

Development of a standardized low-dose double-blind placebo-controlled challenge vehicle for the EuroPrevall project

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Abstract

Background: Double-blind placebo-controlled food challenge (DBPCFC) is the gold standard for diagnosing food allergy. Standardized materials and protocols are essential for comparing DBPCFC results for multicentre studies such as EuroPrevall. This required the development and piloting of a standardized vehicle and low-dose protocol for confirming food allergy and determination of minimum eliciting doses (MEDs).

Methods: A low-dose DBPCFC protocol was developed, with eight titrated protein doses from 3 µg to 1 g. This was delivered using a simple, microbiologically stable food base incorporating allergenic food ingredients manufactured at three sites and centrally distributed to clinical centres. Allergen blinding was assessed by a professional sensory testing panel using a triangle test. Homogeneity and allergen content were confirmed by ELISA and clinical efficacy was assessed in a pilot study, using celeriac and hazelnut as exemplars.

Results: Celeriac and hazelnut ingredients were sufficiently blinded in the dessert. The dessert meals were successfully piloted with hazelnut in allergy clinics in Spain, the Netherlands and Italy and with celeriac and hazelnut in Zurich. The challenges elicited a range of subjective and objective reactions ranging in severity from mild itching of the oral mucosa to bronchospasm.

Conclusions: A standardized challenge vehicle proven to sufficiently blind processed, powdered hazelnut and celeriac ingredients and that can be reproducibly manufactured has been developed. This pilot study shows that the vehicle is promising for the confirmation of food allergy and determination of MEDs in adults and children with body weight >28.8 kg (approximately 7–11 years old).

Double-blind placebo-controlled food challenge (DBPCFC) is considered the gold standard for diagnosing food allergy (1–3) and has been standardized clinically (4). Various matrices have been employed to deliver allergenic foods (5–16), and although sensory analysis to confirm blinding has been conducted in some studies (17–19), this is not routine. Lack

of effective blinding and issues such as poor homogeneity may compromise the quality of a challenge and are especially important when collecting low-dose challenge data to characterize minimum eliciting doses (MEDs).

When selecting ingredients for blinding in a challenge vehicle, issues to consider include taste, smell, texture, appearance

and protein content, whether they are of food-grade quality and microbiological stability. Finely milled powdered materials are less visible, improving homogeneity and blinding. When such finely milled particles cannot be achieved, a texturizing agent, such as oatmeal (5) or rice (7), can be added. If low-odour and blandly coloured ingredients cannot be used, the formulation needs adapting to disguise strong flavours, odours and colours. All materials must be prepared in a food-grade environment and be microbiologically stable. Flavours and textures should also be masked to maximize the amount of allergenic food blinded in the vehicle (13).

Double-blind placebo-controlled food challenge was a cornerstone of the EuroPrevall project (20), being used for the diagnosis of food allergy and definition of MEDs in birth cohorts (21), community surveys in adults and school-age children (22) and an outpatient clinic study. To compare MEDs for different foods in the same matrix and harmonize DBPCFC protocols across centres, a common challenge vehicle capable of blinding a variety of allergenic foods was required. It also needed to be stable under ambient conditions to facilitate centralized production and distribution. Here, we report the development, production and clinical piloting of such a dessert base for DBPCFC with two important European allergenic foods, hazelnut and celeriac.

Materials and methods

Food ingredients and other reagents

Cold swelling starch (Ultratex 4) was purchased from National Starch and Chemical, Manchester, UK; cocoa powder from Cargill, Amsterdam, The Netherlands; powdered sugar from CSM Suiker Dinteloord, The Netherlands; fully refined rapeseed oil from Van der Linden Oliehandel, The Netherlands; and emulsifier (Tween 60) from Kerry Bio-science, Co. Kerry, Ireland. Hazelnut flour (30% protein) was purchased from

Nutt Ranch, Marlborough, New Zealand; and celeriac powder (11.8% protein) from Kraeuter Mix GmbH, Abtswind, Germany. All other chemicals were of analytical grade, and distilled water was used unless otherwise stated.

