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Profilin as a severe food allergen in allergic patients overexposed to grass pollen

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Abstract

Background: Profilins are ubiquitous proteins that act as panallergens in sensitized patients, considered to be mild or incomplete food allergens. The aim of the study was to evaluate the role of profilins as severe food allergens in allergic patients overexposed to grass who were referred for severe food reactions and were sensitized to profilins.
Methods: After a careful *in vitro* screening, 26 patients were included, classified into two groups, mild (17) and severe reactors (9), based on clinical history and subsequently provoked orally with purified profilin in a double-blind placebo-controlled food challenge setup.
Results: A significant number of patients presented severe positive food challenge test reactions at low doses of the allergen profilin. Patients prone to suffer from severe reactions had lower IgG4/IgE ratio to major grass allergens than those who did not.
Conclusion: Profilins are complete food allergens in food-allergic patient populations that are exposed to high levels of grass pollen. This type of patient constitutes an optimal model to understand the link between respiratory and food allergies. The nature of the observed reactions and the low level of allergen eliciting the reactions suggest that intake through the oral mucosa might constitute a relevant route of exposure to food allergens.

Profilins are ubiquitous proteins (1) that control the polymerization of actin and are present in all eukaryotic cells. They were first identified as pollen allergens in 1991 (2) and have been reviewed recently (3).

Plant profilins present a highly conserved structure that provokes multiple positive sIgE responses in sensitized patients in both *in vivo* and *in vitro* extract-based diagnosis. As a consequence, profilin is considered to be an important confusion factor in extract-based diagnosis (4–6).

There is controversial evidence on its role in inducing allergic symptoms, either respiratory or food related.

Profilin almost never monosensitizes allergic patients and in general is considered to be a minor respiratory allergen. Various recent publications have demonstrated its capacity to induce respiratory symptoms (bronchial, nasal, and ocular) in a specific provocation test setup (7, 8). With regard to its capacity to induce food allergy reactions, due to its reduced enzymatic and thermal stability (9), it is accepted as a mild or incomplete food allergen, which can only induce local symptoms (3), such as oral allergy syndrome (OAS). Profilin food allergy is thus considered to be a secondary effector of a primary respiratory allergic disease.

In recent molecular epidemiological studies (10), it was demonstrated that the prevalence of profilin sensitization grew in tandem with the grass pollen gradients and is not statistically associated with other pollens or total pollen counts. In a related study performed in Vienna (D. Barber, R. Jarisch and W. Hemmer, unpublished), a similar association with grass (but not birch) pollen was detected. The

Abbreviations
DBPCFC, DOUBLE-blind placebo-controlled food challenge; HRP, horseradish peroxidase; LTP, lipid transfer proteins; OAS, oral allergy syndrome; OPD, o-phenylenediamine dihydrochloride SPT, skin prick test.

explanation for this association was the higher relative content of profilin in grass pollen (5) compared with other pollens. In fact, there are particular geographical areas in Spain, as is the case of Coria (Extremadura), with high ground moisture and scarce rainfalls during pollen season, where profilin prevalence in pollen-allergic patients is higher than 60%. In these areas, the odds ratio of profilin sensitization was dependent on the sIgE level for the pollen allergen Phl p 5, reaching values higher than 17 when sIgE for Phl p 5 was higher than 50 kU/l. This region is characterized by long grass pollen seasons with pollen peaks in the range of 2000 grains and sustained pollen levels above 300 grains. Previous studies have shown that there is an association between pollen exposure levels and the prevalence of minor pollen allergens and that different clinical olive allergic phenotypes were identified based on minor allergen sensitivity. Patients sensitized to minor olive allergens have an increased incidence of lower respiratory symptoms and increased risk of side reactions during specific intervention (10, 11).

Based on the above-mentioned facts, we formulated the hypothesis that the high exposure to grass in this region could explain the role of profilin as a major respiratory allergen, leading to more severe clinical allergies in that region. As we suspected that profilin food allergies in the area were linked to more severe symptoms than those described in the literature, we planned a systematic approach to investigate this hypothesis in a profilin Double-blind placebo-controlled food challenge (DBPCFC) test design.

