

Combining Analyses of Basophil Allergen Threshold Sensitivity, CD-sens, and IgE Antibodies to Hydrolyzed Wheat, ω -5 Gliadin and Timothy Grass Enhances the Prediction of Wheat Challenge Outcome

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Key Words

Allergen stimulation · Children · Flow cytometry · Oral food challenge · Wheat allergy

Abstract

Background: Wheat is a common food causing allergy which has implications on the quality of life. The diagnosis of IgE-mediated wheat allergy is based on the clinical history and presence of IgE antibodies (IgE-Ab) in skin or blood, and the results of an oral food challenge which is time consuming and associated with risks. An improved diagnostic workup is needed for wheat allergy. The objective was to examine the relationship between wheat challenge, CD-sens and IgE-Ab to related allergens in wheat-allergic children and investigate if a combination of different markers could enhance the prediction of challenge outcome. **Method:** Twenty-four children (aged 1–15 years) with a wheat allergy diagnosis underwent an open wheat challenge. CD-sens and IgE-Ab to wheat, hydrolyzed wheat protein (HWP), ω -5 gliadin and timothy grass were analyzed

and related to the challenge outcome. **Results:** A positive challenge was seen in 12/24 children. Children reacting to the challenge had higher IgE-Ab concentrations to wheat, ω -5 gliadin and HWP ($p < 0.01$) and a tendency to higher wheat CD-sens values ($p = 0.08$) than nonreacting children. Combining wheat CD-sens >150 and IgE-Ab to wheat >20 kU_A/l, or ω -5 gliadin >0.1 kU_A/l predicted the challenge outcome in 83% of the patients. Most children with IgE-Ab to wheat also had IgE-Ab to timothy. Seven of 9 challenge-positive children had a positive CD-sens to HWP and IgE-Ab to HWP >8 kU_A/l. **Conclusion:** Combining CD-sens and IgE-Ab to wheat or wheat components could be useful in the diagnosis and follow-up of wheat-allergic children.

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Introduction

Wheat is the most widely consumed food grain and is one of the six most common foods causing allergy in children worldwide [1, 2]. Wheat allergy is defined as an

adverse immunological reaction to wheat [3] and the diagnosis is currently based on the clinical history and the presence of IgE antibodies (IgE-Ab) in the skin or blood and the results of an oral food challenge. However, a challenge is time consuming and associated with the risk of severe allergic reactions and does not measure the degree of allergen sensitivity [4].

The prevalence of IgE sensitization to wheat in Swedish 4-year-old children was reported to be 4% [5] and was shown in German children to increase with age from 2% in 2-year-old to 9% in 10-year-old children [6]. The high prevalence in school age can be a consequence of pollen sensitization due to the close taxonomic relationship between cereal grains and grasses within the Poaceae family. Jones et al. [7] have demonstrated that IgE-Ab in sera from patients with wheat allergy alone showed extensive in vitro cross-reactivity to other grains but little to taxonomically related grasses, whereas patients with grass allergy alone showed extensive in vitro cross-reactivity to cereal grains.

There are numerous proteins involved in wheat allergy. Among the gliadins, ω -5 gliadin (Tri a 19) was identified as a major allergen of wheat-dependent, exercise-induced anaphylaxis (WDEIA) as well as in immediate reactions to ingested wheat in children [8–10]. IgE-Ab to ω -5 gliadin correlate better with challenge outcome compared to IgE-Ab to wheat [11, 12] and can be useful when monitoring tolerance development over time [13].

IgE sensitization to hydrolyzed wheat proteins (HWP) has recently been reported to cause contact urticaria, anaphylaxis and WDEIA [14–17]. These proteins are used in cosmetics, shampoo and hair conditioner but also as dairy substitute in creamers, cookies, breads and sauces, due to their emulsifying properties. The HWP are a mixture of polypeptides with various sizes depending on the type and extent of hydrolysis using acids or proteolytic enzymes. The IgE-Ab cross-reactivity between HWP and wheat in patients with wheat allergy remains unclear.