DBPCFC material and allergens

A chocolate dessert base formulation was developed by the Institute of Food Research, based on a system devised by Unilever R&D BV (Vlaardingen, Netherlands). For each allergen, a 'high' and 'low' allergen dessert base was produced to enable delivery of the required dose range. In brief, rapeseed oil and Tween 60 were mixed, warmed to approximately 40°C until the mixture became homogenous, and the resultant was added to dry ingredients and mixed by large domestic mixer (Kenwood Chef; Kenwood Ltd, Havant, UK) (2 min minimum speed, 5 min maximum speed). The resulting paste was dispensed in 30 g aliquots into sterile 125 ml containers (VWR International, West Sussex, UK) and packed into kits comprising 'high-allergen' (three pots), 'low-allergen' (one pot) and placebo (allergen-free; five pots) dessert bases, dosing spoons and instructions for storage and use.

Prior to use, dessert base was hydrated with still, bottled mineral water (70 ml per 30 g of dessert base), mixed until it became smooth and chilled at 2–5°C, for at least 30 min, to allow the cold swelling starch to fully hydrate. The resulting 'mousse-style' dessert was mixed again to ensure homogeneity and stored at 2–5°C for a maximum of 24 h prior to DBPCFC. The recipes for hydrated (as-eaten) placebo, high-allergen and low-allergen desserts are described in Table 1a (hazelnut), b (celeriac).

Microbiological stability

Desserts are classed as a category 1 food, according to UK guidelines for microbiological quality (23), and had to pass

Table 1 Composition of the hydrated (as-eaten) a) hazelnut challenge desserts b) celeriac challenge desserts

Ingredient	% Ingredient (by weight)		
	High-allergen	Low-allergen	Placebo
(a)			
Hazelnut flour [30% (w/w) protein]	2.04 (approximately 0.6% protein)	0.021 (approximately 0.006% protein)	0
Cold swelling starch	4.53	4.5	4.86
Cocoa	7.695	7.68	8.256
Sugar	6.255	8.319	6.714
Rapeseed oil	8.37	8.37	8.976
Tween 60	0.21	0.21	0.21
Oats	0.9	0.9	0.9
(b)			
Celeriac powder (11.8% (w/w) protein)	4.26 (approximately 0.5% protein)	0.048 (approximately 0.006% protein)	0
Cold swelling starch	4.5	5.7	5.7
Cocoa	7.68	7.68	7.68
Sugar	4.08	7.02	7.14
Rapeseed oil	8.37	8.37	8.37
Tween 60	0.21	0.21	0.21
Oats	0.9	0.9	0.9

microbiological testing prior to use. Dessert bases were analysed in triplicate at Unilever (Colworth, Bedford, UK) for total colony counts (acceptable limits: $<10^4$ CFU/g), presumptive coliform (acceptable limits: $<10^3$ CFU/g) and yeast and moulds (acceptable limits: $<10^3$ CFU/g), according to ISO 4811 and standard Unilever procedures. The dessert base water activity a_w was <0.5 and hence low enough to inhibit bacterial (a_w : ≤ 0.91) and mould (a_w : ≤ 0.8) growth, providing microbiological stability for at least 6 months at ambient temperature.

Allergen dose verification

The allergen-free status of the placebo doses and homogeneity of allergen distribution were determined by immunoassay. For each batch of desserts, samples were taken from three randomly selected pots of hydrated high- and low-allergen desserts, extracted and analysed for hazelnut using the Bio-kits Hazelnut Assay Kit (Tepnel Biosystems, North Wales, UK) according to manufacturer's instructions. Celeriac was determined by an in-house immunoassay developed using an antibody reactive to celeriac profilin (kind gift of Laurian Zuidmeer; AMC, Amsterdam, The Netherlands), using the celery spice ingredients incorporated into the dessert matrix as the standard.

