3 (0.8)
		PM-Scl-100	2 (0.5)
		Ku	2 (0.5)
		Rib-P0	2 (0.5)
		Mi-2	1 (0.3)
		PL-7	1 (0.3)
		PL-12	1 (0.3)
		SP-100	1 (0.3)

HOM, homogeneous; FS, fine speckled; CS, coarse speckled; NUC, nucleolar; CENT, centromere; HOM with NUC, homogeneous with nucleolar; FS with NUC, fine speckled with nucleolar; MP; mixed pattern; FS with MND, fine speckled with multiple nuclear dots; MND, multiple nuclear dots; CS with FND, coarse speckled with few nuclear dots.

tibodies. Coarse speckled and homogeneous were overly represented by antibodies SSA/Ro 60 (13%) and nucleosomes (5.8%), respectively. Homogeneous with the nucleolar pattern was detected only by the Scl-70 antibody, while other patterns like coarse speckled were detected by fourteen different antibodies (**table II**). Fluorescent images of common and rare patterns are shown in **figure 2** and **figure 3**.

Incidentally, while studying the correlation of IIF patterns and specific autoantibodies, we found the autoantibody distribution in different ADs (**figure 4**, **figure 5**). In hypothyroidism, coarse speckled is the common pattern along with SS-A Ro52 being the most common antibody found. Out of 41 SLE samples, homogeneous (15/41) is the most common pattern and SSA Ro 60, SSA Ro 52, nucleosomes, SS-B and dsDNA antibodies are the predominant antibodies. Homogeneous with nucleolar pattern was found to be associated with Scl-70 in scleroderma. A coarse speckled pattern with SSA Ro 60 was predominant in Sjögren's syndrome.

Figure 2 *- Common ANA patterns by IIF: a, negative sample; b, homogeneous; c, fine speckled; d, coarse speckled; e, nucleolar; f, centromere.*

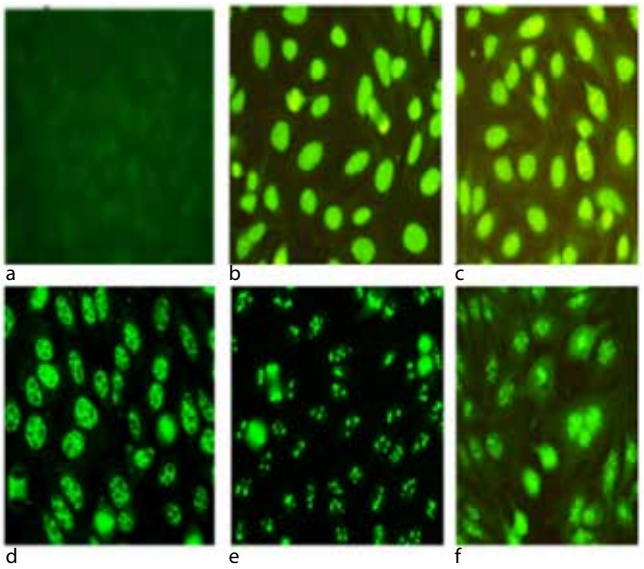
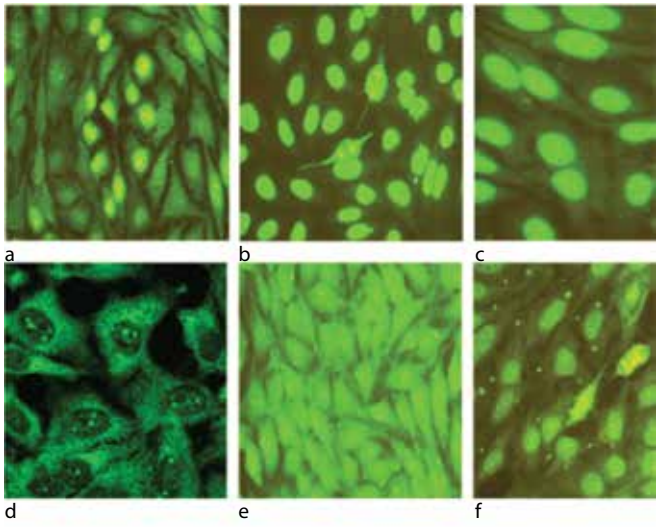


Table II *- Correlation of IIF patterns and specific autoantibodies (data is expressed in percentage).*

Autoantibody	FS	NUC	CS	CENT	HOM	MP	MND	HOM with NUC	FS with NUC
nucleosome	0	0.7	1.4	0.3	5.8	1.0	0	0	0
Scl-70	0	0.3	0	0	1.4	0	0	2.7	0
PM-Scl 100	0.7	0	0	0	0	0	0	0	0.3
Mi-2	0	0	0	0	0.3	0	0	0	0
SRP-54	0	0	0	0	0.7	0	0	0	0
ribosomes P0	0	0	0.3	0	0	0.7	0	0	0
CENPA/B	0.7	0	0.3	1.4	0	0	0	0	0
dsDNA	0	0.3	1.0	0	4.8	0	0	0	0
PCNA	1.4	0.3	0.3	0.3	1.4	0.3	0	0	0
sp100	0	0	0	0	0	0	0.7	0	0
M2 recombinant	0	0	0.3	0.3	0	0	0	0	0
F-actin	0	0	1.4	0.3	0.3	0	0	0	0
histones	0	0	1.4	0	2.4	0	0	0	0
Sm	0	0	1.4	0	1.4	1.0	0	0	0
RNP68kD/A/C	0	0	2.1	0	1.0	0.7	0	0	0
Sm/RNP	0	0	3.1	0	1.7	1.0	0	0	0
SSA Ro 60kD	3.1	0	13.0	0	5.5	1.0	0	0	0
SSA Ro 52kD	1.4	0	10.3	0.3	4.8	0.7	0	0	0.3
SSB	1.0	0	4.4	0	3.0	1	0	0	0

FS, fine speckled; HOM, homogeneous; CS, coarse speckled; CENT, centromere; HOM, homogeneous; MP, mixed pattern; HOM with NUC, homogeneous with nucleolar; FS with NUC, fine speckled with nucleolar.

Figure 3 *- Rare ANA patterns by IIF: a, PCNA (proliferating cell nuclear antigen); b, NUMA (nuclear mitotic apparatus); c, homogeneous with nucleolar; d, nuclear dots; e, Jo-1 pattern; f, mid body pattern.*



Comparison of results of IIF and immunodot assay

Out of 993 samples, 55.9% (555) were ANA-IIF positive at 1:80 serum dilution. Of these positive ANA-IIF, 45.9% (255) were also immunoassay positive. ANA pattern observed in 555 (55.9%) cases were mostly fine speckled 203 (36.6%); homogeneous 131 (23.6%); coarse speckled 112 (20.2%); nucleolar 38 (6.8%); fine speckled with nucleolar 18 (3.2%), homogenous with nucleolar 17 (3.1%). Eight (1.4%) cases exhibited a mixed pattern, and 5 (0.9%) cases showed fine specked with multiple nuclear dot pattern. (**figure 6**)

Out of 324 IIF negative samples, 48 (14.8%) were positive with the immunodot assay. Twelve sera showed significant value for SSA/Ro-60 (25%) with a mean intensity of 47 on dot assay; 15 sera showed positivity for nucleosomes (31%), although the mean intensity of 10 is not significant (**table III**). Clinical features of these IIF negative samples were hypothyroidism (2/48), myositis (4), arthritis (3/48), SLE (2/48), spondyloarthritis (1/48), CTD (1/48), diabetes mellitus (1/48), SLE + vasculitis (1/48), RA + vasculitis (1/48), RA + diabetes mellitus (1/48), RA + hypothyroidism (1/48).