In the early 90s, it was reported that when the basophils empty their histamine containing granules, a structure on the granule vesicle, CD63, becomes exposed on the cell surface [18]. Appearance of CD63 on the surface of basophils, measured with flow cytometry, is now an established procedure to detect allergen triggering of basophils, and the accessibility of the basophil-specific marker CD203c [19] has made it possible to more easily identify basophils. Basophil allergen threshold sensitivity, CD-sens [20], combines CD203c (for basophil identification) and CD63 (for basophil activation) and has been shown to mirror the target organ allergen sensitivity, and

an excellent correlation was found with bronchial allergen threshold sensitivity [21]. Previous studies comparing CD-sens and skin prick test titration [20], nasal allergen challenge [20] and food challenges also indicated a good agreement [22]. Basophil activation tests measuring cell reactivity have also been evaluated in milk, egg and wheat allergy [23–25], and, recently, Chinuki et al. [16] showed that basophil reactivity could be useful in differentiating between subtypes of WDEIA caused by wheat and/or by HWP.

The aim of this descriptive and explorative study was to examine the relationship between wheat challenge, CD-sens and IgE-Ab to related allergens in wheat-allergic children and to investigate if a combination of different markers could enhance the prediction of challenge outcome.

Methods

Study Population

Twenty-nine children with a suspected wheat allergy (aged 1–19 years) were invited to the Astrid Lindgren Children's Hospital, Stockholm, Sweden, for an oral wheat challenge. The inclusion criteria were doctor's diagnosed wheat allergy, wheat IgE-Ab ≥ 0.35 kU_A/l and children's adherence to a wheat-free diet. Exclusion criteria were antihistamines or oral steroids taken within 4 days prior to the challenge. Five children were excluded from the study, because it was deemed inappropriate to perform a wheat challenge since they had a recent history of anaphylaxis to wheat, thus the study population consisted of 24 children (table 1). Information about the child's clinical symptoms to timothy grass during the timothy pollen season was based on questionnaires as well as medical records.

The study was approved by the ethics committee (Stockholm, Sweden) and the parents provided written consent.

Challenge Test

Information about the child's health was collected, a clinical examination was performed and a peripheral venous catheter inserted before the challenge. Open oral challenge tests were performed by experienced personnel using wheat bread in increasing doses every 30 min in 5 or 7 steps from 0.034 to 11.4 g of wheat. The cumulative dose was 22.8 g. Symptoms were scored according to Astier et al. [26]. A negative test was defined as no objective allergic symptoms during the 2 h after the test was completed. The outcomes of the challenge were reported as positive or negative.

Blood Sampling

Blood samples were collected and stored at +4°C for a maximum of 24 h before cell analyses. Serum was separated and stored at –20°C pending analyses.

Basophil Analyses

Basophil analyses were performed essentially as described [20, 27]. Aliquots of 100 μ l of blood were incubated for 20 min at 37°C with 100 μ l RPMI-HEPES (negative control; Gibco Ltd., Paisley,

Table 1. Patient characteristics

Patient No.	Age years	Wheat challenge	Months between challenge and blood samples	Years on wheat-free diet	Allergic symptoms to grass
1	7	+	0	5	–
2	5	+	0	5	–
3	6	+	0	5	+
4	9	+	0	7	+
5	15	+	6	13	+
6	7	+	6	6	+
7	2	+	0	1.5	–
8	4	+	6	3	–
9	11	+	0	1	–
10	4	+	6	5	–
11	15	+	6	16	–
12	11	+	0	1	–
13	4	–	0	4	–
14	2	–	0	2	–
15	7	–	0	6	+
16	13	–	0	13	–
17	5	–	7	4	–
18	6	–	6	5	–
19	4	–	7	4	–
20	3	–	2	3	–
21	4	–	10	3	+
22	3	–	0	3	–
23	2	–	1	1	–
24	2	–	0	2	–

+ = Positive; – = negative.