To ensure the relevance of the food challenge test, a ELISA for profilin was developed to assess the amount of profilin present in a piece of melon, the food most frequently involved in profilin-mediated reactions (12, 13), and which was going to be given as maximum dose for provocation challenge.

Methods

Patients

Consecutive adult patients (18–55 years) who had a clinical history of suspected food allergies sensitized to profilin, and having a negative skin prick test (SPT) to lipid transfer proteins (LTPs), were studied in the allergy ward of the Coria Hospital, Caceres, Spain, between January 2011 and September 2013. All subjects underwent a thorough interview to ascertain possible food allergies. Patients already sensitized to LTP, and those who had received any pollen extract immunotherapy in the last 5 years, were excluded. The patients were separated into two groups according to Ortolani et al. (14): 19 patients were included in the Group 1, with a history of mild reactions, OAS, 12; (Grade 1) or OAS plus digestive symptoms, 7; (Grade 2). Nine patients were assigned to Group 2 (Grade III, 4; (OAS plus systemic symptoms) and Grade IV, 5; (OAS plus uvula edema or life threatening reactions)). These latter had attended urgency consultation with severe food-induced adverse reactions. From those, four referred OAS and systemic symptoms: asthma and/or urticaria; three had OAS and uvula edema and two anaphylaxis. The food most frequently involved was melon. The clinical features of the study population sample are shown in Table 1.

All subjects provided written informed consent, and protocol approval was obtained from the ethics committee.

Skin prick tests

All study patients underwent SPTs with a battery of commercial pollen and food extracts including palm tree pollen profilin from ALK-Abello S.A., Madrid. Patients were tested directly with the offending food.

Oral allergen challenge

The oral challenges were performed from September to March, outside the grass pollen season, using single 2-ml dose vials with increasing concentrations of purified nPho d 2 (palm tree profilin) (0.037, 0.37, 3.7, 37, and 370 μg/ml) and placebo (phosphate buffer, pH = 6.5). Placebo and active were administered on different days.

The double-blind placebo-controlled food challenge was performed according to EAACI guidelines (15). The starting dose was selected as the lowest dose eliciting a positive SPT reaction, and the dose was increased every 20 min thereafter. The provocation test was considered to be positive when at least one of the following symptoms was detected. Objective symptoms: uvula edema, urticaria and/or angioedema, respiratory symptoms with FEV1 decrease, gastrointestinal symptoms (vomiting, diarrhea,), or blood pressure drop. Subjective symptoms (persisting more than 45 min): abdominal pain, oral pruritus, and dyspnea without associated FEV1 decline. Side reactions during provocation tests were treated with antihistamines, corticoids, beta-agonist medications, or adrenaline, according to the clinical guidelines.

In vitro tests

Specific IgE and IgG4 were analyzed using a microarray-based panel of 112 allergens, ImmunoCAP-ISAC (Thermo fisher scientific), in accordance with the manufacturer's instructions.

Profilin ELISA

Plates (Costar) were coated with 5 μg/ml mouse monoclonal antibody Pho d 2 1.41.1 (hybridoma clone derived from mice immunized with nPho d 2) overnight at 4°C and then saturated with 1% BSA in PBS-Tween. Serial threefold dilutions of samples and standard (affinity-purified nPho d 2) were prepared in dilution buffer and incubated in coated plates for 1 h at RT. Bound Pho d 2 was detected by incubation with a rabbit polyclonal anti-Pho d 2 antibody. The secondary antibody was an horseradish peroxidase (HRP)-conjugated goat polyclonal anti-rabbit IgG antibody (Calbiochem), and the signal was visualized with o-phenylenediamine dihydrochloride.