Figure 4 *- Distribution of IIF patterns in ANA-positive patients (n = 394).*

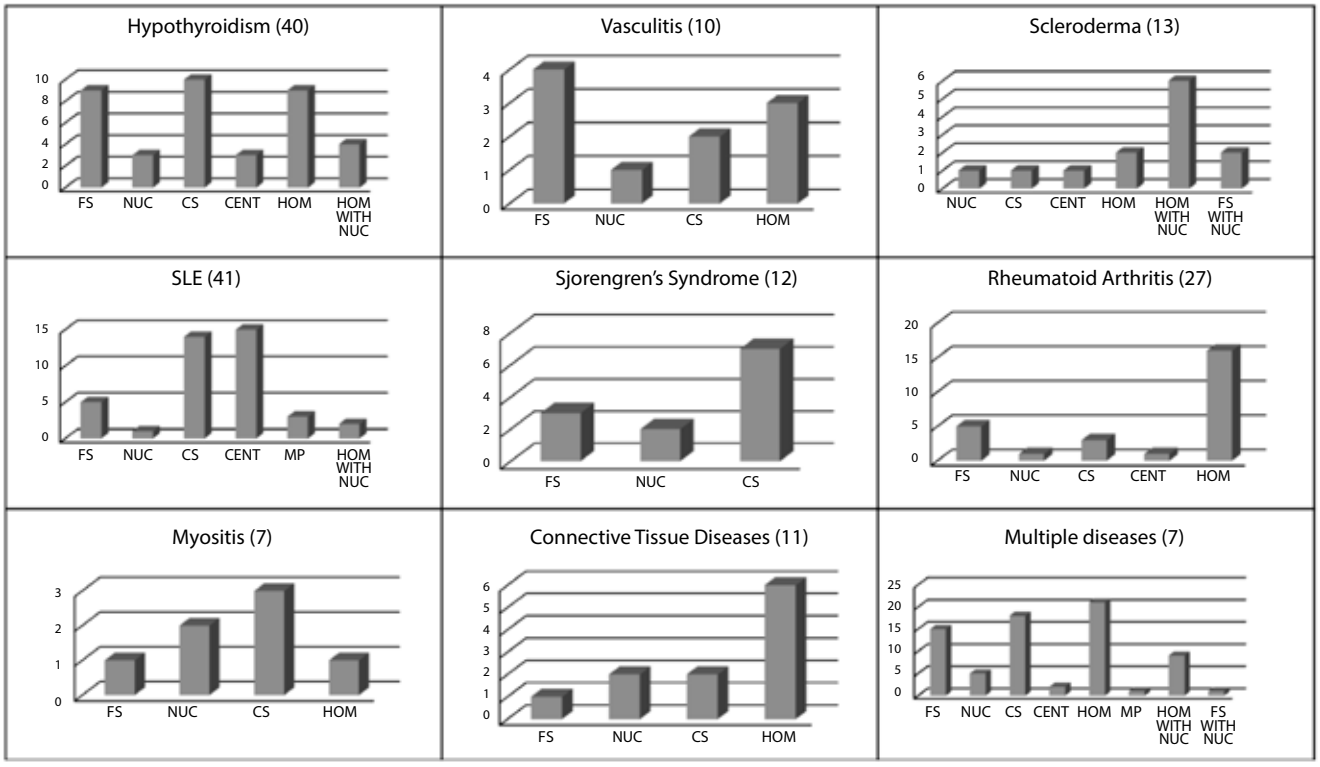


Figure 5 *- Distribution of autoantibodies in ANA-positive patients (n = 394).*

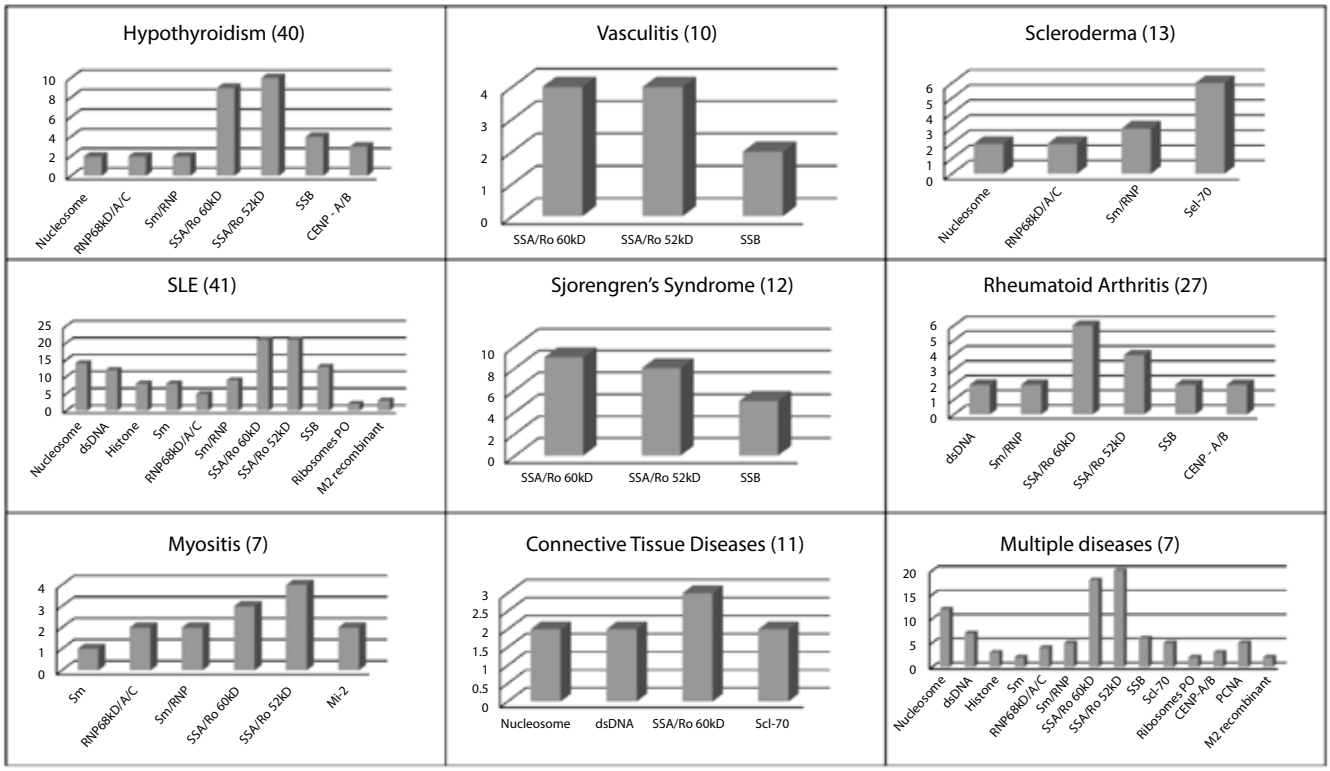


Figure 6 *- Summary of immunoassays (n = 993)*

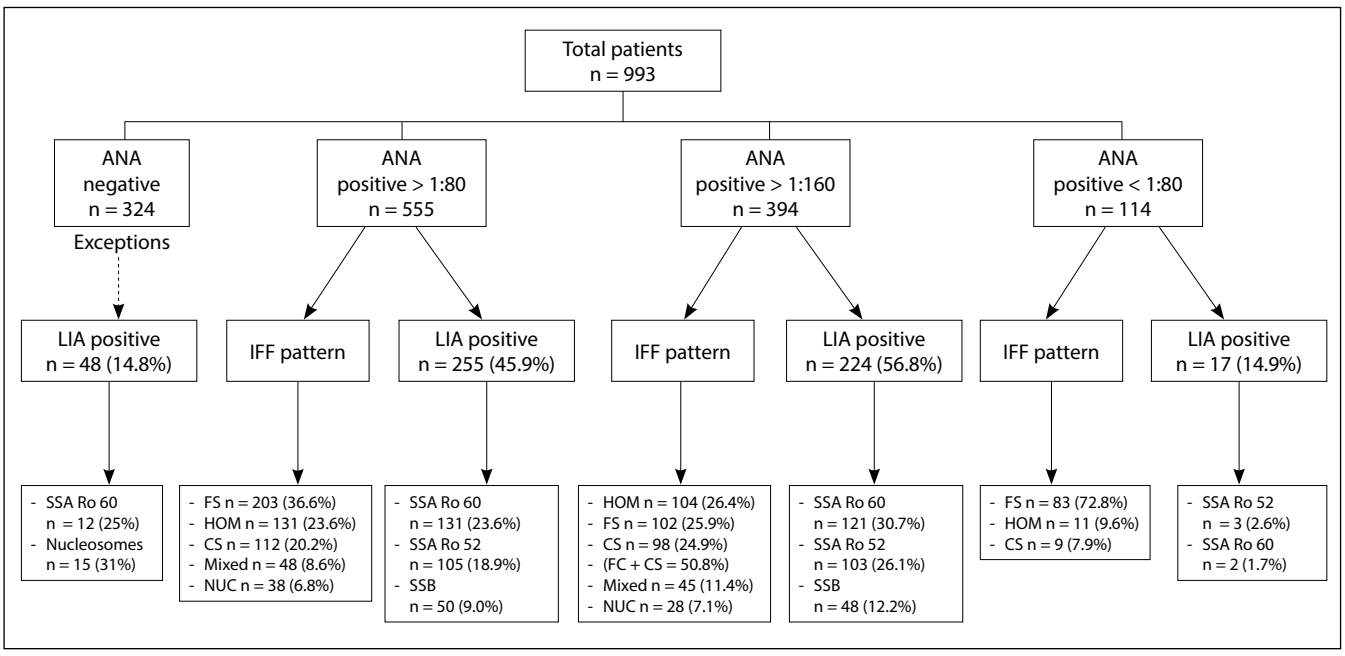


Table III *- Autoantibody intensity in immunodot positive samples and IIF negative samples (n = 48).*

Autoantibody	Number of positive samples by immunodot assay	Results in mean intensity (AU/ml)
nucleosomes	15	10
dsDNA	3	9
histones	2	10
Sm	1	11
RNP 68 KD/A/c	1	11
SSA Ro 60	12	47
SS A Ro 52	5	51
SS-B	2	26
PM-Scl	1	10
Jo-1	3	94
SRP-54	8	12
centromere	2	14
M2 recombinant	2	44
F-actin	3	14

Discussion

Serum ANA are important biomarkers in the diagnosis of ADs. IIF is considered as a gold standard screening assay due to its high sensitivity. In the present study, we focused on the incidence of IIF patterns and specific autoantibodies in patients clinically suspected of ADs. IIF was compared with immunodot assay to predict the specific antibodies associated with a specific pattern. The most common IIF patterns observed were speckled (50.8%) [fine speckled (25.9%) + coarse speckled (24.9%)], homogeneous (26.4%), nucleolar (7.1%), centromere (4.3%), which were similar to study conducted in Bangladeshi population where speckled pattern (50.8%), peripheral pattern (21.64%), homogenous (18.1%) and nucleolar pattern (9%) were observed (23). Another study reported speckled (42.5%) as the most common fluorescence pattern, followed by homogeneous (41.4%) and nucleolar (10.6%) (24). Our results are different when compared to study by Kun-Yi Wang et al. which reported common patterns as homogeneous (42%), mixed (23.9%), speckled (16.9%), centromere (9.3%), and nucleolar (7.9%) except nucleolar prevalence which was similar to our study (25-26). These differences may be due to the ethnic variations and biological heterogeneity of serological immune response (17). Of the 45 patients with rare and mixed patterns (**figure 3**), 16 were diagnosed with an autoimmune disease: six patients with scleroderma, six patients with SLE, one with rheumatoid arthritis, one patient with Sjögren's syndrome, two patients with SLE and scleroderma overlap, indicating the importance of rare patterns. However, Pieter Vermeersch et al. concluded that the observation of a rare ANA pattern could be helpful for the diagnosis of specific ADs, but the clinical significance is low when found as part of a routine clinical investigation (27), and may be considered as not important. It is important to remember that there are some non-autoimmune causes of rare ANA patterns on IIF, which include carcinoma, hepatitis C infection, and transplantation (28-30). In the present study, out of 15 cases of malignancies, 2 cases were positive for common antibody SSA-Ro60, and rare antibody SRP-54 (1), CENP-A/B (1), sp-100(1) and F-actin (3) were found.

Anti-SSA Ro60 (30.7%), is the most common autoantibody including anti-SSA Ro52 (26.1%) and anti-SSB (12.2%), which is comparable to study in Korean patients where anti-Ro52 (66.7%) was the most frequently detected antibody, followed by anti-Ro60 (52.1%) and anti-La (49.0%) (31). Study on US population also reported anti-Ro-52 as the common antibody which is similar to the findings of this study (32). Being the gold standard technique, ANA detection by IIF did not match with 48 samples which were positive by immunodot assay, and out of these, 18 samples were of autoimmune etiology (**table III**) and 30 samples were of multiple diseases. SS-A/Ro 60 (12/48), and SS-A/Ro 52 (5/48) are significantly missed by IIF. This difference is also observed in the literature, that SSA R060 is missed by Hep-2 cells due to the low cellular abundance of this particular protein on Hep-2 cells (33). These differences may also be due to the subjectivity and the inter-operator variability of performing the assay. These technical differences have been observed earlier in literature, and this is one of the major limitations of using IIF as a screening assay for the detection of autoantibodies. Although the validation studies have been performed for automated IIF (34,35), they are not routinely used in developing countries due to the expenses involved.

To understand the association of IIF patterns with specific autoantibodies, our finding was compared to standard reference as well as with earlier studies, and found to be very similar with published literature (**table IV**). The speckled pattern showed an association with SS-A/ Ro 60 and SS-A/Ro 52, which was similar to other studies. Likewise, the centromere pattern is shown in association with the CENP-B antibody, in accordance with other published studies. However, there are some exceptions, like histones in a coarse speckled pattern, Scl-70 in the homogeneous pattern, which may be due to the use of different detection techniques. This correlation is very helpful in predicting a specific antibody with a particular ANA pattern.

Table IV *- Comparison of association of IIF patterns with specific autoantibody in different studies.*

Patterns	Common autoantibody associations in present study	Western textbook (36,37)	Indian study (38)	Standard reference (16)
fine speckled	SSA Ro 60 kD, SSA Ro 52 kD	Sm, RNP, Scl-70, SSA/Ro 52, SSB, RNA pol I and II, and other antigens	Sm, RNP, SSA/Ro 52, SSB	SSA/Ro, SSB/La, Topo-1, common to many antigens
nucleolar	SSA Ro 60 kD, SSA Ro 52 kD, nucleosomes	Nucleolar RNA	Scl-70, SSA/Ro 52, SSB	PM/Scl, RNA-polymerase, URNP, U3-RNP, To/Th
coarse speckled	SSA Ro 60, SSA Ro 52 kD, SS-B, Sm/RNP, RNP 68 kD/A/C, Sm, histones	Sm, RNP, Scl-70, SSA/Ro 52, SSB, RNA pol I and II and other antigens	Sm, RNP, SSA/Ro 52, SSB	U1-SnRNP, U2-6snRNP (Sm), nuclear matrix
centromere	CENP-A/B	centromere protein	centromere protein-B	kinetochore, CENP-A, B, C, F
homogeneous	SS A Ro 60 kD, SSA Ro 52 kD, nucleosomes, dsDNA, SS-B, histones, Scl-70, Sm	DNA-histone complex	dsDNA, nucleosomes, histones, SSA/Ro 52, RNP/Sm, RIB-P	dsDNA, histones, chromatin/nucleosomes, HMG

Conclusions

In conclusion, ANAs detection is very crucial for the evaluation of patients suffering from various ADs. This study provided the correlation of IIF patterns and specific autoantibodies, along with incidence in a tertiary care centre. The presence of a particular IIF pattern is predictive of a specific autoantibody in the sample. Association of IIF patterns and specific autoantibody are relevant for a more accurate diagnosis of disease. The immunodot assay is very helpful in the diagnosis of clinically suspected cases of an overlap syndrome. These findings should be kept in mind by physicians while assessing ANA results, and will be useful in deciding further investigation for the diagnosis of specific ADs.

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Disclosure of interest

The authors report no conflict of interest.

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Isoforms of group 1 allergens from a tropical/subtropical para grass (*Urochloa mutica*) display different levels of IgE reactivity and cross-reactivity

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Summary

Group 1 grass pollen allergens, or beta-expansins, are the most important major allergens from tropical/subtropical grasses. This study aimed to investigate the sequence similarity and immunoreactivity of group 1 allergens from para grass (Urochloa mutica). Three isoforms (Uro m 1.01, Uro m 1.02, and Uro m 1.03) were cloned from cDNA of para grass pollen. The acidic-neutral isoforms rUro m 1.01 and rUro m 1.02 could effectively inhibited beta-expansins in pollen extract of Bermuda and Johnson grasses, suggesting that these isoforms could be major cross-reacting allergens among these grasses. In contrast, the basic isoform rUro m 1.03 had limited IgE reactivity. Thus, group 1 allergens both acidic-neutral and basic isoforms could have markedly different IgE reactivity.

