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Incomplete digestion of codfish represents a risk factor for anaphylaxis in patients with allergy

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Abstract

Background—Fish represents one of the most important allergenic foods causing severe allergic reactions. Nevertheless, it has been shown that gastric digestion significantly reduces its allergenic capacity.

Objective—In this study, we assessed the absorption kinetics of fish proteins and investigated the clinical reactivity of patients with fish allergy to codfish digested at physiological or elevated gastric pH.

Methods—Healthy individuals were openly challenged with codfish and blood samples were evaluated by histamine release for absorbed fish allergens. Patients with allergy were recruited on the basis of previously diagnosed codfish allergy. Fish extracts were digested with gastric enzymes at pH 2.0 and 3.0 and used for histamine release, skin prick tests, and titrated double-blind placebo-controlled food challenges.

Results—Ingestion experiments in subjects without allergy revealed absorption of biologically active fish allergens only 10 minutes after ingestion with maximal serum levels after 1 to 2 hours. Incubation of fish proteins with digestive enzymes at pH 2.0 resulted in a fragmentation of the proteins leading to a reduced biological activity evidenced by a significantly smaller wheal reaction and reduced histamine release. Fish digested at pH 3.0 revealed comparable reactivity patterns as undigested extracts. Moreover, these test materials triggered reactions at 10-fold to 30-fold lower cumulated challenge doses in patients with allergy.

Conclusion—Our data indicate the paramount importance of gastric digestion for fish allergens because the quantitatively significant absorption and elicitation of symptoms seemed to take place in the intestine.

Clinical implications—Hindered digestion puts patients with fish allergy at risk to develop severe allergic reactions at minute amounts of allergens.

Keywords

Food allergy; gastric digestion; anaphylaxis; skin tests; double-blind placebo-controlled food challenge; threshold levels; codfish; protein absorption

Food allergies represent a major health concern in Western countries, as 3% to 4% of the general population is affected with a rising tendency.^{1,2} After intake of triggering foods, the patients show clinical reactivity ranging from mild local symptoms to severe systemic, even anaphylactic reactions.³⁻⁵ Food allergens were revealed to be the most frequent causes for potentially life-threatening anaphylaxis,^{6,7} which may even occur after ingestion of minimal quantities of the respective food compound.^{8,9}

These severe reactions in highly sensitized individuals represent major problems for patients and a great challenge for regulatory authorities. Hidden to many consumers, allergenic foods such as egg white, casein, fish proteins, or wheat gluten may be used as food supplements—for example, as clarifying agents in the wine fining treatment.^{10,11} Thus, a first approach for patients' safety is the accurate labeling of the content of major elicitors of food allergic reactions, which has been obligatory since the beginning of January 2005 in the European Community and since January 1, 2006, in the United States.^{12,13} Nevertheless, these labeling regulations have limitations because accidental cross-contamination with allergens through production in shared equipment or errors in labeling of products might occur.^{14,15}

Knowledge of personal allergen threshold levels is of great importance for patients with allergy for developing avoidance strategies (ie, eliminating any trace of fish-derived products versus avoiding visible fish in the diet), and population-based studies of food-allergic cohorts are important for authorities and for the food industry.¹⁶ Using titrations in double-blind placebo-controlled food challenge (DBPCFC) tests¹⁷ enables allergologists to define lowest observed adverse effect levels (LOAELs) or no observed adverse effect levels (NOAELs).¹⁸ However, the determined threshold levels show great variations within patients or food proteins.¹⁹⁻²¹ For broader implications of threshold levels, a consensus protocol has been established ensuring a uniform determination and calculation of LOAELs and NOAELs.¹⁶

We have previously shown that elevation of pH levels in simulated gastric fluid (SGF) to 2.75 and above resulted in complete abrogation of codfish digestion. Nevertheless, gastric digestion has a paramount function because it reduced the allergenic capacity of codfish proteins up to 10,000-fold.²² These findings have major effect for patients with allergy in situations of incomplete digestion, because threshold levels might be lowered when gastric digestion is impaired. Moreover, we have earlier demonstrated that biologically active peanut allergens can be absorbed via the buccal mucosa, suggesting an explanation for very acute clinical reactions occurring shortly after ingestion of offending food by patients with allergy.²³

Thus, in the current study, we aimed to combine these previous observations to a clinical investigation of the role of digestibility for absorption and allergenicity of fish allergens.