UK), fMLP 10^{-7} M (non-IgE-dependent positive control; Sigma Chemical Co., St. Louis, Mo., USA), 25 μ l anti-Fc ϵ RI (IgE-dependent positive control; Böhmann Laboratories, Basel, Switzerland) or 100 μ l allergen extract of wheat (IDD Thermo Fischer Scientific, Uppsala, Sweden), recombinant ω -5 gliadin (rTri a 19; IDD Thermo Fischer Scientific), HWP (Meripro 711®; Tate & Lyle, Aalst, Belgium) and timothy grass (IDD Thermo Fischer Scientific) in decreasing concentrations. After stimulation, cells were incubated for 25 min on ice in the dark with 18 μ l CD203c-PE (Immunotech, Marseille, France) for basophil identification and 18 μ l CD63-FITC (Immunotech) for detection of basophil activation. After antibody staining, the red blood cells were hemolyzed by adding 2 ml +4°C isotonic NH₄Cl-EDTA lysing solution. The leukocyte suspensions were washed with 2 ml of PBS at 4°C. After centrifugation, the leukocyte pellet was resuspended in 300 μ l of PBS and counted in a Navios flow cytometer (Beckman Coulter Inc., Fullerton, Calif., USA). The cutoff determining a positive allergen test was set to 5% of CD63-positive basophils.

Individuals with basophils, which after anti-Fc ϵ RI stimulation (positive control) responded with 0–5% CD63 upregulation were regarded as nonresponders. For individuals with a response between 5 and 16% (low responders), the results should be interpreted with caution. The cutoff 16% was calculated (mean 76% $\pm$ 3 SD) from the positive controls of an in-house reference material of 264 allergic children and adults [22].

Basophil allergen threshold sensitivity, CD-sens, was defined as the inverted value for the lowest allergen concentration giving 50% (LC₅₀) of maximum CD63 upregulation multiplied by 100, i.e. $1/\text{LC}_{50} \times 100 (= 100/\text{LC}_{50})$ and was used to describe the patient's allergen sensitivity [27].

Serological Analyses

IgE-Ab to wheat, ω -5 gliadin, HWP (Meripro) and timothy grass were determined in serum with ImmunoCAP® (IDD Thermo Fischer Scientific) according to the manufacturer's instructions. A positive test was defined as an IgE-Ab level >0.1 kU_A/l.

Statistics

The results are presented as medians and ranges. Spearman's rank order correlation (r_s) was used to assess the relationship between the challenge, CD-sens and IgE-Ab measurements and the Mann-Whitney test to assess differences between the children with positive and negative challenges. Significance was considered at a value of $p < 0.05$. No adjustment for multiple testing has been performed. Thus, significant results should be regarded as descriptive and explorative. Analyses were performed using GraphPad Prism 5.

Results

Patient Characteristics

All patients in the study were included on the basis of doctor's diagnosed wheat allergy and had at inclusion IgE-Ab to wheat ≥ 0.35 kU_A/l. Of the 24 children included, 16 were boys and the median age was 5 (2–15) years. The median time of wheat-free diet for all children was 4.0 (1–6) years. In 13/24 children, the challenge and the blood sampling for CD-sens and IgE-Ab measurements was performed at the same occasion. In 11 cases, blood samples were collected 1–10 months after the challenge test (table 1).

Challenge Outcome

Of the 24 patients, 12 had a positive reaction at the oral wheat challenge (table 1). The median age of children with a positive challenge test was 7 (2–15) years and that of children with a negative challenge 4 (2–13) years. All symptoms in the oral challenge test were scored, but the outcome of the challenge was only reported as positive or negative (tables 1, 2). Of the 12 patients with a positive challenge, 8 had mild-to-moderate symptoms (grade 1–3) and 4 patients experienced severe symptoms (grade 4). The most common clinical reactions were urticaria ($n = 10$) and respiratory symptoms ($n = 9$). Two children reacted with rhinoconjunctivitis. The median cumulative dose of wheat in those with a positive challenge was 11.4 g compared with the predefined highest dose of 22.8 g in those with no reaction.