Introduction

Grass pollen (GP) is among the most significant sources of outdoor airborne allergens worldwide. Nonetheless, information about tropical / subtropical GP allergens is relatively limited despite the high diversity of grasses in these regions. Allergenicity and cross-reactivity of GP are often presumed from prior knowledge in the temperate regions, ignoring the genuine sources of sensitization and the complexity / redundancy of multiple allergens and their isoforms. This, combined with the inadequate patient profiles from large scale studies, hinders the expansion of our understanding of tropical / subtropical GP allergy. Hence, identification of local tropical / subtropical grass species and their GP major allergens should be encouraged.

The dominating grass species in the tropical / subtropical regions mostly belong to the subfamily Chloridoideae (e.g. *Cy-*

nodon dactylon, Bermuda grass) and Panicoideae (e.g. *Paspalum notatum*, Bahia grass; *Sorghum halepense*, Johnson grass; and *Zea mays*, maize), while Pooideae grasses (e.g. *Lolium perenne*, rye grass; *Phleum pratense*, Timothy grass; *Dactylis glomerata*, orchard grass; *Phalaris aquatica*, Canary grass; *Poa pratensis*, Kentucky blue grass; and *Anthoxanthum odoratum*, sweet vernal grass) are largely absent in these regions. The main difference relevant to allergenicity is that the group 5 GP allergens are not present in Chloridoideae and Panicoideae GP. On the other hand, the group 1 GP allergens have been identified as the most prevalent and potent allergens in all grass species (1,2). Group 1 GP allergens have been categorized as a subclass of the beta-expansin family (3). These glycoproteins are highly expressed in GP and secreted at pollen walls to assist pollen-tube penetration (4). Like other expansins, beta-expansins loosen plant cell walls by disrupting hydrogen bonds between cellulose microfibrils and cross-linking glycans in plant cell wall (5).

Group 1 GP allergens have been reported as major allergens in several grass species. The most notable members are Cyn d 1 in Bermuda grass, Sor h 1 in Johnson grass, Zea m 1 in maize, Pas n 1 in Bahia grass, Ory s 1 in rice, Phl p 1 in Timothy grass, and Lol p 1 in rye grass (6-12). Group 1 allergens in Pooideae grasses, have highly conserved amino acid sequences (85 - 95% identity) and were shown to have comparable levels of allergenicity among species (13). Sequence homology group 1 allergens from different subfamilies such as Phl p 1 (Pooideae) and Cyn d 1 (Chloridoideae) was considerably lower (67 - 70% identity) and these allergens were shown to have incomplete inhibition (14). Therefore, it was largely assumed that the degree of similarity and, consequently, cross-reactivity of group 1 allergens largely corresponded to the taxonomic relationship of the grass species. However, as more genomic, transcriptomic, and proteomic information became available, it was clear that several genes encoding beta-expansins could be present in a given genome, giving rise to several isoforms (isoallergens) from a single species. For example, two isoforms with 60% sequence identity have been reported in Zea m 1 and Sor h 1 (7,8,15). Different amino acid sequences might affect IgE binding and allergenicity, but direct comparison between isoforms from a single species has been scarce.

Para grass or *Urochloa mutica* (formerly *Brachiaria mutica*) had been reported as one of the top allergenic grass species in Thailand (16,17). Of the 2,383 AR patients attending the ENT Allergy Clinic at Siriraj Hospital, Bangkok, between 2005 - 2014, 53.2% had positive skin prick test reaction to para grass pollen (PGP) extract (17). However, allergenicity of PGP has rarely been reported in other countries. This grass species is native to Africa, but had been introduced to tropical and subtropical regions of the world as fodder grass. It is currently considered one of the worst grass species in several regions, including the United States, Central America, Australia, and Asia (18). With a wide range of tolerable climates and soil conditions and its aggressively invasive nature, this species has been rapidly increasing. Due to the large distribution area of para grass, it is possible that the GP is an important allergen source and the incidence of sensitization could be rising in tropical/subtropical regions.

In our previous study, beta-expansin had been reported as a major cross-reactive allergen among Bermuda, Johnson, and para grass (19). In this study, we aimed to clone the different isoforms of beta-expansins from PGP, characterize their IgE reactivity and determine their ability to inhibit IgE binding to crude extracts of other grasses. This study provided information about the major allergenic components of an unreported allergenic grass species, and compared the IgE-reactivity of the different isoforms of group 1 GP allergens. The knowledge obtained from this study may be useful for improving diagnosis and immunotherapy for tropical/subtropical GP allergy.

Materials and methods

Serum samples

Serum samples were obtained as a part of the "Development of Siriraj Pollen Allergen Vaccine (SPAV)" project, in accordance with the approved ethics for research in humans by the Siriraj Institutional Review Board, Mahidol University, Bangkok, Thailand (SiEc100/2012). Forty patients with AR history were skin prick tested (SPT), and seventeen of them were sensitized to PGP. Six sera with IgE reactivity to Uro m 1 were included in this study based on the criteria as described previously (19) (**table A**).

Pollen protein extraction and SDS-PAGE

GP were collected from various natural sites within Bangkok metropolitan area and extracted in phosphate buffer saline (PBS) (19). PG extracts were separated by 12% sodium dodecyl

Table A *- Demographic of individual sera from allergic rhinitis patients of this study.*

no.	sex	age	SPT (mean wheal diameter, mm)			sIgE (kUA/L)	
			BGP	JGP	PGP	BGP	JGP
9	F	46	9	5	3	30.3	34.7
12	M	52	4	3.5	3	0.16	0.05
17	M	30	4	4	2	4.16	5.19
29	M	21	5	3	2	4.02	3.05
33	M	10	8.5	4.5	3.5	> 100	85.3
36	M	18	4	5	4.5	9.1	13.6

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For visualization, SDS-PAGE gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Merck, USA).

Cloning of cDNA encoding beta-expansins from PGP

Total RNA was extracted from PGP using TRIzol™ Reagent kit (Invitrogen, USA). RNA was converted into cDNA using oligo-dT primer and iScript reverse transcriptase (Bio-Rad, USA). Coding sequences of beta-expansins were amplified by PCR using Platinum® Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, USA). Three beta-expansins cDNAs were amplified by primers designed from group 1 GP allergens or beta-expansins from related grasses e.g. Cyn d 1, Zea m 1, Sor h 1, beta-expansins of *Setaria italica* (**table I**). The gel purified PCR products were elongated A-tail, and then inserted into pGEM®-T easy vector (Promega, USA). The recombinant plasmids were transformed into *Escherichia coli* DH5α, and then submitted to sequence by the AIT biotech Pte Ltd, Singapore.
The obtained sequences were manually edited and subjected to homology search using BLAST against the Genbank database. The nucleotide sequences were deposited in GenBank database: Uro m 1.01 (MH820172), Uro m 1.02 (MH820171), and Uro m 1.03 (MH820173). Deduced amino acid sequences were analyzed by ExPaSy proteomic tools such as ProtParam (20), ScanProsite (21), and CFSSP (22). N-terminus signal sequence and expansin domains were characterized by SignalP (23) and ScanProsite (21). The conserved regions of Uro m 1 and the 67-100% homologous sequences were analyzed by ConSurf (24). Nucleotides and amino acid sequences were compared with grass pollen allergens reported by the International Union of Immunological Societies (IUIS) Sub-committee for Allergen Nomenclature. Multiple sequence alignment and percent identity matrix were performed using Clustal Omega (25). A phylogenetic tree was reconstructed based on amino acid sequences. The evolutionary analyses were conducted in MEGA7 (26) using the Neighbor-Joining method (27) and the evolutionary distances were computed using the Poisson correction method (28).

Expression and purification of recombinant proteins

PCR products of beta-expansins contained endonuclease enzyme recognition sites were amplified using primers listed in **table I**. The PCR products were double digested for Uro m 1.01 or Uro m 1.02 and Uro m 1.03 constructions using Eco RI/Xho I or Nde I/Hind III (NEB, USA), respectively, then gel-purification and sub-cloning of PCR pieces into the expression vector pET-28a(+) (Novagen, Germany). The DNA sequence was confirmed by sequencing of both strands. Expression vectors were transformed into expressing host *E. coli* BL21 (DE3) pLysS cells.
The recombinant protein expression was induced by addition isopropyl β-D-thiogalactopyranoside (IPTG) (Amresco, USA and Canada) at 37 °C. Bacterial cells were harvested and the

Table I *- Primer list for cDNA cloning and expression vector construction of three Uro m 1 isoforms.*

Name	Sequence1	Ta (°C)	Product (bp)
	cDNA cloning		
U1F1	5'-GCC AAG CTT GAA TTC GAA CAG GCT ATG CTC GCG-3'	55	Uro m 1.01 (829 bp)
U1R1	5'-GCC AAG CTT CTC GAG TCA GAA CTG GAT CTT GGA CTT GTA GAC-3'		
U2F1	5'-GAC GGC AAG TGG CTG GAC-3'	57	Uro m 1.02 (695 bp)
U2R1	5'-GGA ATT AGA ACT GGA GCT TGG AG-3'		
U3F1	5'-GCA ACA GCC ACA CAC AAC AAC-3	55	Uro m 1.03 (984 bp)
U3R1	5'-GTG AGC CCG GAT TAC AGA TTA G-3		
	expression vector construction		
U1F2	5'-GCC AAG CTT GAA TTC GAA CAG GCT ATG CTC GCG-3' (Eco RI)	60	Uro m 1.01 (829 bp)
U1R2	5'-GCC AAG CTT CTC GAG TCA GAA CTG GAT CTT GGA CTT GTA GAC-3' (Xho I)		
U2F2	5'-CTA TTA CAT ATG TGG CTG GAC GCC AAG GCG ACG TG-3' (Nde I)	62	Uro m 1.02 (708 bp)
U2R2	5'-CGT GCG AAG CTT GGA ATT AGA ACT GGA GC-3' (Hind III)		
U3F2	5'-GGC AGC CAT ATG TGG CTC CCC GCC AGG GCC AC-3' (Nde I)	60	Uro m 1.03 (776 bp)
U3R2	5'-GTG AGC CCG GAT TAC AGA TTA G-3 (Eco RI)		

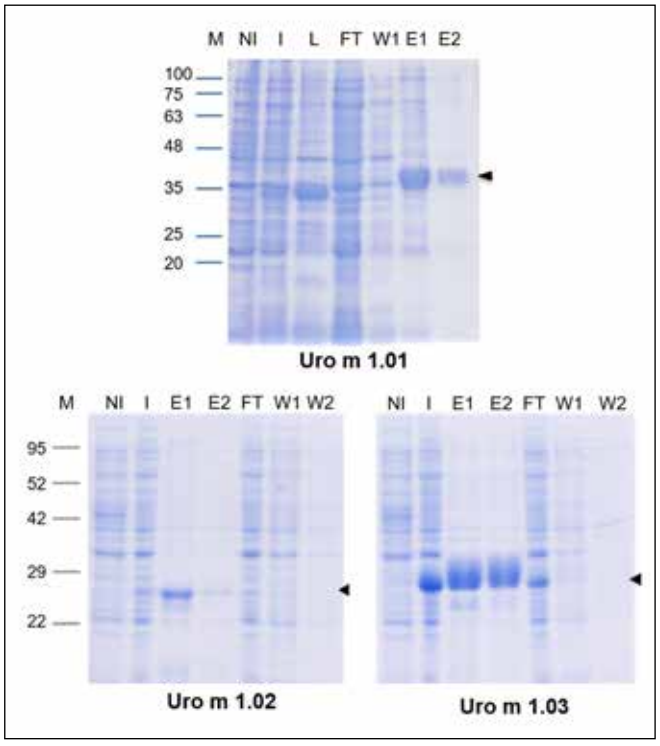
[1]Underlined letters indicate endonuclease recognition sites. Restriction enzymes specific to each primer are presented in parentheses.

recombinant proteins were found mostly in the insoluble fractions of bacterial extracts. Therefore, recombinants having a 6-His tag at the N-terminus were purified from the inclusion body in cell pellets under denaturing condition using Ni-NTA resins (Novex®, USA). The recombinant proteins had 71%, 89%, and 85% purity for rUro m 1.01, rUro m 1.02, and rUro m 1.03, respectively, analysed by measuring intensity of eluted protein bands from SDS-PAGE gel using ImageJ (**table V**).