Sensory testing

Sensory evaluation of the hydrated 'high-allergen' dessert was carried out by a professional tasting panel at Unilever (Colworth, UK) using a triangular testing protocol with and without nose clips (18). A triangle test is a discriminative form of sensory analysis, which aims to identify differences between two products. The test has a forced-choice procedure, whereby panellists must guess which sample is odd when an odd sample is not detected. All panellists were screened following ISO standard protocols (24–26) for ability to detect and discriminate between many stimuli and to describe differ-

ences between sensations, prior to recruitment. ISO 4120:2004 triangle test methodology (27) was employed. In brief, approximately 40 panellists were presented with three samples (combinations of placebo and high-allergen desserts); two samples were the same, and the third was different. Panellists were asked to identify the odd sample and describe why this sample was different based on visual, taste and texture observations. Water and cream crackers were used as palate cleansers. Test results were collated and analysed using standard binomial approaches and a 95% confidence level.

Double-blind placebo-controlled food challenge

Clinical centres and patient details

Table 2 summarizes the clinical histories of patients recruited for pilot challenges. For hazelnut, patients were from Clinica San Carlo Milano, Italy; Hospital Clinico San Carlos Madrid, Spain; Allergy Unit of the Department of Dermatology, University Hospital Zürich, Switzerland; and University Medical Centre Utrecht, The Netherlands. In each centre, patients with a clinical history of hazelnut allergy underwent DBPCFCs with hazelnut desserts. Celeriac desserts were piloted in Zürich in four patients with convincing histories of celeriac allergy. The study was approved by local ethical committees, and all subjects provided written informed consent before enrolment.

DBPCFC protocol

Active and placebo provocations were performed on two different days (in randomized order) with eight increasing doses (Table 3), containing 3 µg, 30 µg, 300 µg, 3 mg, 30 mg, 100 mg, 300 mg and 1 g of hazelnut or celeriac protein administered at 20-min intervals. Doses were administered by feeding the required amounts of high- or low-allergen dessert, with further dilution of low-allergen dessert in placebo dessert to deliver the two lowest doses (Table 3). Challenges ended after the dose that provoked the first objective symptoms or ingestion of all eight doses. Objective symptoms leading to discon-

Table 2 Clinical characteristics of patients and symptoms upon ingestion of hazelnut or celeriac, respectively, as reported by the patients

Patient identifier	Country	Food allergy	Gender	Age (years)	Case history
2010078	NL	Hazelnut	F	23	OAS, AE, Dph
2010099	NL	Hazelnut	F	41	OAS, Dph,
4510006	I	Hazelnut	M	46	OAS, E
4510010	I	Hazelnut	F	57	OAS, D
0710001	ES	Hazelnut	F	10	OAS
0710040	ES	Hazelnut	M	32	N, Dph, TT
0610001	CH	Hazelnut	F	32	OAS
0610033	CH	Hazelnut	F	56	OAS
0610056	CH	Celeriac	F	37	OAS
0610103	CH	Celeriac	F	59	OAS
0610204	CH	Celeriac	F	17	OAS
0610227	CH	Celeriac	M	38	OAS, AE, F, R, C, Lpru

CH, Switzerland; ES, Spain; I, Italy; NL, the Netherlands f, female; m, male, AE, angioedema; C, conjunctivitis; D, dyspnea; Dph, dysphagia; E, emesis; F, flush; Lpru, local pruritus; OAS, itching and tingling sensation on lips, oral cavity, auditory canal, throat; R, rhinitis; N, Nausea, TT, Tightness of the throat.