Table 2. Serological and cellular results

Patient No.	Wheat challenge	IgE-Ab, kU _A /l				CD-sens			
		wheat	ω-5 gliadin	HWP	timothy	wheat	ω-5 gliadin ¹	HWP	timothy
1	+	37	0.18	20.2	0.59	1,659	+	n.t.	150
2	+	11	0	6.0	2.0	303	–	n.t.	6.9
3	+	3.7	0.27	0.47	6.4	5.0	+	0	71.7
4	+	2,500	14	160.4	20	1,305	+	191	3.5
5	+	255	4.3	108.4	6.6	9,896	+	814	169
6	+	40	0.62	13.6	49	1,678	–	49.6	583
7	+	35	0.34	14.6	0.43	1,046	–	11.8	1.87
8	+	22	0.12	8.2	0.36	515	+	75	+
9	+	93	0.13	26.6	0	n.r.	n.r.	n.r.	n.r.
10	+	940	4.1	47.3	0.17	+	–	0	0
11	+	72	0.29	28.9	1.0	5,326	+	13	7.56
12	+	57	0	10.8	13	177	–	23.7	4.26
13	–	14	0	2.8	0.97	1,145	–	32.4	8.2
14	–	5.3	0.11	1.3	0	319	–	0	0
15	–	7.8	0	0	5.9	12.7	–	0	345
16	–	6.7	0	2.1	0	621	–	8.32	1.98
17	–	1.3	0	0.2	0.96	n.r.	n.r.	n.r.	n.r.
18	–	69	0.17	3.8	4.5	55.5	–	3.05	3.18
19	–	31.8	0.05	4.0	0	62.1	+	0	0
20	–	6.6	0	5.9	1.6	17.3	–	0	1.94
21	–	74	0	37.3	3.0	1427	–	23.9	3.1
22	–	18	0	3.0	0.12	514	–	n.t.	0
23	–	2.9	0.21	0.41	0.73	51.7	–	n.t.	0
24	–	0.6	0	0.15	0	l.r.	l.r.	l.r.	l.r.

n.t. = Not tested; n.r. = nonresponder; l.r. = low responder; + = positive; – = negative.

¹ Due to solubility difficulties, we chose to describe CD-sens to ω-5 gliadin only as positive or negative.

IgE-Ab versus Challenge Outcome

Children positive in the challenge had significantly higher concentrations of IgE-Ab to wheat ($p < 0.01$), ω-5 gliadin ($p < 0.005$) and HWP ($p < 0.005$) compared to children with a negative outcome in the challenge (fig. 1a). There was no significant difference between IgE-Ab levels to timothy in the challenge-positive as compared to the challenge-negative group (fig. 1a).

CD-sens versus Challenge Outcome

All patients ($n = 24$) were tested in CD-sens to wheat, ω-5 gliadin and timothy, and the results could be evaluated in 21 cases (table 2). All 21 patients had a positive CD-sens to wheat and there was a tendency ($p = 0.08$) for the wheat CD-sens value to discriminate between children positive or negative in wheat challenge (fig. 1b). Six of 11 children with a positive challenge also had a positive CD-sens to ω-5 gliadin and 9/10 children with a negative challenge were negative in CD-sens to ω-5 gliadin (fig. 1b). Due to possible solubility problems with ω-5 gliadin, the

results were only reported as positive or negative, and no further CD-sens calculation was made.

CD-sens for HWP was performed only in 20/24 patients due to scarcity of material and was possible to evaluate in 17 patients (table 2). Eleven of 17 patients had a positive CD-sens reaction to HWP, and 7 of these 11 were positive in wheat challenge (table 2; fig. 1b).