Immunoblotting and inhibition

Protein extracts were separated by electrophoresis in 12% SDS-polyacrylamide gels using 0.5 and 5 µg of recombinants and GP proteins, respectively, per well. The separated proteins were electro-transferred from gels to nitrocellulose membranes using a Mini Tran-Blot® Electrophoretic Transfer Cell (Bio-Rad, USA). The membrane was incubated overnight at 4°C with patient's sera diluted at 1/100 - 1/5,000 in phosphate buffer saline (PBS) PBS containing 3% (w/v) skim milk. Washed membrane then incubated with a 1:10,000 diluted HRP-labeled mouse IgG anti-human IgE antibodies (KLP, USA) for 2 hr at room temperature. Bound IgEs were detected with Immobilon™ Western Chemiluminescent HRP substrate (Millipore, Germany) and emitted signal was captured by x-ray films.
The inhibition assays were performed similarly, except that the diluted sera were pre-incubated with 50 ng/µl recombinant proteins (19). The recombinant D7 protein from mosquito *Aedes aegypti* was used as a negative control. Recombinant D7 protein was expressed and purified in a system comparable to that of recombinant Uro m 1.

Table V *- Purification of 6X-His tag proteins using Ni-NTA resins (Novex®, USA) under denaturing conditions. The arrowheads indicated recombinant protein bands. NI, Un-induced cells; I, induced cells; L, induced cell lysate; E1-E2, recombinant elution fraction; FT, flow-through fraction; W1-W2, wash fraction.*



Enzyme-linked immunosorbent assay (ELISA) and inhibition ELISA

GP proteins were diluted with PBS and coated on ELISA plates at 1 µg/well at 4°C overnight. The coated plate was washed and incubated with 1/4 - 1/64 diluted sera in PBS containing 3% (w/v) skim milk. After sera incubation, bound IgEs were detected by incubation with 1/5,000 diluted HRP-labeled mouse IgG anti-human IgE anti-human antibodies. 3,3'5,5-tetramethylbenzidine (TMB) substrate (KPL Inc., USA) was added to each well and the color reaction stopped using 1 N HCl. Light absorbance was measured at a wavelength of 450 nm. All determinations were performed in duplicate.
The inhibition assay was performed in a similar manner. The serum from each individual was tested at one dilution at 1/4 - 1/64, which was determined from a preliminary experiment to show sufficient IgE binding and inhibition. The inhibitors were tested in the range of 15-200 ng/µl recombinant proteins. The experiment was performed in duplicate (19).

Results

Sequences and characteristics of (Uro m 1) beta expansin isoforms from Para grass

Molecular cloning of beta-expansins from PGP was performed based on conserved regions to reported beta-expansins from other grass species. Several clones were obtained and sequenced and were classified into three separate isoforms: Uro m 1.01, Uro m 1.02, and Uro m 1.03. Information about these three isoforms were deposited in the IUIS Sub-committee for Allergen Nomenclature database. ORF length, homologous sequence, and sequence variation of all identified clones of three Uro m 1 are presented in **table II**. The highest sequence identity among clones was seen with Uro m 1.01. Interestingly, these Uro m 1.01 clones were nearly identical to Cyn d 1 isoforms 2 and 4 (99 - 100%), differing only in one or two nucleotides. Uro m 1.02 and Uro m 1.03 clones had higher clone variation than Uro m 1.01. The three isoforms shared only 70-79% nucleotide sequence identity.

Table II *- Sequence variation of Uro m 1 peptidic epitopes predicted from well-known allergens*

Allergen	Position	% Epitope conservancy[1]	Epitope	Sequence
			Pas n 1 epitope	
Pas n 1	123-142	-	T cell	IAPYHFDLSGKAFGAMAKPG
Uro m 1.01	119-138	90		IA**A**YHFDLSGKAFGAMAK**K**G
Uro m 1.02	86-105	80		IA**A**YHFDL**A**G**T**AFGAMAK**K**G
Uro m 1.03	124-143	95		IAPYHFDLSGKAFGA**L**AKPG
Pas n 1	177-196	-	T cell	GSNPNYLAMLVKFVADDGDI
Uro m 1.01	173-192	75		GSNPNYLA**L**LVK**YAAG**DGN**I**
Uro m 1.02	140-159	65		G**C**NPNY**FALLIKYAAG**DGDI
Uro m 1.03	178-197	85		**AC**NPNYLA**V**LVKFVADDGDI
Pas n 1	240-259	-	T cell	GKKVIAQDVIPVNWKPDTVY
Uro m 1.01	237-256	65		G**GH**V**EQE**DVIP**ED**WKPDTVY
Uro m 1.02	204-223	50		G**TTLVQD**DA**I**P**EG**WK**A**DTVY
Uro m 1.03	242-261	65		GKK**LVAN**DVIP**A**NWK**AN**T**A**Y
			Cyn d 1 epitope	
Cyn d 1	109-128	-	T cell	SGKAFGAMAKKGQEDKLRKA
Uro m 1.01	127-14	95		SGKAFGAMAKKG**E**EDKLRKA
Uro m 1.02	94-113	80		**A**G**T**AFGAMAKKG**EEE**KLRKA
Uro m 1.03	132-151	75		SGKAFGA**L**AK**P**G**LN**DKLR**H**A
Cyn d 1	181-209	-	T cell	PKDSDEFIPMKSSWGAIWRIDPKKPLKGP
Uro m 1.01	199-227	83		**S**K**G**SDEF**L**PMK**Q**SWGAIWRIDP**P**KPLKGP
Uro m 1.02	166-194	72		**E**K**GSE**EFIP**LKH**SWGAIWRID**SP**KP**I**KGP
Uro m 1.03	204-232	66		**EKASAEWK**PMK**L**SWGAIWR**V**D**TP**K**A**LKGP
Cyn d 1	217-241	-	T cell	EGGAHLVQDDVIPANWKPDTVYTSK
Uro m 1.01	235-259	68		E**SGGH**V**EQE**DVIP**ED**WKPDTVY**K**SK
Uro m 1.02	202-226	76		EGG**TT**LVQDD**A**IP**EG**WK**A**DTVYTSK
Uro m 1.03	240-264	60		E**SGKK**LV**AN**DVIPANWK**AN**T**AYPSN**
Cyn d 1	88-97	-	IgE and IgG_4	CGSCYEIKCK
Uro m 1.01	88-97	100		CGSCYEIKCK
Uro m 1.02	55-64	90		CGSCYEIKC**D**
Uro m 1.03	93-102	90		CGSCYEI**R**CK
Cyn d 1	119-128	-	IgE	IAAYHFDLSG
Uro m 1.01	119-128	100		IAAYHFDLSG
Uro m 1.02	86-95	90		IAAYHFDL**A**G
Uro m 1.03	124-133	90		IA**P**YHFDLSG
Cyn d 1	162-171	-	IgG_4	SGTKITFHIE
Uro m 1.1	162-171	70		S**D**TKI**A**FH**V**E
Uro m 1.2	129-138	60		**AN**TKI**A**FH**V**E
Uro m 1.3	167-176	60		**GGQ**KI**V**FH**V**E
Cyn d 1	177-185	-	IgE and IgG_4	HYLALLVKY
Uro m 1.01	177-185	88		**N**YLALLVKY
Uro m 1.02	144-152	66		**NYF**ALL**I**KY
Uro m 1.03	182-190	66		**N**YLA**V**LVK**F**
Cyn d 1	190-199	-	IgE and IgG_4	GNIVSVDIKS
Uro m 1.01	190-199	100		GNIVSVDIKS
Uro m 1.02	157-166	70		G**D**IV**A**VDIK**E**
Uro m 1.03	195-204	40		G**D**IV**NMEL**K**E**
Cyn d 1	209-218	-	IgG_4 binding	KSSWGAIWRI
Uro m 1.01	209-218	90		K**Q**SWGAIWRI
Uro m 1.02	176-185	90		K**H**SWGAIWRI
Uro m 1.03	214-223	80		K**L**SWGAIWR**V**

[1]Epitope analysis tool obtained from IEDB.org was epitope conservancy analysis (38).
Nucleotide sequences from cDNA clones of different Uro m 1 isoforms.

Deduced amino acid sequences and their predicted features

The full-length mRNA transcripts of Uro m 1.01 and Uro m 1.03 could be translated to 262 and 267 amino acids, respectively, while the partial mRNA region of Uro m 1.02 coded for 229 amino acids (**table III**). The deduced amino acid sequence alignments of Uro m 1 are presented in **figure 1**. All isoforms of Uro m 1 contained conserved features of expansins such as signal peptide, glycosylation site, HFD motifs and cysteine (C) residues in expansin family-45 endoglucanase-like (expansin-like EG45) domain, and tryptophan (W) residue in expansin cellulose-binding like (expansin-like CBD) domain. The high sequence homology to other group 1 grass pollen allergens and the presence of conserved amino acid residues suggest that these sequences encode beta-expansins group 1 grass pollen allergens of Para grass.

Based on the amino acid sequences, Uro m 1.01 and Uro m 1.02 were predicted to be acidic glycoproteins, while Uro m 1.03 was a basic glycoprotein with the approximate molecular mass of 28 kDa. Each Uro m 1 could harbour three disulfide bridges in expansin-like EG45 domain, which is the putative catalytic region. The percentage of alpha-helix forming regions is higher than beta-sheet forming regions in all Uro m 1. Based on the homologous sequence comparison using ConSurf algorithm (24), most conserved amino acid regions of Uro m 1 are located in the sites important for protein function rather than structure. So far, the only beta-expansins with characterized IgE and IgG4 antibody-binding epitopes/T cell epitope are Cyn d 1 and Pas n 1 (29, 30, 31). The corresponding epitopes were found within the three isoforms of Uro m 1, with some amino acid changes (**table II**). These differences might confer the unique and cross-reactive IgE-reactivity of Uro m 1 isoforms. Details of all Uro m 1 peptide sequences are presented in **table IV**.

Homology to previously characterized grass group 1 allergens

To determine the similarity between Uro m 1 isoforms and previously reported beta-expansins, the beta-expansin sequences with high similarity to Uro m 1 were obtained from GenBank and used for clustering analysis with Neighbor-Joining method. The resulting dendrogram is presented in **figure 2**. As expected, grass group 1 allergens are divided into two classes (A and B) (8, 15). Class A allergens can be further divided into four sub-groups, largely corresponding to the grass sub-family: (A-I) Pooideae allergens (e.g. Lol p 1, Phl p 1), (A-II) Ehrhartoideae allergen (Ory s 1), (A-III) Panicoideae allergens (e.g. Sor h 1, Zea m 1), and (A-IV) Chloridoideae allergens (Cyn d 1). Class B allergens were separated into two sub-groups: (B-I) Ehrhartoideae allergen (Ory s 1), and (B-II) Panicoideae allergens (e.g. Pas n 1, Sor h 1). Importantly, Uro m 1.01, Uro m 1.02 and Uro m 1.03 were clustered into different classes and subgroups (A-IV, A-III, and B-II, respectively). Other beta-expansins from a single plant species that were clustered into separate groups are Ory s 1, Sor h 1, and Zea m 1. In comparison with beta-expansins found in rice and maize genome Uro m 1.01 was most similar to OsEXPB1 (73%), ZmEXPB10 (71%), and ZmEX-

Table III - *Nucleotide sequences from cDNA clones of different Uro m 1 isoforms.*

Gene	No. of clones	Seq. identity among clones	Percentage of allergen identity (%)1	Sequence length
Uro m 1.01	3	99.8 - 99.9%	Cyn d 1 AF177380.1 (100%) Cyn d 1 AF177378.1 (99%) Cyn d 1 AF177379.1 (96%) Cyn d 1 AF177030.1 (93%) Cyn d 1 AF159703.2 (93%) Cyn d 1 S83343.1 (87%)	789 bp (262 aa)
Uro m 1.02 (partial)	4	87.9 - 100%	Sor h 1 KF887425.1 (83%) Zea m 1 DQ421827.1 (81%) Zea m 1 DQ421828.1 (80%) Cyn d 1 AF177030.1 (80%) Cyn d 1 AF159703.2 (80%) Cyn d 1 AF177380.1 (79%)	690 bp (229 aa)
Uro m 1.03	5	95.2 - 100%	Pas n 1 EU327342.1 (87%) Zea m 1 NM001111739.1 (86%) Zea m 1 L14271.1 (85%) Sor h 1 KF887426.1 (85%) Ory s 1 AF220610.1 (81%) Cyn d 1 AF177380.1 (70%)	804 bp (267 aa)

[1]Percentage of sequence identity was calculated to compare the sequence similarity between well-known allergens and Uro m 1. The NCBI accession numbers are presented following allergens' name. Percentages of sequence identities were presented in parentheses.