Table 3 a) Hazelnut dosing scheme. b) Celeriac dosing scheme

Dose no.	Dose of protein	Cumulative protein dose	Equivalent hazelnut flour mass	Equivalent* hazelnut kernel mass	Amount of 'low-allergen' ready-to-eat dessert
(a)					
1	3 µg	3 µg	10 µg	21 µg	Thoroughly mix 1 ml of the 'low-allergen' dessert with 19 ml of blank dessert. Feed 1 ml to the patient
2	30 µg	33 µg	100 µg	210 µg	Thoroughly mix 5 ml of the 'low-allergen' dessert with 5 ml of blank dessert. Feed 1 ml to the patient
3	300 µg	333 µg	1 mg	2.1mg	5 ml
4	3 mg	3 mg	10 mg	21.3 mg	50 ml
					Amount of 'high-allergen' ready-to-eat dessert
5	30 mg	33 mg	100 mg	213 mg	5 ml
6	100 mg	133 mg	333 mg	709 mg	17 ml
7	300 mg	433 mg	1 g	2.1 g	50 ml
8	1 g	1.4 g	3.3 g	7.1 g	170 ml
Dose no.	Dose of protein	Cumulative protein dose	Equivalent celeriac powder mass	Equivalent* celeriac tuber mass	Amount of 'low-allergen' ready-to-eat dessert
(b)					
1	3 µg	3 µg	25.4 µg	250 µg	Thoroughly mix 1 ml of the 'low-allergen' dessert with 19 ml of blank dessert. Feed 1 ml to the patient
2	30 µg	33 µg	254 µg	2.5 mg	Thoroughly mix 5 ml of the 'low-allergen' dessert with 5 ml of blank dessert. Feed 1 ml to the patient
3	300 µg	333 µg	2.54 mg	25 mg	5 ml
4	3 mg	3 mg	25.4 mg	250 mg	50 ml
					Amount of 'high-allergen' ready-to-eat dessert
5	30 mg	33 mg	254 mg	2.5 g	6 ml
6	100 mg	133 mg	847 mg	8.3 g	20 ml
7	300 mg	433 mg	2.5 g	25 g	60 ml
8	1 g	1.4 g	8.5 g	83 g	200 ml

*The equivalent food mass assumes a protein content of 14.1% in hazelnut kernels and 1.2% in raw celeriac tuber. These values are taken from 2002 McCance and Widdowson's The Composition of Foods integrated dataset.

tinuation were blisters of the oral mucosa, skin symptoms such as flush, urticaria and angioedema, rhinitis, conjunctivitis, blood pressure drop of at least 20 mmHg, drop in FEV1 > 12% or PEF of at least 20 %, laryngeal oedema, diarrhoea, emesis or severe persistent subjective symptoms lasting more than 45 min such as severe itching of palms, soles, head or severe gastric/abdominal pain. If all DBPCFC doses were negative, patients underwent open challenges with 20 shelled raw hazelnuts (approximately 27 g hazelnuts = 4 g protein) or 100 g raw shredded celeriac in three doses of 12.5 g (125 mg protein), 25 g (250 mg protein) and 62.5 g (625 mg protein).

Results

Matrix development

A simple, allergen-free dessert base was developed with good microbiological stability at ambient temperature. This was achieved using a cold swelling starch base hydrated with bottled water in the clinic to form a mousse-style dessert. Cocoa powder and sugar were added to impart flavour and blind

the taste of the allergenic ingredients, and the texture was improved by the inclusion of rapeseed oil and a permitted food emulsifier (Tween 60, acceptable daily intake 25 mg/kg body weight). Consumption of the eight doses would result in an intake of 720 mg of Tween 60, which was acceptable for persons with a minimum body weight of 28.8 kg corresponding to a child 7–11 years old (NDNS 1997). Because the fat content of a challenge matrix may affect the nature of an allergic reaction (28), the matrix was formulated with a relatively low fat content (<9% of hydrated dessert as eaten). Hazelnut flour and celeriac powder were incorporated into the dessert at as high a level as was compatible with blinding to ensure that the smallest portion size possible was fed to patients. This was achieved by adjusting the cocoa mass and addition of oatmeal to disguise the fibrous texture of celeriac powder and coarse hazelnut flour in the verum and placebo formulations.

Allergen analysis confirmed the absence of allergens in the placebo dessert base and showed that the 'low-allergen' base contained 0.025–0.06 mg/g of protein and the 'high-allergen' base 3.0–5.6 mg/g. The recoveries of protein were within the range expected for ELISAs (29). Sensory analysis of the pla-

cebo compared with the 'high-allergen' hazelnut dessert found 12/37 panellists correctly identified the odd sample without nose clips, reducing to 8/37 panellists with nose clips. Because approximately 33% of panellists in a triangle test will identify the correct sample by chance, no significant difference between the two samples ($P = 0.607$ and $P = 0.959$, respectively) could be detected by the panellists. Thus, hazelnut flour was successfully blinded in the high-allergen dessert. For the comparison of placebo with the 'high-allergen' celeriac dessert, 30/37 panellists correctly identified the odd sample without nose clips and 27/35 panellists with nose clips ($P \leq 0.001$). However, the panellists observed only minor changes in sensory character, reporting the 'high-allergen' dessert as having a more grainy, less smooth texture and less sweet, more bitter taste and, consequently, less chocolate flavour. No celery-type flavour or vegetable note was detected by any panellist. All comments made by the panellists are provided in Table 4.