METHODS

Study subjects

Five healthy subjects (2 female, 3 male; age 24-57 years) served as controls. Exclusion criteria were diagnosed codfish allergy, atopic family history, and medication during the tests. Five patients with codfish allergy (4 female, 1 male; age 36-53 years) were recruited

for the study on the basis of their previously diagnosed IgE-mediated codfish allergy. Inclusion criteria were histories of clinical reactivity on codfish ingestion and allergologic diagnosis of codfish allergy based on specific IgE antibodies and positive skin tests. Exclusion criteria were any severe illness, medication with antiallergic or anti-inflammatory drugs during the test period, and refusal to participate. The study protocol was approved by the local ethics committee (number: KF 01-144/04), and all study subjects gave written informed consent.

Kinetics of codfish absorption determined by histamine release

Three of the healthy control subjects fasted overnight and were openly challenged with a meal of 100 g cooked codfish with water *ad libitum*. To evaluate the time kinetics of antigen absorption, blood samples were taken from each test person before the meal and 10, 30, 60, 120, and 180 minutes after the end of the meal, which lasted a maximum of 10 minutes.

Thereafter, histamine release assays from basophil leukocytes were performed.²⁴ In brief, basophils from healthy donors were isolated using Lymphoprep (Fresenius Kabi Norge AS for Axis-Shield PoC AS, Oslo, Norway). After intensive washing with piperazine-N,N'-bis(2-ethanesulfonic acid) buffer (3.02 g/L), 19.05 g/L sodium acetate, 0.49 g/L potassium acetate, 1.0 mol/L TRIS buffer, 0.088 g/L sodium chloride, and 0.224 g/L magnesium chloride, cell-bound IgE was stripped from cells with a buffer containing 20 g/L potassium chloride and 0.37 g/L sodium hydrogen phosphate at pH 3.55. After passive sensitization with the serum of an individual with codfish allergy (codfish specific IgE, 477 kU_A/L) for 1 hour at 37°C, the cell suspension was incubated with the serum samples obtained from the test subjects at 0, 10, 30, 60, 120, and 180 minutes in flat-bottomed microtiter plates for 1 hour at 37°C. Thereafter, piperazine-N,N'-bis(2-ethanesulfonic acid) was added, the plates were centrifuged for 5 minutes at 2000g and 11°C, and the supernatant was discarded. Consequently, cells were lysed by perchloric acid (7%), and 50 µL cell lysate was transferred to the wells of a glass microfiber-coated microtiter plate (Reference Laboratory, Copenhagen, Denmark), which was incubated for 1 hour at 37°C. After washing steps, the released, glass fiber-bound histamine was determined by the fluorometric o-Phthal-di-aldehyde method,²⁴ and the amount of released histamine was expressed as a percentage of the total histamine content of the cells. The release induced by cell incubation with the sera was assessed in a 6-fold determination and calculated after subtraction of background values.

Preparation of skin test and food challenge substances

Codfish skin prick test (SPT) substances (ALK 726; ALK-Abelló, Hørsholm, Denmark) and frozen codfish filets (Sigma Seafood, Esbjerg, Denmark) were purchased. The filets were used for preparation of protein extracts as previously described²⁵ for DBPCFCs, and the protein content of both preparations was determined according to the method of Bradford.²⁶ Both test substances were subjected to SGF digestion using a pharmaceutical enzyme tablet (Enzynorm forte; Pharmaselect, Vienna, Austria) following a modified protocol.²⁷ In short, the SGF contained 1 gastric tablet of 180 Fédération Internationale Pharmaceutique (FIP) units pepsin and 1.37 mval acid dissolved in 175 mL 0.9% sodium chloride. For digestion, the substances were incubated with SGF for 1 minute, 15 minutes, and 2 hours at pH 2.0. For simulating hypoacidic gastric conditions, the pH of the SGF was elevated to 3.0 with 0.1 N NaOH, and the substances were incubated for the respective periods. The digestion reaction was quenched by neutralizing the pH with 1 N NaOH and by cooling the samples on ice.