Figure 1 *- Alignment of deduced amino acid sequences among three Uro m 1. Opened, dark grey, and light grey boxes indicate N-terminus signal sequence, expansin family-45 endoglucanase-like (expansin-like EG45) domain, and expansin cellulose-binding like (expansin-like CBD) domain, respectively, predicted by SignalP (23) and ScanProsite (21). Bold letters indicate the highly conserved amino acid residues of each Uro m 1 and the 67-100% homologous sequences using ConSurf (24).*

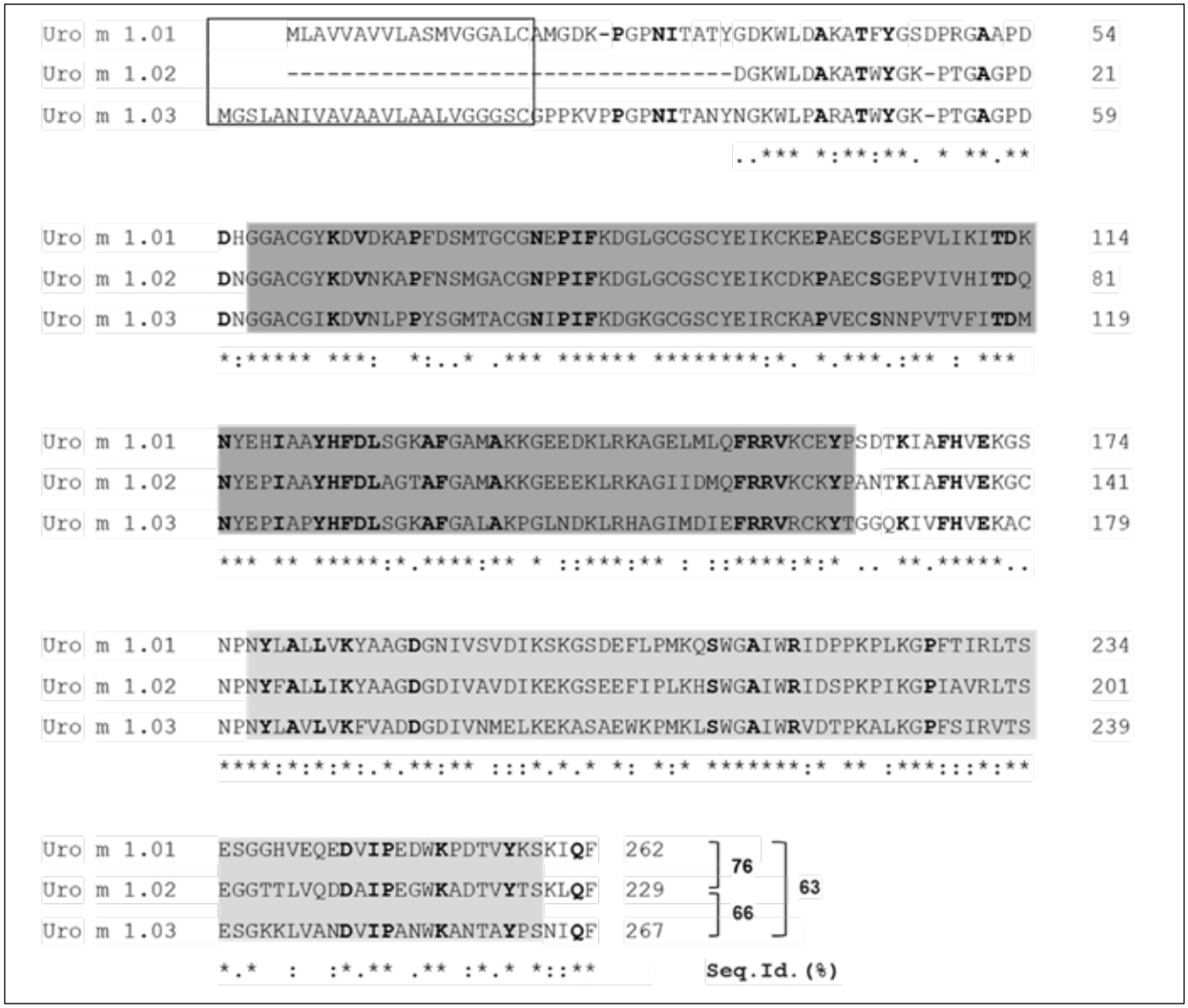
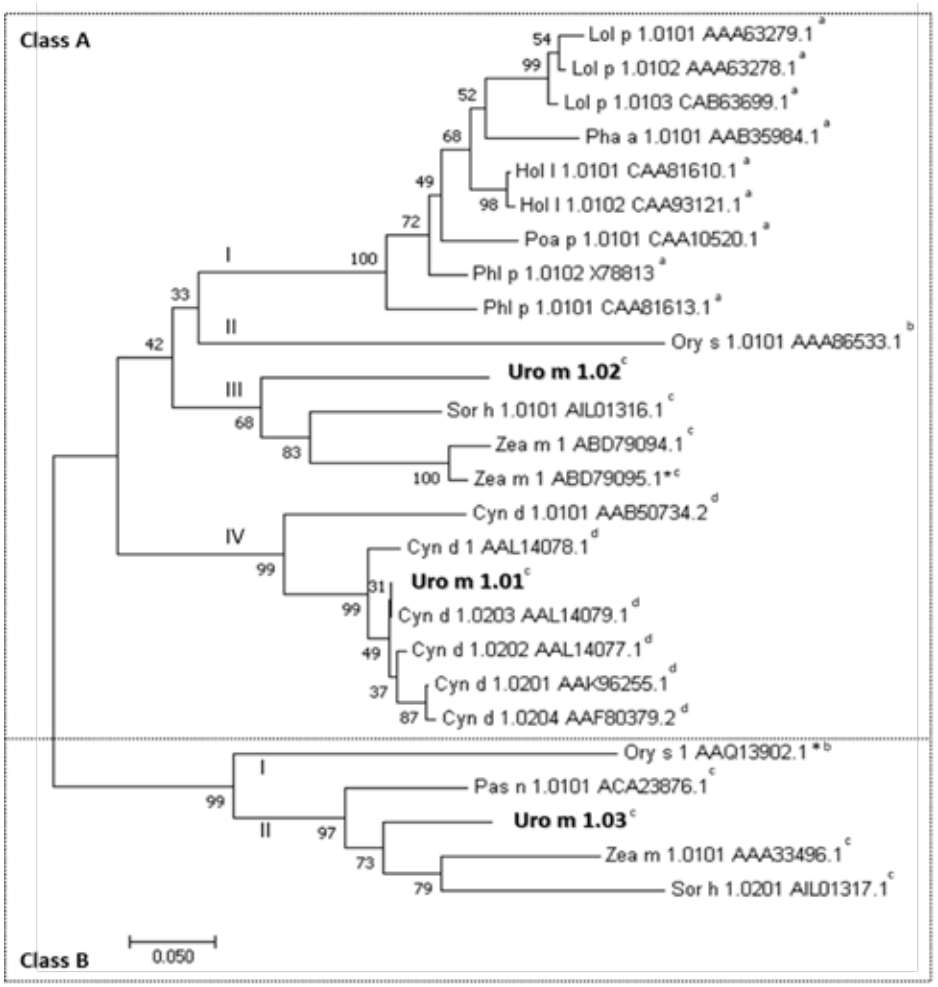


Table IV *-Amino acid sequence analysis using proteomic tools[1].*

Name	Molecular mass (kDa)	PI	No. and positions of disulfide bridges2/	Beta sheet/alpha helix (% / %)
Uro m 1.01	28.4	5.8	aa. 60-88, 91-158, and 96 - 102	51.5 / 66.8
Uro m 1.02 (partial)	24.8	6.5	aa. 27-55, 58-125, and 63 - 69	45.4 / 70.7
Uro m 1.03	28.6	9.1	aa. 65-93, 96-163, and 101 - 107	52.8 / 60.7

[1]Proteomic tools analysed Uro m 1 sequence were obtained from ExPASy.org such as ProtParam (Gasteiger et al. 2005), ScanProsite (de Castro et al. 2006), and CFSSP (Chou and Fasman 1974); [2]The numbers in parentheses indicate the amino acid regions forming a disulfide bridge.

Figure 2 - *The phylogenetic tree of group 1 GP allergens. The evolutionary analyses were conducted in MEGA7 (26) using the Neighbor-Joining method (27) and the evolutionary distances were computed using the Poisson correction method (28). Bootstrap values are shown at each node. Allergen sequences were retrieved from the WHO/IUIS Sub-Committee for Allergen Nomenclature database and the homolog proteins from Blastp analysis. The GenBank accession numbers are presented after allergen names. Superscript letters indicate grass subfamily (a, Pooideae; b, Ehrhartoideae; c, Panicoideae; d, Chloridoideae) and * indicates unpublished reference.*



PB11 (71%). Uro m 1.02 was similar to ZmEXPB11 (79%) and OsEXPB1 (77%), while Uro m1.03 was a close homolog of ZmEXPB1 (85%) and OsEXPB9 (80%).

Variation of IgE reactivity among different isoforms of rUro m 1

The three isoforms of Uro m 1 were cloned and expressed as recombinant proteins using the *E. coli* expression system. An immunoblot of purified recombinant Uro m 1 is shown in **figure 3**. Uro m 1.01 and Uro m 1.02 could interact with IgE in sera from all six atopic donors with PGP sensitization chosen for this study. On the other hand, there was no detectable IgE reactivity to Uro m 1.03 in sera from four patients, and only a relatively weak signal could be detected with serum number 33. No IgE reactivity to the 30 kDa protein in the pollen crude extract or recombinants was detected when sera of negative control individuals were used. These data showed that the three isoforms of rUro m 1 had different levels of IgE reactivity. Moreover, more than one isoform of beta-expansins could contribute, albeit not equally, to the overall IgE reactivity, and possibly to the allergic symptoms. Because Uro m 1.03, the class B beta-expansin, only showed weak IgE binding, this isoform was not used in further experiments.

Recombinant Uro m 1 isoforms with specific and dose-dependent IgE-reactivity

To confirm the specificity and IgE-binding properties of rUro m 1.01 and rUro m 1.02, we conducted immunoblot inhibition experiments. As shown in **figure 4a**, the IgE-binding signals of the 30 kDa proteins were largely reduced by serum pre-incubations with rUro m 1.01 and rUro m 1.02. The inhibition was not observed when recombinant D7 protein, a major allergen from mosquito, was used for pre-incubation, confirming that this inhibition was specific to rUro m 1. In ELISA inhibition, both rUro m 1.01 and rUro m 1.02 showed the inhibitory effects in a dose-dependent manner with all patient sera (**figure 4b**).

The inhibition by Uro m 1.01 calculated from log-concentration scale was relatively more effective than that of Uro m 1.02 at IC_{50} of 38.2 and 52.5 ng/µl, respectively. Of note, we could obtain the highest levels of inhibition at 70.1 ± 3.7% for Uro m 1.01 and 79.6 ± 9.5% for Uro m 1.02, which were not the maximum levels possible. Higher level of inhibition could not

Figure 3 *- Profiles of IgE bound proteins from PGP and rUro m 1. The arrows and arrowheads indicate the IgE-binding proteins of PGP and rUro m 1, respectively. Lane 1: PGP proteins; Lane 2: Uro m 1.01; Lane 3: Uro m 1.02; Lane 4: Uro m 1.03; P9-P36: Sera of PGP sensitized donors; N: Serum of non-sensitized donor.*