Table 4 Summary of comments made by panellists during triangle testing of the celeriac dessert

Placebo dessert	'High-allergen' celeriac dessert
More smooth (x11)	More gritty/grainy (x6)
Less grainy/bits (x4)	Less smooth
More thick	Less creamy
More silky	More dry
More sweet (x7)	More powdery
Less nutty (x2)	More rough
Less coconut	More bitter (x3)
Less bitter	Less sweet (x3)
More chocolate	Weaker (x2)
Less solid	Less chocolate
Less chocolate	Less nutty
	Less coconut

Clinical piloting

Double-blind placebo-controlled food challenges were positive in 6/8 patients with reported hazelnut allergy and in 3/4 with reported celeriac allergy (Table 5). Three hazelnut and one celeriac allergic patients ($n = 4$) experienced subjective symptoms, such as itching and paraesthesia of the oral mucosa and lips (OAS) or nausea, respectively. In those patients, challenges were continued until the cumulative top dose of 1.433 g protein was consumed apart from one Italian patient (4510006), who experienced severe nausea after 3.333 mg of hazelnut protein and refused further intake. Objective symptoms such as blistering of the oral mucosa, rhinoconjunctivitis, angioedema, urticaria, flush and bronchospasm were observed in four patients. The most sensitive patients reacted to 30 µg hazelnut protein and 30 mg celeriac protein, respectively with subjective symptoms, the doses eliciting objective symptoms were 300 µg hazelnut protein and 100 mg celeriac protein in this pilot population. One patient with a history of celeriac allergy (0610103) did not respond to the dessert challenge, but responded to subsequent open food provocation. She developed OAS at 30 g of raw celeriac (approximately 0.45 g celeriac protein). Two patients with a history of hazelnut allergy (2010078 & 0710040) were negative under DBPCFC and to the following open food challenge or introduction into the diet. We also observed one placebo reaction from a Dutch patient (2010099) who experienced irritation of the oral mucosa similar to an OAS when ingesting doses 3 and 8 of the placebo dessert.

Discussion

To date, only a limited number of DBPCFC studies have formally confirmed either the blinding or the allergen concentration in challenge meals before use. We have developed a simple, microbiologically stable chocolate dessert vehicle and

Table 5 Symptoms under double-blind placebo-controlled food challenge with the active meal and eliciting dose

Patient Identifier	Meal	Symptoms under provocation with the active meal							
		3 µg	30 µg	300 µg	3 mg	30 mg	100 mg	300 mg	1 g
2010078	Hazelnut	–	–	–	–	–	–	–	–
2010099	Hazelnut	–	–	OAS	–	OAS	OAS	OAS	OAS
4510006	Hazelnut	–	–	OAS	OAS, N	nd	nd	nd	nd
4510010	Hazelnut	–	–	–	OAS	OAS	OAS, BI	nd	nd
0710001	Hazelnut	–	–	–	OAS, BI, Lpru	nd	nd	nd	nd
0710040	Hazelnut	–	–	–	–	–	–	–	–
0610001	Hazelnut	–	OAS	OAS	OAS	OAS	OAS	OAS	OAS
0610033	Hazelnut	–	–	OAS, BI	nd	nd	nd	nd	nd
0610056	Celeriac	–	–	–	–	–	–	OAS	OAS
0610103	Celeriac	–	–	–	–	–	–	–	–
0610204	Celeriac	–	–	–	–	OAS	–	–	OAS, AE
0610227	Celeriac	–	–	–	–	–	OAS, C, F, R, AE, BS, U, Lpru, BI Dph, Co	nd	nd