Combination of IgE-Ab and CD-sens in Relation to Challenge Outcome

There was a significant correlation between the levels of IgE-Ab and the CD-sens values to wheat ($r = 0.64$, $p < 0.003$; fig. 2a). If a wheat CD-sens value >150 was combined with a wheat IgE-Ab concentration >20 kU_A/l, or IgE-Ab to ω-5 gliadin >0.1 kU_A/l, 83% of the wheat challenge outcomes were predicted. If the non- and low responders were excluded, the agreement was 91%.

There was also a significant correlation between the level of IgE-Ab to HWP and the CD-sens to HWP ($r = 0.59$, $p < 0.05$; fig. 2b). All challenge-positive children, ex-

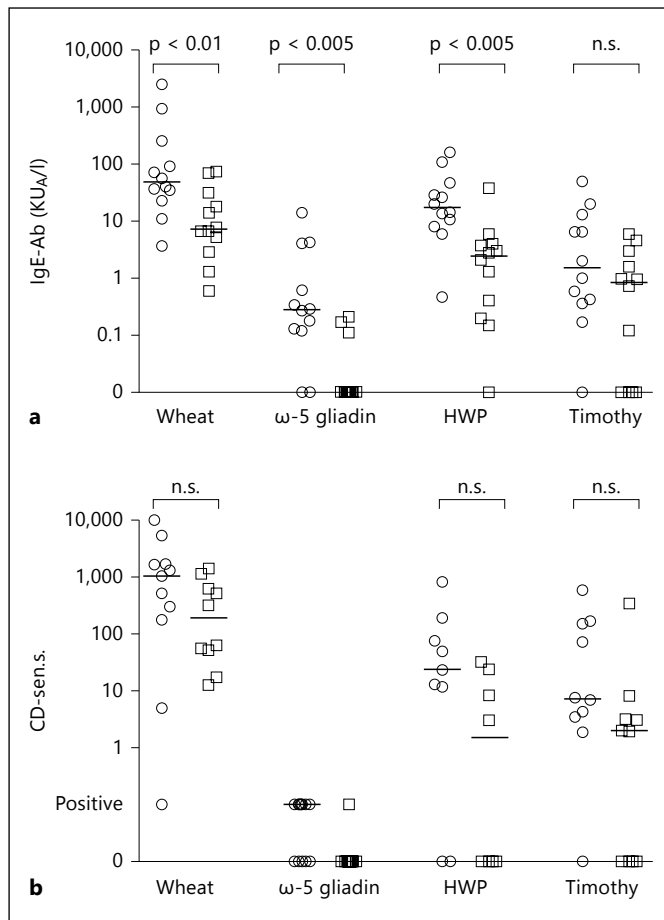


Fig. 1. IgE-Ab (a) and basophil allergen threshold sensitivity, CD-sens (b), to wheat, ω -5 gliadin, HWP and timothy grass in relation to outcome of wheat challenge. $\circ$ = Positive challenge; $\square$ = negative challenge.

cept 2 nonresponders, had a CD-sens value to HWP >10 and an IgE-Ab level to HWP >8 kU_A/l (table 2). Five children had IgE-Ab to HWP but were negative in CD-sens to HWP. Of these 5 children, 3 were also negative in the wheat challenge (fig. 2b).

IgE-Ab and CD-sens to Timothy in Relation to Timothy Symptoms

Twenty of 24 patients had IgE-Ab to timothy >0.1 kU_A/l. CD-sens to timothy was performed in all patients and was possible to evaluate in 21/24 (table 2). Five of the 21 patients were negative in CD-sens and 3 of them had low levels (<0.8 kU_A/l) of IgE-Ab to timothy. These children were between 2 and 4 years old (table 2). Children with IgE-Ab to timothy >3 kU_A/l were more likely to have allergic symptoms during the grass