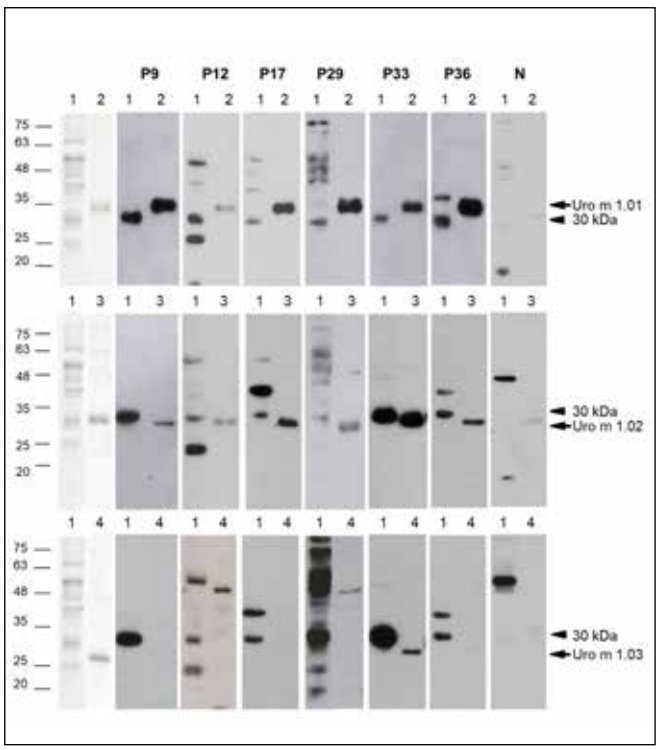
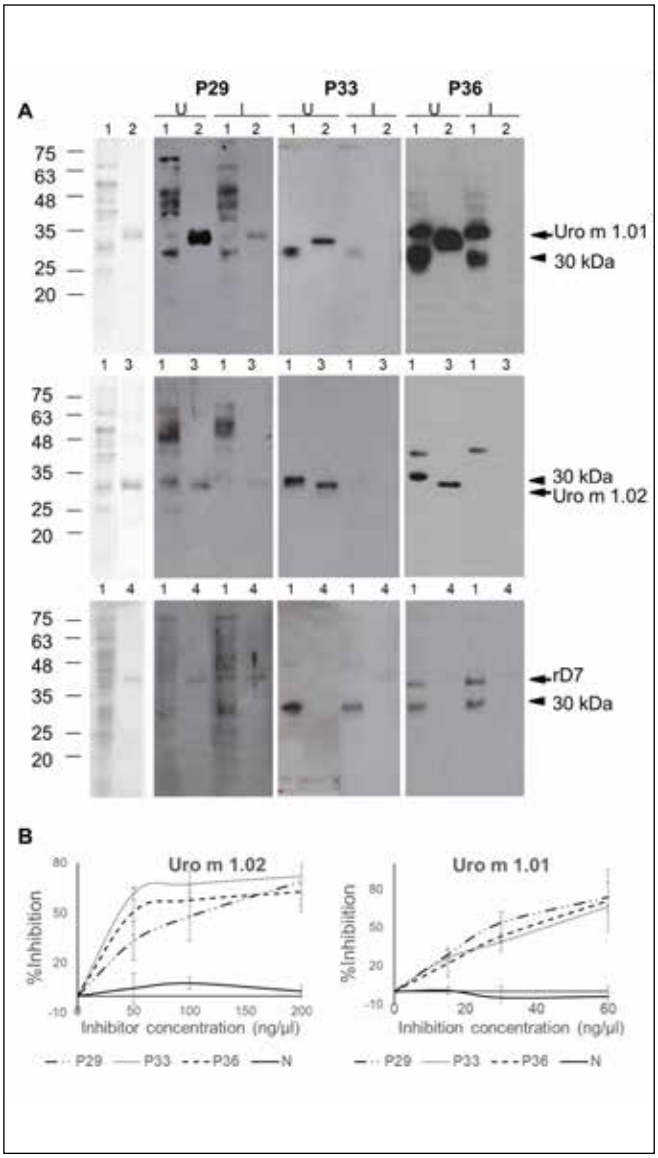


Figure 4 *- Inhibition of specific IgE binding to PGP with rUro m 1. The immunoblot inhibition of IgE-binding was performed using recombinant pre-incubated sera (I) in comparison with buffer pre-incubated sera (U) (A). The recombinant D7 protein from mosquito Aedes aegypti was used as a negative control. The arrows and arrowheads indicate the IgE-binding proteins of PGP and recombinant proteins, respectively. Percentage inhibition of specific IgE binding to PGP with rUro m 1 were detected in ELISA analysis (B). The data were performed in duplicate and presented as mean±SD. Lane 1: PGP proteins; Lane 2: Recombinant Uro m 1.01; Lane 3: Recombinant Uro m 1.02; Lane 4: Recombinant D7; P29-P36, Sera of PGP sensitized donors; N: Serum of non-sensitized donor.*

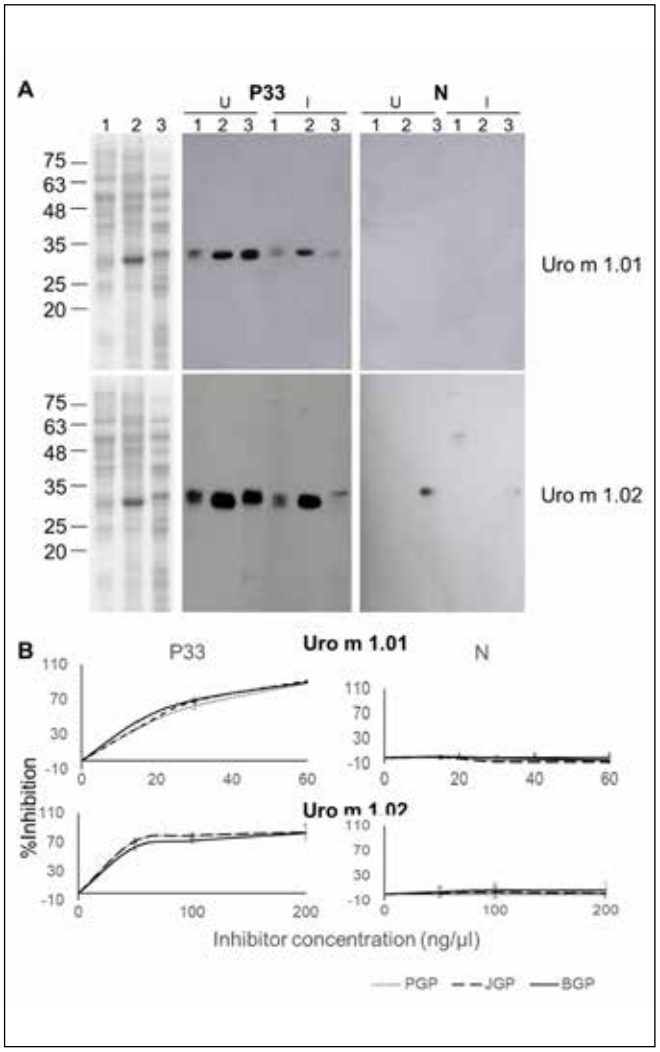


be achieved mostly due to the low concentrations of expressed recombinant proteins. These data confirmed that 1) the major IgE binding proteins in para grass crude extract were beta-expansins; 2) both isoforms of rUro m 1 could sufficiently inhibit the IgE binding to the native beta-expansin, suggesting significant cross reactivity; and 3) because the inhibition by each isoform was not complete, it is possible that other beta-expansins (or other 30 kDa proteins) were involved in the IgE binding.

Cross reactivity between rUro m 1 isoforms with Johnson and Bermuda GP proteins

Because group 1 GP allergens or beta-expansins are major allergenic proteins in all grass species, we would like to investigate the extent of IgE cross-reactivity among subtropical grasses using rUro m 1. The immunoblot and ELISA inhibitions were performed with crude extracts from pollen of Para, Johnson, and Bermuda grasses (PGP, JGP, and BGP). Serum number 33 was used to investigate the IgE-cross reactivity due to high specific IgE among these three grass species. Without inhibition, the IgE-binding signals of 30 kDa proteins were observed in all crude pollen protein extracts, but the signal was stronger for BGP and JGP than for PGP (**figure 5a**). When sera were pre-incubated with rUro m 1.01 and rUro m 1.02, sIgE binding was partially inhibited for JGP, and mostly removed for BGP and PGP. In ELISA inhibition assay, both Uro m 1.01 and Uro m 1.02 had strong inhibitory effects in dose-dependent manner for all GP proteins (**figure 5b**). Levels of inhibition by Uro m 1.01 and Uro m 1.02 were similar: IC_{50} calculated from log-concentration scale was of 24.3 ng/µl for Uro m 1.01, and extrapolated calculated from equation y = 18.712 ln(x) - 4.8524 was 18.8 ng/µl for Uro m 1.02. Therefore, these two beta-expansins might be the potent isoforms that play a major role in cross-reactivity among subtropical grass pollens.

Figure 5 *- Cross-inhibition of specific IgE binding to PGP, JGP, and BGP with rUro m 1. The immunoblot inhibition of IgE-binding was performed using sera pre-incubated with recombinant (I) in comparison with sera pre-incubated with buffer (U) (A). Percentage inhibitions of specific IgE binding to coated PGP, JGP, and BGP extracts were detected in ELISA analysis (B). The data were performed in duplicate and presented as mean ± SD. Lanes 1, 2, and 3 are PGP, JGP, and BGP extracts, respectively.*



Discussion

Urochloa mutica or para grass is a tropical/subtropical grass with extremely wide range of distribution. Its pollen extract had been shown to trigger positive skin prick test results in a considerably large number of AR patients in Thailand (16,17). Curiously, there had been no report of PGP allergy from other countries so far. It is unclear whether this is due to the relatively less prevalent distribution, pollen dispersion, or simply lack of information. More attention should be paid to the allergenicity of this species due to its invasive nature and the ability to adapt to new environment.

This study confirmed that the grass group 1 allergen (beta-expansin) was a major allergen of PGP. Furthermore, three different isoforms of the PGP allergen Uro m 1 were cloned and expressed as Uro m 1.01, Uro m 1.02, and Uro m 1.03 and were found to differ in their IgE reactivity. Although there have been reports of isoallergens found in other grass species such as *Brachypodium distachyon*, maize, Johnson grass, and rice (4,7,8,32), few other isoallergens from the same species had been tested for IgE reactivity in parallel. Similar to beta-expansin isoallergens in Bermuda grass (Cyn d 1) and Bahia grass (Pas n 1), Uro m 1 with

acidic to neutral pI (Uro m 1.01 and Uro m 1.02) had higher IgE reactivity than the basic isoform (Uro m 1.03) (6,33,34). Although Uro m 1.03 did not show potent IgE-binding function, this isoform could still be clinically relevant if sera from a larger or different population were tested. It is also possible that Uro m 1.03 was not expressed in a form that allowed IgE binding to be detected. In fact, Uro m 1.03 had 87% identity with Pas n 1.0101, which had been shown to be a major allergen in Bahia grass pollen (9). rPas n 1.01 which is a basic isoform shows frequency of 85% spIgE reactivity allergic rhinitis patient sera (9). Therefore, the basic isoforms are allergenic in some cases.

The three Uro m 1 isoforms obtained in this study were cloned based on PCR amplification of cDNA using specific primers designed from conserved regions of previously reported group 1 GP allergens, and were by no means exhaustive. In comparison with the most related species, maize, Uro m 1.01 and Uro m 1.02 could potentially be UmEXPB10 or UmEXPB11 and Uro m 1.03 could possibly be UmEXPB1. ZmEXPB1, ZmEXPB9, ZmEXPB10, and ZmEXPB11 are expressed in pollens and anthers and identified as group 1 grass pollen allergens in maize (8,15,35).

Based on the studies in species with complete genome information, including maize, rice, and *B. distachyon*, a number of beta-expansin genes are present within each genome (15,32,36). For example, in maize genomic sequence, three ZmEXPB10s were found on chromosome 3 and 9. Five ZmExpB11s were located in close proximity on chromosome 5, whereas ZmEXPB9 was located in a single cluster with three ZmEXPB1s on chromosome 9 (15). Thus, the three isoforms of Uro m 1 were also likely to be encoded by different loci in the genome and these proteins were possibly present together in the same pollen. Group 1 grass pollen allergens in maize (Zea m 1) were classified into two classes: class A (ZmEXPB10 and 11) and class B (ZmEXPB1 and 9), sharing about 60% sequence identity between classes (8,15). Likewise, our clustering analysis showed that Uro m 1 were divided into class A (Uro m 1.01 and 1.02) and class B (Uro m 1.03), along with isoforms of Sor h 1 and Pas n 1 (Panicoideae), and Ory s 1 (Ehrhartoideae). However, only class A isoforms had been reported for most allergenic grass species from Pooideae such as Phl p 1, Lol p 1, Poa p 1, Pha a 1, and Hol l 1; and Cyn d 1 from Chloridoideae, despite the high possibility that all Pooideae and Chloridoideae grass genomes also harbor class B beta-expansin genes. The absence of reported allergenic class B beta-expansins from Pooideae and Chloridoideae could suggest that only class A proteins have clinical relevance in these subfamilies. Until further information becomes available, it is unclear whether the IgE-reactive class B proteins are limited to grasses in Panicoideae subfamily.