–, no symptoms; AE, angioedema; BI, blistering of the oral mucosa; BS, (objective) bronchospasm with positive lung auscultation and/or significant decrease in basal FEV1 (>12%) or PEF (>20%); C, conjunctivitis; CE, celeriac; Co, cough; Dph, dysphagia; F, flush; Ha, hazelnut; Lpru, local pruritus; N, nausea; OAS, oral allergy syndrome; R, rhinitis; U, urticaria; nd, not done.

demonstrated by professional sensory testing that hazelnut was completely, and celeriac sufficiently, blinded for use in DBPCFC for a maximum top dose of 1 g. Only subtle differences were detected between the 'high-allergen' celeriac dessert and placebo dessert by this professional taste panel. These related to differences in texture and taste, and no panellist identified the presence of celery or celery-type flavour. Because professional taste panellists are better able to differentiate between placebo and active doses than study volunteers (18) and because verum and placebo were given on separate days, the blinding, although not complete, was considered sufficient.

Clinical piloting results show that the protocol, vehicle and chosen allergens were suitable for the confirmation of hazelnut and celeriac allergy and determination of MEDs. Clinical piloting results reflect those previously reported; for example, 6/8 hazelnut DBPCFCs were positive with OAS as the dominant symptom and two patients did not respond. These are consistent with results obtained in a larger study (7) across very similar centres (Milan, Zurich and Copenhagen) reporting 67/86 (77.9%) positive DBPCFCs with OAS as the primary symptom, with eight placebo reactors and 11 nonresponding patients. These pilot challenges were undertaken only to demonstrate the efficacy and utility of the challenge matrix, and thus, the study population was too small to establish criteria such as objective or subjective lowest observed adverse effect levels (o, sLOAEL). However, sensitive patients reacted to 30 and 300 µg of hazelnut protein with subjective and objective symptoms within the range of doses reported by Wensing et al. (5), who established a sLOAEL of <1 mg and oLOAEL of 1 mg protein for hazelnut.

Three of the four celeriac DBPCFCs were positive, consistent with a previous report of 5/5 positive DBPCFCs to celeriac powder (8). This can be attributed to celeriac powder containing processing-resistant allergens to which the majority of patients react (30). The most sensitive patient reacted with subjective symptoms to 30 mg of protein (approximately 0.25 g of celeriac powder) and objective symptoms to 100 mg celeriac protein (approximately 1.18 g of celeriac powder). This is also consistent with the sLOAEL of 0.16 g although higher than the oLOAEL of 0.16 g reported by Ballmer-Weber et al. (8). Only one patient gave a negative DBPCFC but reacted on open challenge with 30g fresh celeriac (approximately 0.45 g celeriac protein).

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This pilot study showed that subjective and objective symptoms could be elicited to two foods in our vehicle in a DBPCFC. Further studies, undertaking repeat challenges comparing different matrices with the same active allergenic ingredient, or allergenic ingredients prepared with different extents of thermal processing, will be required to demonstrate the presence of matrix or processing-related effects.

In summary, a standardized food challenge vehicle proven to sufficiently blind processed, powdered hazelnut and celeriac ingredients has been developed. Its long shelf life and reproducibility combined with simple ingredients and potential to blind a range of allergens make it suitable for use in a multicentre study such as EuroPrevall. Larger-scale studies will be required to demonstrate its effectiveness in the diagnosis of food allergy and determination of MEDs.

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Author's contributions

Stella A. Cochrane, Louise J. Salt, Emma Wantling, Matthew Golding, René Crevel, E.N Clare Mills and Alan R. Mackie were involved in the design of the challenge dessert, sourcing of ingredients, dessert production and delivery; in sensorial and microbiological testing of the challenge dessert; and in drafting and revising the manuscript. Kirsten Beyer, Barbara K. Ballmer-Weber, Philipp Fritsche, Montserrat Fernandez Rivas, Isabel Reig, Andre Knulst, Thuy-My Le and Riccardo Asero were involved in the design of the challenge protocol, in performing DBPCFCs with the challenge materials and in drafting and revising the manuscript. Adrian Rogers and Jacqueline Coutts were involved in the analysis of the allergen content and homogeneity of the challenge dessert and in drafting and revising the manuscript.

Conflict of interest

No author has any conflict of interest to disclose.

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