Determination of protein digestion by SDS-PAGE and histamine release assays

Protein digestion was evaluated in Coomassie brilliant blue stained SDS gels (12% separation gel, 5% stacking gel) by the method of Lammeli²⁸ in a vertical slab gel apparatus.

The histamine release procedure with the test substances was performed as described with slight modifications. Washed cells were passively sensitized with a serum pool from patients with codfish allergy. After intensive washing, cells were incubated with the allergens at adjusted concentrations in glass fiber microtiter plates (Reference Laboratory) under releasing conditions. Thereafter, cells were washed away and the released histamine was quantified by using the described glass microfiber method. Biological activity of the digested test material was calculated by comparison of the obtained values with the histamine release triggered by undigested codfish skin test or food challenge test substances, which was set 100%.

Evaluation of codfish-specific IgE levels and skin testing

Serum samples of all study subjects were screened for codfish-specific IgE in the CAP-FEIA system (Pharmacia & Upjohn, Uppsala, Sweden) according to the manufacturer's instructions, and the results were expressed in kU_A/L.

Skin prick tests were performed on the volar surface of the forearms of patients and controls with digested or undigested skin test substances.²⁹ Glycerol (Merck, Darmstadt, Germany) served as a negative and 10 mg/mL histamine dihydrochloride (ALK-Abelló) as a positive control. A titration of the extract concentration was included for undigested codfish samples (concentrated, 1:10, 1:100, 1:1000) and 1 minute digested extracts (concentrated, 1:10, 1:100). All test substances were applied in duplicates, the tests were read after 20 minutes, and the precise area of the reactions was determined.³⁰

Double-blind food challenges

In an allergy clinic equipped with resuscitation and monitoring of vital signs, food challenges with the codfish protein extracts digested at pH 2.0 or pH 3.0 were performed in 4 of the 5 patients with codfish allergy on 2 separate days according to the European Academy of Allergy and Clinical Immunology guidelines with intravenous lines inserted before challenges.¹⁷ The order of pH 2.0 and pH 3.0 was blind to the patients and the investigator. Before and after each challenge, the baseline parameter was recorded, and the skin and oral cavity were inspected for lesions. Each dose was given at 30-minute intervals. The test material was blinded by adding concentrated blackcurrant juice (Sølgård, Borup, Denmark) and Elemental diet 028 (SHS Ltd, Liverpool, United Kingdom). On the basis of SDS-PAGE and histamine release evaluations of digestion, the following dosage scheme was established: each day the first challenge contained placebo (dose 1: water with blackcurrant juice and elemental diet) followed by increasing amounts of digested proteins and decreasing intervals of digestion (Table I).

Statistical analysis

Diameters of wheal reactions were statistically compared by Mann-Whitney *U* test using the SPSS 12.0 program (SPSS Inc, Chicago, Ill), and a *P* value < .05 was considered significant.

RESULTS

Characteristics of patients with allergy and control subjects

All study patients had a history of clinical reactions on ingestion of codfish and were previously diagnosed as having fish allergy, whereas the controls had never experienced any

allergic reactions. In the allergy testing at the inclusion day, all patients revealed codfish-specific IgE (RAST class 1-6) and positive SPTs, whereas all control subjects exhibited negative results in the allergologic evaluation (Table II).

Absorption kinetics of codfish proteins in healthy volunteers

Three healthy control subjects underwent open challenges with 100 g cooked codfish after fasting overnight. To evaluate the uptake of codfish allergens and the distribution in the circulation, blood samples were drawn and sera were analyzed for codfish proteins by histamine release assays using basophils passively sensitized with the serum of a highly sensitive patient with codfish allergy. The amount of histamine released during stimulation with antigen-containing sera was calculated, and the background values were subtracted. Within 10 minutes of codfish consumption, an increase of histamine release was observed indicating pregastric codfish protein absorption. However, the amount of codfish proteins in the sera indicated by the release was highest in 1 of the control persons after 1 hour and in 2 of them 2 hours after the meal, which slowly returned to the baseline 3 hours after protein consumption (Fig 1). Two repetitions of the histamine release determination of fish allergens in the sera of challenged persons showed comparable results (data not shown).