Of the class A beta-expansins Uro m 1.01 and 1.02, the IgE reactivity was estimated to be high (all 6 of the 17 patients sensitized to PGP) among GP allergic patients. Because these two isoforms had highly similar sequences and pI values, the levels of IgE reactivity were also comparable. Both isoforms had the ability to significantly (up to 90-95%) inhibit IgE binding to beta-expansins in the crude extracts of PGP, BGP, and JGP. However, the actual contribution of each isoform to patient sensitization and elicitation of symptoms could not be discerned easily. Other factors such as gene expression level and protein modification could play important roles. Moreover, IgE reactivity could also differ in other populations. For example, Pas n 1 showed different IgE-reactivity levels in sera from patients of sub-tropical and temperate regions (37).

The ability of Uro m 1.01 and 1.02 to inhibit IgE binding of JGP and BGP extracts suggested that reactivity with Uro m 1 could be due to primary sensitization or co-sensitization with JGP and BGP, and cross-reactivity with PGP. Interestingly, the amino acid sequence of Uro m 1.01 is 100% identical to the sequence of Cyn d 1.0203. This result provided additional evidence that two allergens from different species may be more similar in sequence, and presumably in allergenicity, than two isoforms from the same species. Nonetheless, SPT using PGP and BGP extracts yielded different results. In our study, most patients who tested positive with PGP extract also tested positive with BGP (17/17) and JGP (15/17) extracts, but the reverse was not true (19). This could be due to the additional allergenic proteins present in the BGP and JGP or the different relative levels of allergenic proteins present in the GP crude extracts from these grass species.

In the era of precision medicine, it has been increasingly popular to identify the exact allergenic proteins or even the binding epitopes for each patient. Based on this study and others about grass group 1 allergens, it could be suggested that the class A and class B beta-expansins have different IgE reactivity and both classes should be represented when performing component-resolved diagnostics and allergen-specific immunotherapy.

Conflict of interest

The authors declare that they have no conflict of interest.

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Frequency of allergic sensitization to Can f 5 in North East Italy. An analysis of 1403 ISACs 112 (component resolved diagnosis) collected retrospectively

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Key words
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Summary
Recent studies have shown the increasing relevance of allergic sensitization to Can f 5, a prostatic kallicrein expressed in the prostate and detectable only in male dogs. The aim of the present study was to establish the frequency, level of sensitization and association with other dog allergens of Can f 5, as assessed by component resolved diagnosis (CRD ISAC 112, ThermoFisher Scientific, Uppsala, Sweden) in North East Italy.
A total of 1403 CRD ISAC 112 were examined retrospectively. Five hundred twenty subjects (37%) had a positive IgE response to at least one of the available animal allergens. Among these 520 subjects, 268 (51.5%) showed at least one sensitization to dog allergens. Among dog-sensitized individuals, 183 (69.02%) showed IgE against Can f 5, and 106 (57.92%) were sensitized exclusively against Can f 5. The average Can f 5 specific IgE was 8.810 ISU-E, with 77.6% of individuals showing medium or high values of specific IgE according to manufacturer's specifications.
In conclusions, our data confirmed that there is a high number of patients sensitized to Can f 5, which have a high degree of allergic sensitization. These results should be taken into account by allergists managing dog allergic patients. In fact, clinical consequences of this sensitization regard respiratory allergy (burden of rhinitis/asthma), systemic reactions (anaphylaxis during sexual intercourse from cross-reaction with human prostatic antigen), allergen immunotherapy-AIT (likely ineffective in patients with exclusive sensitization), and preventive measures (possibility to own a female dog and a likely reduction of allergen passive transport). Further studies are needed to better explore these aspects in "real life".

Dog allergens are a common cause of allergic sensitization and triggering respiratory symptoms worldwide, especially in geographical areas characterized by a high level of pet ownership such as US and Northern Europe (1,2). It is well known that common dog allergens belong to lipocalins or albumins families of proteins (3-5).

Can f 5, a newly described dog allergen, is a prostatic kallicrein, an androgen-regulated protein expressed in the prostate and detectable only in male dogs (6). Few studies have shown that the rate of a prevalent or exclusive allergic sensitization to Can f 5 is high, ranging between 37% and 52% in the case of dog

sensitized patients or pet-sensitized individuals living in areas with a high pet ownership (6-8). The peculiar characteristics of Can f 5, in highly or exclusive sensitized patients, can determine positive and negative actions which must be carefully evaluated by allergists managing such patients (9,10).

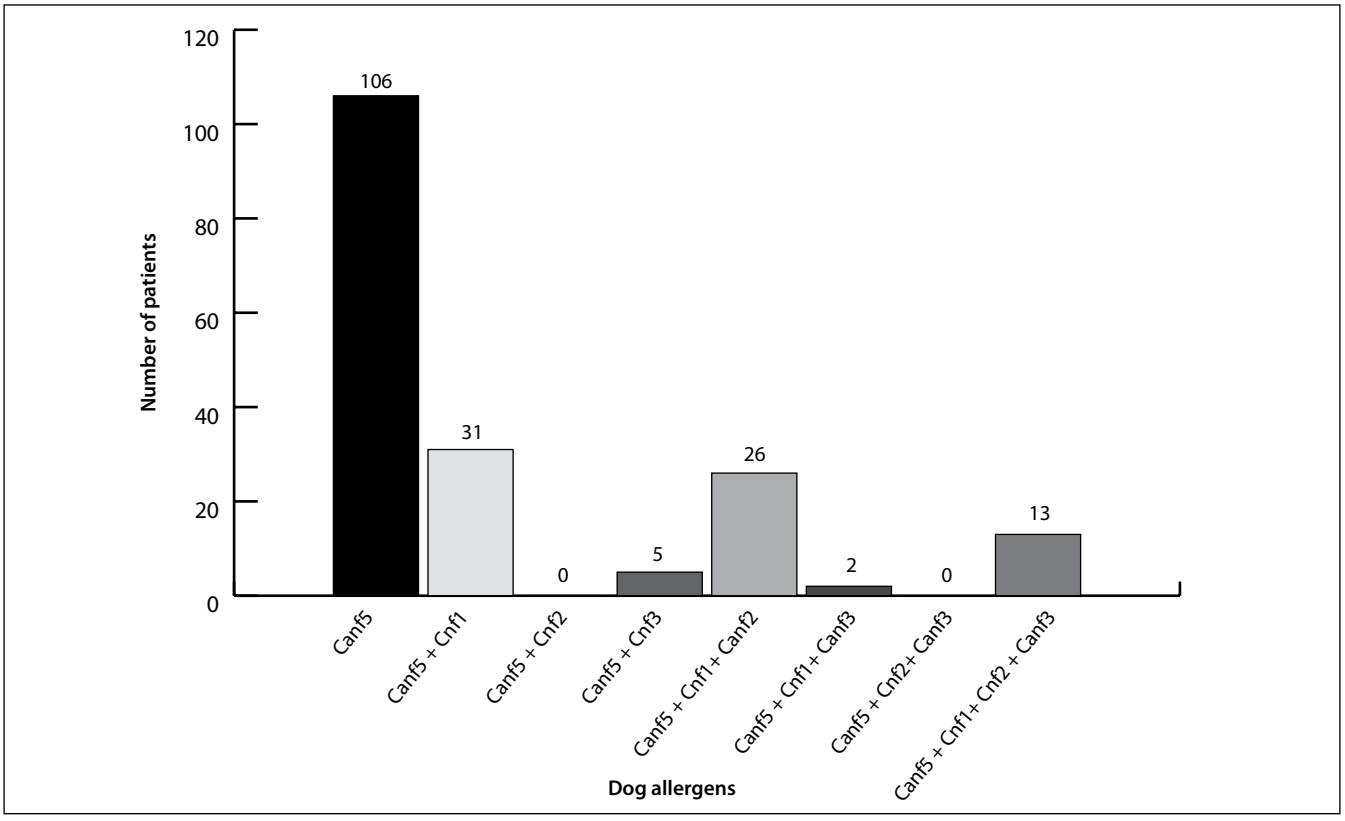
In Italy, data on the frequency of Can f 5 as sensitizing agent in dog allergic individuals are lacking. Therefore, we performed a study in North East Italy to investigate the frequency, the degree of sensitization and the association with other dog allergens, as assessed by Component Resolved Diagnosis (CRD). We retrospectively examined all CRD (ISAC 112, ThermoFisher Scientific, Uppsala, Sweden) carried out from January 1 2013 to December 31 2016 at the Immunology and Allergy Unit of Santa Maria degli Angeli Hospital, Pordenone, Italy. Among CRD results, we have selected those containing IgE against animal allergens including those against dog allergens. The number of individuals with positivity to at least one dog allergen was calculated, as well as the number of Can f 5 sensitizations and average of IgE titration. IgE values were considered as low (0.3 - 0.9 ISU-E), medium (1 - 15 ISU-E) and high (> 15 ISU-E) according to manufacturer's indications.

A total of 1403 consecutive samples from patients with an age range between 4 - 67 years (54% females) were examined with CRD ISAC 112. Five hundred twenty subjects (37%) had a positive IgE response to at least one of available animal allergens (Fel d 1, Fel d 2, Fel d 4, Can f 1, Can f 2, Can f 3, Can f 5, Equ c 3, Mus m 1, Bos d 1). Among these 520 subjects, 268 (51.5%) showed at least one sensitization to allergens of dog. Among dog-sensitized individuals, 183 (69.02%) showed IgE against Can f 5, and 106 (57.92%) were sensitized exclusively to Can f 5.

The association between different dog allergens (Can f 1, Can f 2, Can f 3 and Can f 5) in 183 Can f 5-sensitized patients is described in **figure 1**. The average degree of all 183 sensitizations was 8.810 ISU-E.

The results of our study demonstrated that Can f 5 represents an allergen characterized by high frequency, considering both the number of sensitized individuals (69.02% among 268 dog sensitized ones, and 57.92% as exclusive sensitization) and the level of sensitization (77.6% of individuals showed medium or high values of specific IgE according to manufacturer's specifications). From a general point of view, we believe that the role of Can

Figure 1 *- Association between different dog allergens (Can f 1, Can f 2, Can f 3 and Can f 5) in 183 Can f 5-sensitized patients.*



f 5 in dog - allergic individuals might be more multi - faceted than previously reported. The practical consequences regard respiratory allergy (burden of rhinitis/asthma), systemic reactions (anaphylaxis during sexual intercourse from cross-reaction with human prostatic antigen), allergen immunotherapy-AIT (likely ineffective in patients with exclusive sensitization), and preventive measures (possibility to own a female dog and a likely reduction of allergen passive transport) (**figure 2**) (11-16). The results of this study emphasize the need of an adequate management of patients suffering from dog allergy, especially those with significant clinical symptoms following dog exposure. A detailed collection of anamnestic data (including modalities of exposure to dogs and sex of dogs with more frequent contact), clinical and routine diagnostic examinations integrated by CRD may represent the base for a correct management of these patients. In this scenario, the microarray technique for available animal allergens could be useful to evaluate the possibility of cross-reactions between allergens of different animals and in the management of Can f 5 positive patients, as reported in **figure 2** (17,18).
In conclusions, although our data are limited to North East Italy, they confirm that the frequency of Can f 5 as sensitizing dog allergen is high, as demonstrated by the number of sensitized patients and the level of allergic sensitization. These results should be taken into account by allergists managing dog allergic patients. Further studies are needed to better explore the multiple aspects (with clinical implications) related to Can f 5 sensitization as previously described. Finally, we are also planning further studies to establish the prevalence of allergic sensitization to Can f 5 in Italy.