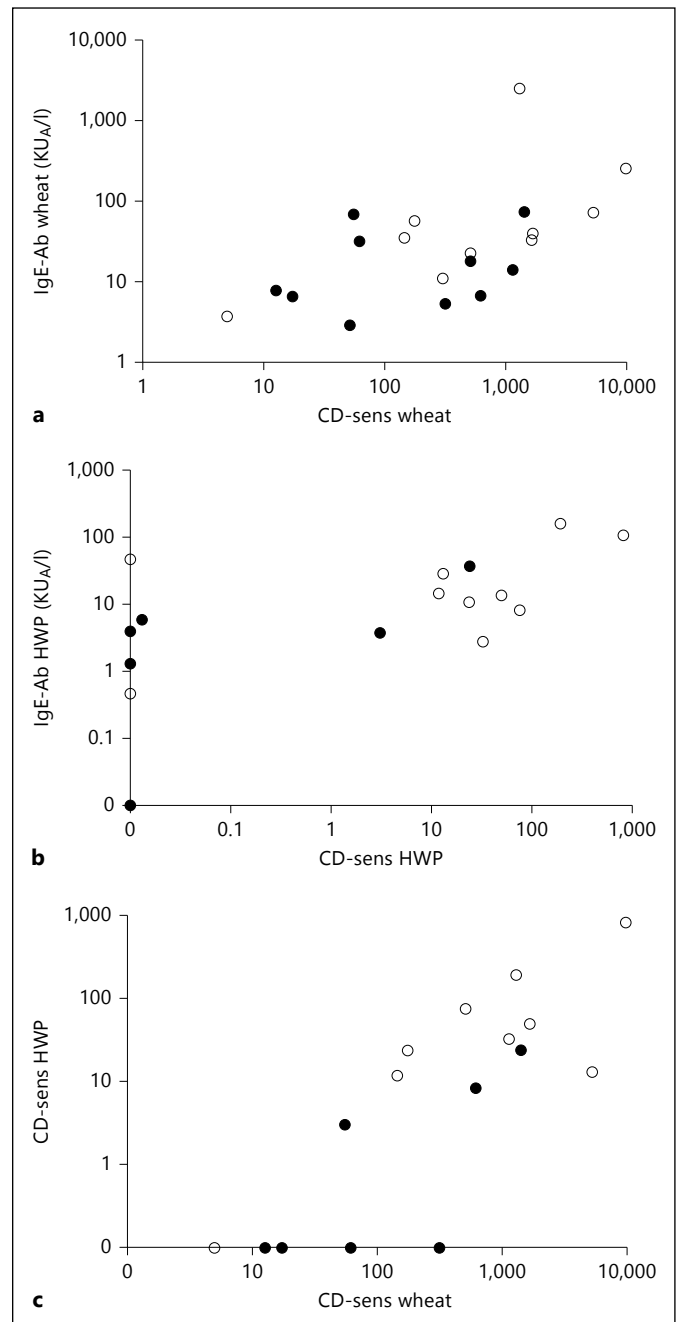


Fig. 2. IgE-Ab in relation to basophil allergen threshold sensitivity, CD-sens, for wheat (a) and HWP (b). c CD-sens to HWP in relation to CD-sens to wheat. $\circ$ = Positive challenge; $\bullet$ = negative challenge.

pollen season compared to children with levels <1 kU_A/l (fig. 3). The children reporting symptoms during the grass pollen season were 7 (6–9) years old compared to children not having symptoms who were 4 (3–7) years old.