***In vitro* evaluation of SPT samples and fish extracts**

After SGF experiments under normal and hypoacidic gastric conditions, SPT and DBPCFC material was evaluated in SDS-PAGE by Coomassie brilliant blue staining. Digestion experiments revealed the digestion capacity of the SGF with the gastric tablet to be time-dependent and pH-dependent. Codfish proteins of the SPT substances were digested to small fragments after incubation with SGF for 1 minute. These fragments were further enzymatically degraded and could not be detected after 2 hours of peptic digestion. When hypoacidic conditions were simulated by elevating the pH of the gastric fluid to 3.0, the major allergens remained stable for as long as 2 hours (Fig 2, A).

The protein extract prepared for DBPCFCs reacted similarly, as degradation of proteins to fragments was observed after 1 minute of incubation and further digestion was seen during the 2-hour incubation period. Moreover, the proteins remained intact after incubation with SGF at pH 3 (Fig 2, B).

These results were confirmed by histamine release assays calculating the biological activity of digested and undigested test materials. Undigested substances were set to 100% biological activity, and the effect of digestion under the 2 pH conditions was calculated accordingly. Interestingly, 2 hours of digestion under normal gastric conditions induced a 70% reduction of the allergic potential of the SPT material and an 83% reduction of the DBPCFC samples, whereas only a marginal reduction of the triggering capacity was observed after the 2-hour incubation with SGF, pH 3.0 (Table III).

Skin testing

Digested and undigested SPT substances were tested in patients with codfish allergy and controls. Already 1 minute of digestion at pH 2.0 significantly reduced the wheal reaction, which was further pronounced after 15-minute or 2-hour incubation. In contrast, wheal reactions of the same size as triggered by undigested codfish allergens were observed after applying SPT substances digested under hypoacidic conditions. Moreover, the size of the skin reaction was significantly increased by the pH shift of the gastric fluid from pH 2.0 to pH 3.0 when comparing each digestion time point (Fig 3). None of the control persons revealed reactivity with any of the digested or undigested codfish samples (data not shown).

DBPCFCs

Four of the 5 patients with codfish allergy underwent DBPCFC with the codfish protein extracts digested either at pH 2.0 or pH 3.0 on 2 different days. The results are given in Table IV. When testing the preparations of codfish extract digested at pH 2.0, 2 patients went all the way through the challenge protocol (cumulated 33.11 mg codfish protein), 1 patient described subjective symptoms at challenge 7 (cumulated dose 13.11 mg codfish), and a highly fish-sensitive patient described a vague, subjective reaction at dose 5 (cumulated 2.11 mg protein). When testing the codfish preparations, which were digested under hypoacidic conditions at pH 3.0, 2 patients reported subjective symptoms at dose 4 (cumulated protein concentration, 1.11 mg), 1 patient revealed an objective reaction with need for medication at a cumulated protein concentration of 2.11 mg (dose 5), and 1 patient with codfish allergy reported first symptoms at dose 6, corresponding to a cumulated dose of 3.11 mg codfish protein. Despite the very low LOAELs observed in all patients, 3 of them revealed a 10-fold to 30-fold reduction of the tolerated cumulated dose when the codfish proteins were digested in SGF under hypoacidic conditions.

DISCUSSION

We have recently shown in murine and clinical studies that medication with antiulcer drugs correlates with the development of food allergy by hindrance of gastric digestion of food allergens, indicating an important role of the stomach in the sensitization phase of food allergy.^{25,31,32} In a next step, the role of digested codfish proteins as elicitors of fish adverse reactions was assessed in an *in vitro* approach revealing an up to 10,000-fold reduced triggering capacity of the allergens after gastric degradation.²² Additionally, we have reported that absorption of peanut allergens can occur exclusively via the oral mucosa.²³

Thus, in the current study, we investigated the absorption kinetics of codfish proteins in healthy volunteers. On the basis of these preliminary data, we evaluated the allergenic potential codfish allergens digested under normal acidic and hypoacidic gastric conditions in patients with codfish allergy.