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Conflict of interest

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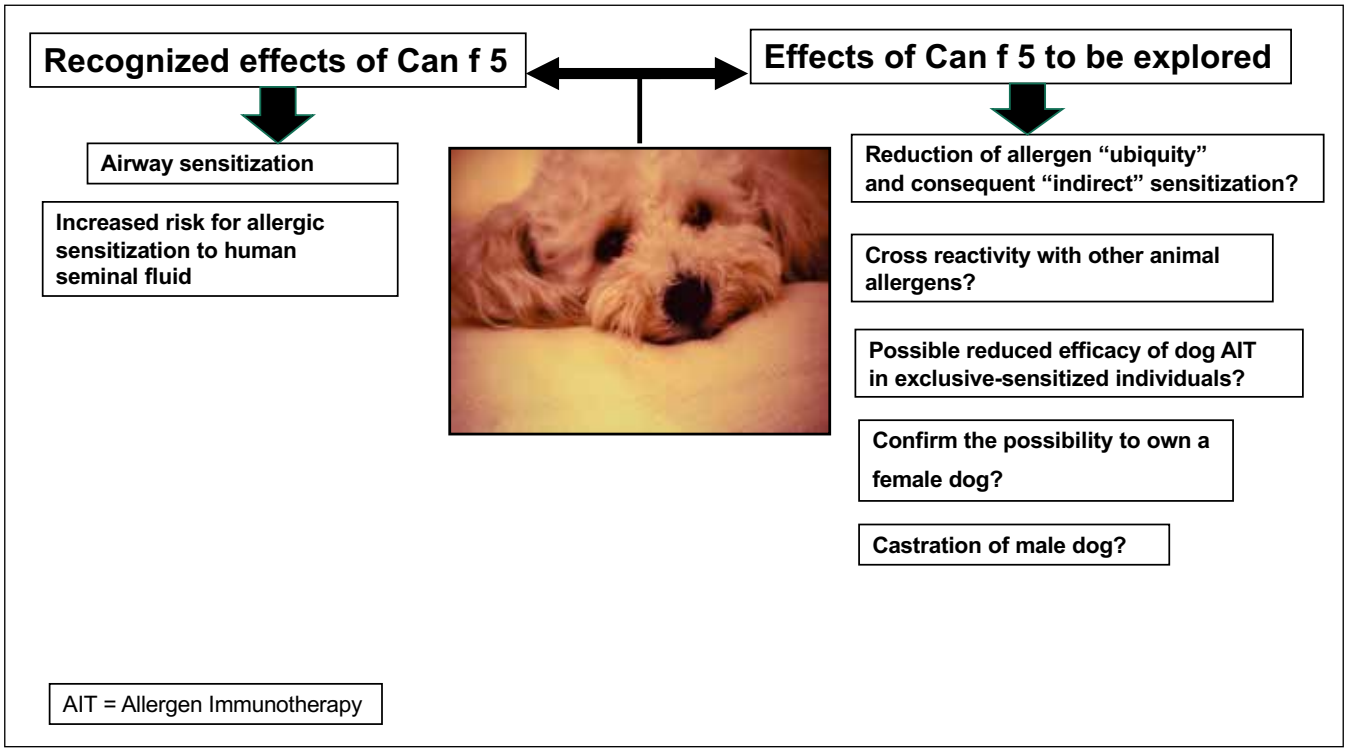
Figure 2 *- Possible topics associated with a prevalent or exclusive allergic sensitization to Can f 5. Adapted from (10). (The picture of the dog "Charlie" 2001 - 2017 is property of GL).*



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Anxiety/depression changes are associated with improved asthma control perception in asthmatic adolescents after adequate management

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Key words
asthma; children; anxiety; depression; asthma control; treatment

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To the Editor

Asthma is a complex disorder where many pathogenic mechanisms are involved. Historically, asthma was defined a psychosomatic disease until the '50s. However, it is well known that anxiety and depression may significantly affect asthma, mainly concerning frequent exacerbations, pharmacoeconomic costs, and uncontrolled asthma. In this regard, Sastre and colleagues explored very recently the relationship between asthma control and anxiety/depression in a Spanish population, and measured changes over a 6-month period of standardized treatment (1). They confirmed the association between anxiety/depression and uncontrolled asthma. Interestingly, specialist-managed treatment improved anxiety/depression, asthma control, and lung function. They concluded that regular specialist care improves both asthma and anxiety/depression. Psychological aspects in asthmatic patients is an emerging topic that deserves adequate attention. There is a body of evidence showing that anxiety and depression significantly worsen asthma outcomes (2). In particular, a real-life study demonstrated that anxiety and depression are a common and relevant comorbidity in asthmatic outpatients and are associated with uncontrolled asthma and lower ACT scores (3). Asthma gender difference consisted mainly in a worse perception of asthma control and more anxiety in females than in males (4). Moreover, emotional disorders may affect also children and adolescents, so new interventional strategies should be developed to empower children and adolescents to improve their asthma self-management (5).

Therefore, a longitudinal real-world study included 54 consecutive adolescents (31 males, 23 females, mean age 13.1 ± 2.2 years) with allergic asthma and visited for the first time at a third-level pediatric clinic. The inclusion criteria were adolescent age (12-17), asthma diagnosis, and anxiety or depression suggested by positive HADS questionnaire as below described. The procedure was approved by the Ethics Committee and the parents signed an informed consent. Asthma diagnosis was performed according with the Global Initiative for Asthma (GINA) document (6). Anxiety and depression were evaluated by the Hospital Anxiety Depression Scale (HADS) questionnaire; a score > 7 (in the two subscales) could define anxiety or depression (7). Children with anxiety (39) or depression (15) were carefully managed and re-evaluated after 6 and 12 months. Asthma control perception was measured by Asthma control test (ACT) questionnaire. Asthma treatment was tailored personalizing the medication options according to the GINA guidelines. Data are reported as median with inter-quartile range. Difference in the median values between at baseline and after 12-months follow-up was evaluated with the Wilcoxon signed

rank test. Correlation between the HADS-A or HADS-D and ACT was evaluated with Spearman rank-order correlation coefficient. Statistica software 9.0 (StatSoft Corp., Tulsa, OK, USA) was used.

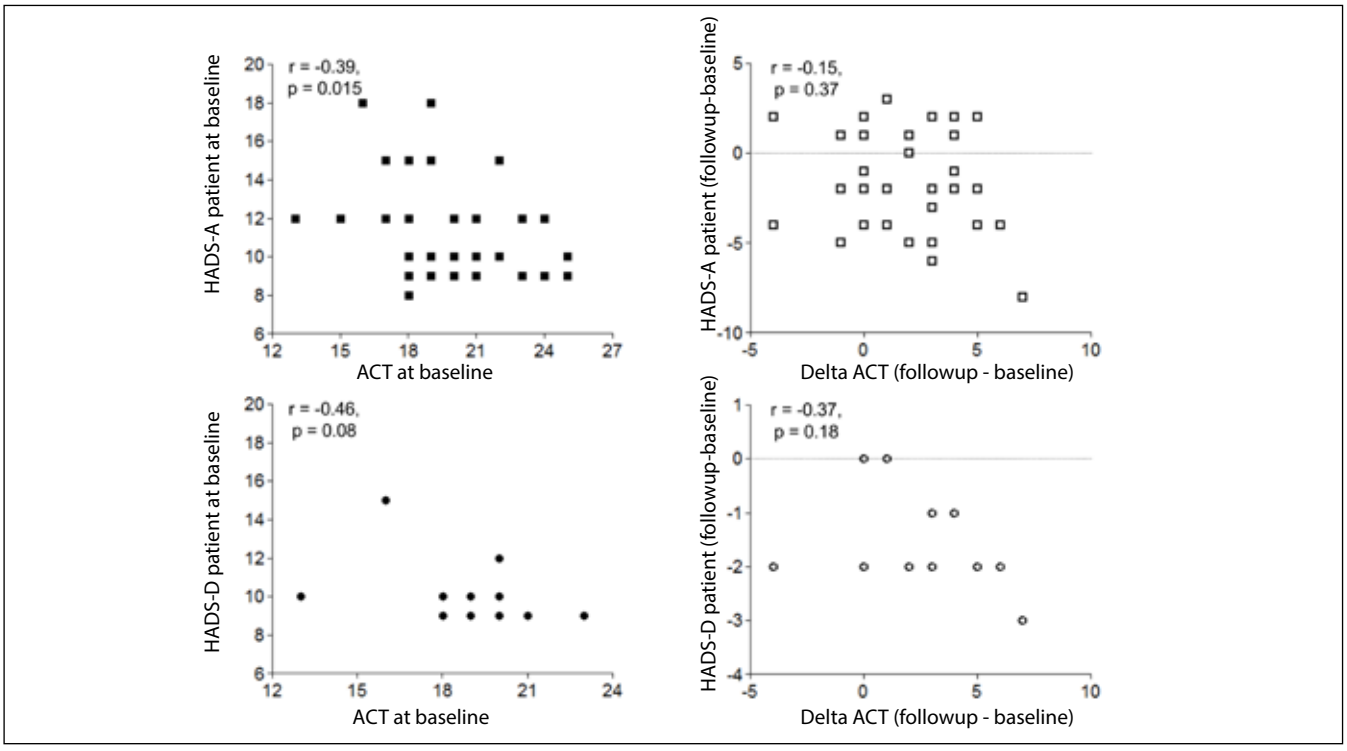
ACT significantly increased in patient with anxiety (p = 0.0002) or depression (p = 0.0085) as well as HADS-Anxiety and HADS-Depression significantly decreased after 12-months follow-up as reported in **table I**. Notably, there were relevant correlations between HADS and ACT score before and after treatment (**figure 1**). In other words, after adequate therapy the perception of asthma control improved in patients with anxiety or depression, as well as the scores of these emotional disorders diminished.

The current study confirms that anxiety and depression are related with the perception of asthma control in adolescents. This outcome underlines the practical relevance of emotional disorders because they are really present at this age. Moreover, this study confirms the results obtained in adult asthmatics. Indeed, adolescents, well managed by pediatricians at a third-level clinic, achieved an improved asthma control as well as emotional symptoms. Notably, asthma control grade significantly correlated with emotional scoring.

Table I *- HADS-A and HADS-D scores and ACT scores in asthmatic adolescents before and after 12-month optimal therapy.*

		Baseline	12-mo follow-up	p value
Pts with an abnormal HADS-A at baseline (n = 39)	ACT	20.00 (18.00 - 23.00)	22.00 (20.00 - 24.00)	0.0002
	HADS-A	10.00 (9.00 - 12.00)	10.00 (8.00 - 10.00)	0.0011
Pts with an abnormal HADS-D at baseline (n = 15)	ACT	20.00 (18.00 - 20.00)	22.00 (20.00 - 24.00)	0.0085
	HADS-D	9.00 (9.00 - 10.00)	8.00 (8.00 - 9.00)	0.0013

Figure 1 *- top left, relationship between HADS-A score and ACT score at baseline; top right, relationship between delta HADS-A and delta ACT after treatment; low left, relationship between HADS-D score and ACT score at baseline; top right, relationship between delta HADS-D and delta ACT after treatment.*



The present study was open designed, but lung function, inflammatory biomarkers, and clinical parameters were measured over time. Another relevant limitation of the study is that the Italian version of HADS questionnaire has been validated for adults. Therefore, the present findings should be cautiously considered. However, HADS questionnaire has been previously validated for use with adolescents aged 12-17 years (8), and consequently it was used in many pediatric studies, including one Italian investigation. Chan and colleagues used HADS in a large community sample of 5,857 adolescents (10-19 years; mean age 13.4 years) in Hong Kong (9). The HADS showed satisfactory psychometric properties as a screening instrument in assessing anxious and depressive states as two correlated but distinct factors in adolescents. Petronyte and Praninskiene enrolled 253 schoolchildren (13-18 years of age) using HADS; they found 20.6% of adolescents with emotional problems (10). Ayaki and colleagues performed a cross-sectional survey in 486 participants aged from 10 to 59 years (11). In particular, children showed the highest probability of sleep and mood disorders as the HADS analysis disclosed the shortest sleep duration in the high myopia group. Catistini and colleagues used the HADS questionnaire in 127 Italian adolescents with cystic fibrosis (12). They reported that cystic fibrosis increased the risk of developing anxiety and depression in female patients, and that their levels depended on clinical status. Chai and coworkers used HADS in 60 children (< 15 years of age) with strabismus (13). The authors concluded that HADS questionnaire was a reliable instrument for determining depression and anxiety status in a hospital outpatient clinic setting. Kabra et al. investigated 22 adolescents (age range 10-19 years) with neurogenic bladder dysfunction using HADS (14). The authors concluded that the prevalence of anxiety in adolescents was striking. Mihalca and Pilecka evaluated a group of 146 chronically ill adolescents (12-16 years) using an adapted version of HADS (15). The authors observed different structures in chronically ill versus healthy adolescents. Pizolato studied 40 children (aged 8 to 12 years) with temporomandibular disorder using HADS; anxiety was a predictor factor (OR = 18.59) for this disorder (16). More interestingly, HADS questionnaire was used in a group of adolescents and young adults (12-35 years) with asthma (17). The authors demonstrated that anxiety and depression were associated with impaired quality of life and asthma control. Therefore, the current findings confirm the previous observation in asthmatic adolescents.

In conclusion, the present study documents the clinical relevance of emotional disorders also in asthmatic adolescents, and overall shows that optimal asthma management improves both asthma control and anxiety and depression.

Conflict of interest

All authors declare that there is no conflict of interest.

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