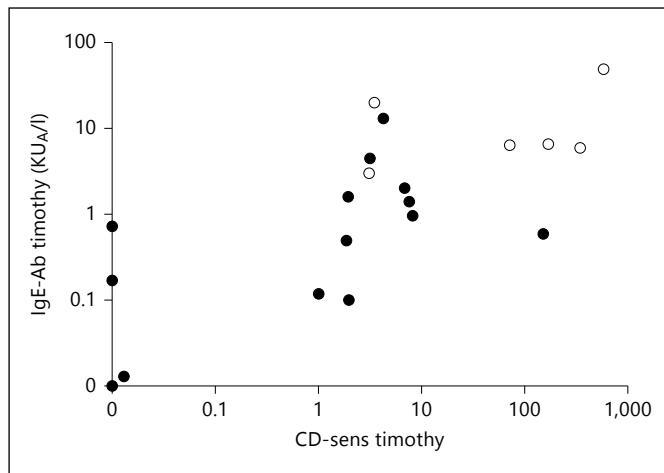


Fig. 3. IgE-Ab and basophil allergen threshold sensitivity, CD-sens, for timothy in relation to case history of grass pollen symptoms. O = Symptoms; ● = no symptoms.

Discussion

The results of this study show that a combination of markers could enhance the prediction of the wheat challenge outcome. Thus wheat CD-sens levels >150 combined with IgE-Ab levels to wheat >20 kU_A/l or ω -5 gliadin >0.1 kU_A/l predicted the challenge outcome in 83% of the patients. Another interesting finding was that the majority (96%) of the children with IgE-Ab to wheat also had IgE-Ab to HWP and that 7/10 challenge-positive children also had a positive CD-sens to HWP.

In our study of Swedish children, we found that 79% of the patients with a positive wheat challenge had IgE-Ab to ω -5 gliadin (>0.1 kU_A/l), which is in contrast to IgE-Ab to wheat, which was positive (>0.1 kU_A/l) in all 24 patients regardless of challenge outcome. This is in agreement with the findings among Finnish as well Japanese subjects [8, 11]. We cannot, however, confirm the findings of Tokuda et al. [28] that the basophil response to ω -5 gliadin predicted wheat allergy in children. They incubated peripheral blood with purified native ω -5 gliadin and recombinant ω -5 gliadin, and basophil reactivity was analyzed by flow cytometry; they found the native form of ω -5 gliadin to discriminate better than the recombinant form. We used the recombinant form but had some solubility problems. The basophils require a physiological pH in contrast to ω -5 gliadin, which should be dissolved in 1% acetic acid. Since we may have encountered solubility problems, we could not guarantee the exact amount of allergen in the solutions used and we therefore chose to

describe CD-sens to ω -5 gliadin only as positive or negative. Further research in this field is required.

The majority (96%) of children with IgE-Ab to wheat had also IgE-Ab to HWP. This is in line with the theory suggested by Leduc et al. [29] that human subjects appear to be easily sensitized to HWPs. It is conceivable that human subjects do not have sufficient tolerance to HWP, which are not natural proteins. Further research is needed to clarify if the raised HWP-specific IgE can be a result of cross-reactions of wheat-specific IgE to HWP or possibly be a double sensitization by wheat and HWP. In addition, Denery-Papini et al. [30] showed recently that this type of allergy to deaminated gluten has a separate entity, which is characterized by a homogeneous IgE-Ab response, and is a direct consequence of present technologies applied to industrial food products. We found that most children with IgE-Ab <8 kU_A/l to HWP did not react in CD-sens to HWP in comparison to those with higher IgE-Ab levels. Based on these data, we can speculate that the basophils only in the severe wheat-allergic patients react to HWP and that this hypersensitivity is not a separate entity as suggested by Chinuki et al. [16]. We suggest that the patients with a strict wheat-free diet also should take precautions to avoid HWP. In many cases, HWP is a hidden allergen due to its emulsifying function and might easily not be detected as a wheat allergen. It could thus constitute a unrecognized risk factor in the daily life of wheat-allergic patients. The HWP that we investigated is a product called Meripro 711. The results for this protein cannot be generalized to other HWP produced by other manufacturers but gives an indication of the potential danger of ingesting this kind of wheat proteins when having a wheat allergy.

The mechanism of the immune responses to the three wheat antigens is due to exposure. When and to what degree the individuals are sensitized differs and are not yet fully understood, but it is most likely due to the way of exposure, i.e. ingestion or inhalation, exposure dose, patient age, i.e. duration of exposure and genetics of the individual, i.e. atopic or nonatopic.