In accordance with the published data on peanut proteins,²³ we found detectable codfish protein amounts in sera of healthy individuals already 10 minutes after the meal, indicating a pregastric absorption in the preliminary ingestion experiments. Interestingly, these findings might explain the rapid reactions observed on ingestion of the offending allergen in some patients with food allergy.⁶ For some time, sublingual application of pharmaceuticals and nowadays even the sublingual therapy of allergies are state-of-the-art.^{33,34} Nevertheless, the largest amount of protein in the sera was found after 1 or 2 hours, respectively, which correlates with the average gastric transit time.³⁵ In line with studies performed at the beginning of the 20th century,^{36,37} these data indicate the absorption of codfish allergens with triggering capacity also after the gastric passage. It has been discussed previously that also linear fragments exhibited IgE binding capacity, even though the affinity might be largely reduced.³⁸ Moreover, we could show previously that gastrically digested codfish extract triggers histamine release if applied in rather high concentrations.²² Thus, it is tempting to speculate that under impaired gastric digestion, the uptake of higher amounts of allergens with an elevated triggering potential might have been observed.

Simulated gastric fluid experiments with gastric enzyme tablets confirmed the importance of low pH for the activation of pepsinogens into pepsins to initiate gastric digestion. Only under acidic conditions, a conformational change of the prosegment of pepsinogen and the disruption of the electrostatic interactions between the prosegment and the active enzyme take place, initiating the activation reaction.³⁹ In our experiments, codfish allergens lost their biological activity only at pH 2.0, whereas protein patterns and histamine releasing

capacity of the proteins were maintained even after 2 hours of digestion when incubated at hypoacidic conditions (pH 3.0). Furthermore, skin testing revealed wheal reactions of the same size as induced by undigested allergens when the test substances were incubated with SGF at elevated pH. In contrast, reactivities were significantly reduced in a time-dependent manner when proteins were subjected to physiological gastric digestion, which is in line with our previous observations.²²

To assess ultimately the allergenic capacity of the different codfish preparations, DBPCFCs were performed, which are considered to be the gold standard of food allergy diagnosis.¹⁷ For ethical reasons, the patients with allergy could not be medicated with acid-suppression drugs during food challenges for impairment of gastric digestion. Nevertheless, on the basis of the kinetic evaluations, we could assume a partial pregastric absorption of the test material without further exposure to gastric digestive enzymes. Interestingly, in our study, we observed in all patients adverse reactions to minute amounts of codfish proteins with cumulated doses ranging between 1.11 mg and 3.11 mg for the preparations digested at pH 3.0. The lowest amount of codfish eliciting clinical reactions previously observed was 6 mg,⁴⁰ indicating that already calculated NOAELs or LOAELs would not account for patients of these study, especially in conditions of impaired gastric digestion. In these situations, the use of fish proteins as nutrition supplements¹¹ might put patients with allergy at risk to encounter adverse reactions. Despite the negligible protein concentration of fining agents⁴¹ and the safety of fish gelatine,⁴² our data indicate that strict labeling regulations represent an important legislative tool⁴³ to ensure full patient safety.

This study clearly underlines the important gate-keeping function of the stomach in food allergy. We could show that protein degradation at hypoacidic conditions is a risk factor for triggering allergic reactions similar to undigested proteins at very low antigen concentrations. Patients with food allergy have to be aware of the high risk to accidentally encounter severe allergic reactions on ingestion of traces of hidden food proteins in situations of impaired gastric digestion.

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Abbreviations used

DBPCFC	Double-blind, placebo-controlled food challenge
LOAEL	Lowest observed adverse effect level
NOAEL	No observed adverse effect level
SGF	Simulated gastric fluid SPT: Skin prick test

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FIG 1. Time dependency of codfish absorption in healthy individuals. Serum samples drawn 10, 30, 60, 120, and 180 minutes after a codfish meal were analyzed for histamine release capacity with passively sensitized basophils. The quantity of circulating codfish antigen is relatively represented by the amount of released histamine. Data from 3 subjects are presented as means $\pm$ SDs.

FIG 2.

Codfish proteins are exclusively digested in SGF at low pH. Codfish SPT substance (**A**, *I*) and codfish protein extract for DBPCFC (**B**, *I*) were subjected to gastric digestion assays at acidic (pH 2.0; 2-4) and hypoacidic conditions (pH 3.0; 5-7) for 1 minute (2, 5), 15 minutes (3, 6), and 2 hours (4, 7).

FIG 3.