Since none of the tests alone provided a satisfactory prediction of the challenge outcome we combined the different markers. We found that a combination of CD-sens to wheat >150 together with IgE-Ab to wheat >20 kU_A/l or IgE-Ab to ω -5 gliadin >0.1 kU_A/l gave an 83% correct prediction of the challenge outcome and if the CD-sens non- and low responders were excluded, it further increased to 91%. This is a diagnostic improvement as Ebisawa et al. [12] showed for recombinant ω -5 gliadin a sensitivity of 72% and a specificity of 79% at

an estimated cutoff of 0.41 kU_A/l based on challenge in Japanese children.

In our study, 1 patient with a positive challenge test was a nonresponder in CD-sens, and in the group of patients negative in the challenge test 1 was a nonresponder and 1 a low responder. This varied response has been described previously [22–24]. The consequence of this is that an oral challenge has to be performed on individuals who are low responders or nonresponders in the CD-sens test [22].

This study shows that most of the children with IgE-Ab to wheat also had IgE-Ab to timothy grass and that they had a positive CD-sens to the same allergens. To our knowledge, this has not been shown before and is in contrast to findings of Jones et al. [7] that showed little in vitro cross-reactivity to grass among wheat-allergic patients. One of our most interesting findings is that 65% of the children that had IgE-Ab and a positive CD-sens to timothy had no symptoms to grass pollen. Hatzler et al. [31] have recently investigated the evolution of IgE sensitization in children affected by seasonal allergic rhinitis due to grass pollen. They found that the IgE-Ab response to grass pollen components can start years before the appearance of the first symptoms and they found increases in serum concentration before and during the disease progress. Our findings indicate that inflammatory cells like basophils can also be sensitized to IgE long before the target organ responds. Probably, a local, non-IgE-mediated, inflammation is necessary for the reaction and thus for symptoms. In our study, 3/4 of the children with low levels (<0.8 kU_A/l) of IgE-Ab to timothy had a negative CD-sens to timothy and no symptoms during the pollen season. These children were all very young, between 2 and 4 years old. Interestingly, children who did not react to the wheat challenge had lower, but detectable, CD-sens values and lower concentrations of IgE-Ab to wheat compared to reacting children, and they were also younger (median age in nonreacting children was 4 vs. 7 years in reacting children). Thus, it seems that IgE-Ab-sensitized basophils precede target organ sensitivity and thus might indicate later development of clinical symptoms of food allergy as well as future airway allergy. We are now planning to study if our observed wheat and timothy sensitization may derive from cross-reactions between these two allergens or if the patients may be sensitized independently of both allergens. If these findings can be further documented early laboratory testing may motivate a closer follow-up or even introduction of allergen-specific immune therapy in these children.

The strength of this study is that the wheat challenges and CD-sens analyses have been performed and evaluated by the same experienced personnel. However, there

are a couple of minor problems that can be discussed. The time between challenge and blood sampling should not have exceeded 6 months due to the increased possibility that the child's target organ sensitivity might have changed, i.e. developed tolerance. Three children exceeded this time interval, but they were not excluded because they all had a negative challenge, which had no consequence for the results.

Furthermore, when performing the oral challenge test, we have used ordinary wheat products which have been heated when produced. Hence, we cannot exclude that the wheat proteins have undergone changes in this process. In contrast, at the time IgE-Ab and CD-sens investigations were performed, we used the standardized tests using pure, native wheat components.

Since this is a rather small study, the results should be interpreted with caution but we believe that it raises interesting questions that need long-term follow-up.

We conclude that wheat allergy seems to be a complex disease with immunological cross-reactivity among allergens. However, a combination of CD-sens and IgE-Ab to wheat components could be useful in the diagnosis and follow-up of wheat allergy in children. Since a positive CD-sens seems to precede target organ sensitivity in young children it might open the possibility of early prevention and treatment.

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