Gastric digestion reduces capacity of codfish allergens to trigger skin reactions. SPTs were performed with undigested substances (1-4) or samples digested at pH 2.0 (5-9) and pH 3.0 (10-14) for 1 minute (5-7 and 10-12), 15 minutes (8, 13), and 2 hours (9, 14; dark grey), diluted 1:10 (2, 6, 11), 1:100 (3, 7, 12), and 1:1000 (4; light grey). Boxes represent the size of wheal reactions of 5 patients with codfish allergy (* $P = .009$; ** $P = .016$).

TABLE I

Design of DBPCFCs *

Protein concentration	Codfish extract, 2 hours digested	Codfish extract, 15 minutes digested	Codfish extract, 1 minute digested
0.01 mg	Dose 2		
0.1 mg	Dose 3		
1 mg	Dose 4	Dose 5	Dose 6
10 mg	Dose 7	Dose 8	Dose 9

*
Dose #1 was placebo.

TABLE II

Reactivity to codfish allergens in patients (P) and controls (C)

No.	Previous evaluations				Current testing	
	Codfish specific IgE	SPT	Reported symptoms	DBPCFC	Codfish specific IgE	SPT
P1	>100 kU _A /L	Positive	OAS, Ec, A, RC, GI	Positive/0.005 g	>100 kU _A /L	Positive
P2	2.61 kU _A /L	Positive	OAS, Ec, GI	Positive/0.5 g	1.03 kU _A /L	Positive
P3	0.82 kU _A /L	Positive	OAS, AE, Er, GI	Positive/1.5 g	1.49 kU _A /L	Positive
P4	2.62 kU _A /L	Positive	OAS, A, RC, GI	ND	3.25 kU _A /L	Positive
P5	0.98 kU _A /L	Positive	OAS	ND	0.64 kU _A /L	Positive
C1	ND	ND	ND	ND	<0.35 kU _A /L	Negative
C2	ND	ND	ND	ND	<0.35 kU _A /L	Negative
C3	ND	ND	ND	ND	<0.35 kU _A /L	Negative
C4	ND	ND	ND	ND	<0.35 kU _A /L	Negative
C5	ND	ND	ND	ND	<0.35 kU _A /L	Negative

A, Asthma; AE, angioedema; Ec, eczema; Er, erythema; GI, gastrointestinal symptoms (nausea, vomiting, diarrhea, abdominal pain); ND, not determined; OAS, oral allergy syndrome; RC, rhinoconjunctivitis.

TABLE III

Biological activity of SPT and DBPCFC substances determined by histamine release tests with sera from patients with codfish allergy*

SPT substances	Biological activity	DBPCFC substances	Biological activity
Undigested	100%	Undigested	100%
Digested pH 2.0; 1 min	87.1%	Digested pH 2.0; 1 min	87.4%
Digested pH 2.0; 15 min	35.8%	Digested pH 2.0; 15 min	84.1%
Digested pH 2.0; 2 h	22.6%	Digested pH 2.0; 2 h	16.5%
Digested pH 3.0; 1 min	110.9%	Digested pH 3.0; 1 min	103.4%
Digested pH 3.0; 15 min	113.2%	Digested pH 3.0; 15 min	111.1%
Digested pH 3.0; 2 h	99.8%	Digested pH 3.0; 2 h	84.8%

* Histamine release values were measured at 12 antigen concentrations. Biological activity was determined from 12 values and expressed in percentages.

TABLE IV
Outcome of DBPCFC with codfish extracts digested at low and elevated pH in patients with codfish allergy

No.	Challenge 1 Codfish extract, digested at pH 2.0				Challenge 2 Codfish extract, digested at pH 3.0			
	Highest dose number given	Protein amount, digestion time	Cumulated dose	Symptoms	Highest dose number given	Protein amount, digestion time	Cumulated dose	Symptoms
P1	5	1 mg, 15 min	2.11 mg	Blocked nose	6	1 mg, 1 min	3.11 mg	Gastric pain
P2	9	10 mg, 1 min	33.11 mg	—	5	1 mg, 15 min	2.11 mg	Gastric pain, nausea, vomiting
P3	9	10 mg, 1 min	33.11 mg	—	4	1 mg, 2 h	1.11 mg	Pruritus
P4	7	10 mg, 2 h	13.11 mg	OAS, diarrhea	4	1 mg, 2 h	1.11 mg	OAS

OAS, Oral allergy syndrome.