Table 1 Detailed information on the study population

							Skin prick test results		
		Pat.no.	Age/sex	[+]Self-reported foods	[+]Off. food	Self-reported reaction	*Pollens (POS/9)	Off. food	Pho d 2 (mm)
Group 1	Grade 1	1	27/M	M, B, P	M	OAS	8/9	+	9
		2	24/F	M, B, P	M	OAS	5/9	+	8.5
		3	33/F	M, W, O, A, Ch	M	OAS	7/9	+	9.5
		4	20/F	M, P, K, Ch, T	T, M	OAS	7/9	+	8
		5	30/F	W, O, K, Che	W	OAS	7/9	+	10
		6	38/M	M, W	M	OAS	8/9	+	7
		7	20/F	M, W, B, O, P	M	OAS	7/9	+	8
		8	29/M	B, A, Wa, Al, H, Pn	B	OAS	6/9	+	17.5
		9	46/F	M, P, Pn, Z	M	OAS	6/9	+	8
		10	18/F	W	W	OAS	3/9	+	10
		11	20/M	M, W, B, T, G	M	OAS	2/9	+	6
		12	25/F	M, B, K, P	M	OAS	4/9	+	12
	Grade 2	13	18/F	M, W, B, T, O, A, K, S, Pi, T, Au	T	OAS, DS	8/9	+	11
		14	41/F	M, W	M	OAS, DS	4/9	+	5
		15	22/M	M, W, O, A, G	O	OAS, DS	8/9	+	10
		16	24/M	M, T, O, B, S	M	OAS, DS	7/9	+	6.5
		17	38/F	M, P, Pg	M	OAS, DS	5/9	+	15
Group 2	Grade 3	18	30/F	M	M	OAS, U	5/9	+	8
		19	18/F	M, W, T, K	M	OAS, U	8/9	+	10
		20	21/F	P, K, T	P	OAS, U	7/9	+	8
		21	20/M	M, W, B T, S, Pe, O, Wa, A, Al, Ch, H	S, O	OAS, AST	5/9	+	10
	Grade 4	22	18/F	P, O, K, Po	O	OS, U, AST	5/9	+	10
		23	34/M	M, Ti, Wa, C	TI	OAS, UE	4/9	+	10
		24	19/F	M, W	W	OAS, UE	2/9	+	7
		25	32/F	M, W, B, P, O	M, W	OAS, U, AST	3/9	+	7
		26	21/F	M, W, B, K, Chi	K	OAS, UE	3/9	+	5

Reactions: OAS, oral allergy syndrome; DS, digestive symptoms; UE, uvula edema; AST, asthma. [+]Food: M, melon; W, watermelon; B, banana; T, tomato; S, strawberry; O, orange; K, kiwi; P, peach; Pe, pear; A, apple; G, grape; Pg, pomegranate; Pi, pineapple; Che, cherry; Wa, walnut; Al, almond; Ch, chestnut; Ti, tigernut; H, hazelnut; Pn, peanut; Chi, chickpea; C, carrot; Po, potato; Au, aubergine; Z, zucchini.
*Pollens: *Olea europeae*, Gras mix (*Phleum pratense, Lolium perenne, Cynodon, Poa, Secale), Salsola Kali, Parietaria judaica, Artemisa vulgaris, Plantago lanceolata, Betula Alba, Platanus acerifolia, Cupressus arizonica*

Food extract preparations

The pulp from fresh slides from different melon pieces, or pulp from tomatoes or oranges, was homogenized and extracted at a 5% ratio (w/v) in phosphate buffer pH 6.5 or in saline buffer for 90 min at 6°C. After clarifying by centrifugation, the samples were filtered through 0.22-μm Ministart filters and stored in 50% glycerol at −20°C.

Results

Skin prick tests

All patients presented sensitization to grass. As it could be expected based on their positive response to profilin, most of these were positive to multiple pollen extracts (English plantain: 96.1%; plane tree: 84.6%; olive: 76.9%; and Russian thistle: 69.2%). Most of the patients had positive SPT reactions to melon and tomato, and all the patients were positive to the offending food. Results are summarized in Table 1.

Quantification of Profilin in melon, tomato, and orange

Figure 1 shows the dose–response titration curves of profilin. Parallel dose–response curves were obtained. The mean amount of profilin per gram of melon was 2.9 μg. The measured profilin was similar in orange (2.1 μg/g), while tomato contained only 280 ng/g.

Interestingly, the profilin content in orange and tomato could only be measured when the extracts were prepared in phosphate buffer. If extraction into nonbuffered solution was carried out with a final pH lower than 4, profilin was undetectable, suggesting either low solubility or rapid degradation. On the other hand, melon pH was high, and the pH remains above 6 even under NaCl extraction condition, which likely facilitated profilin extraction.

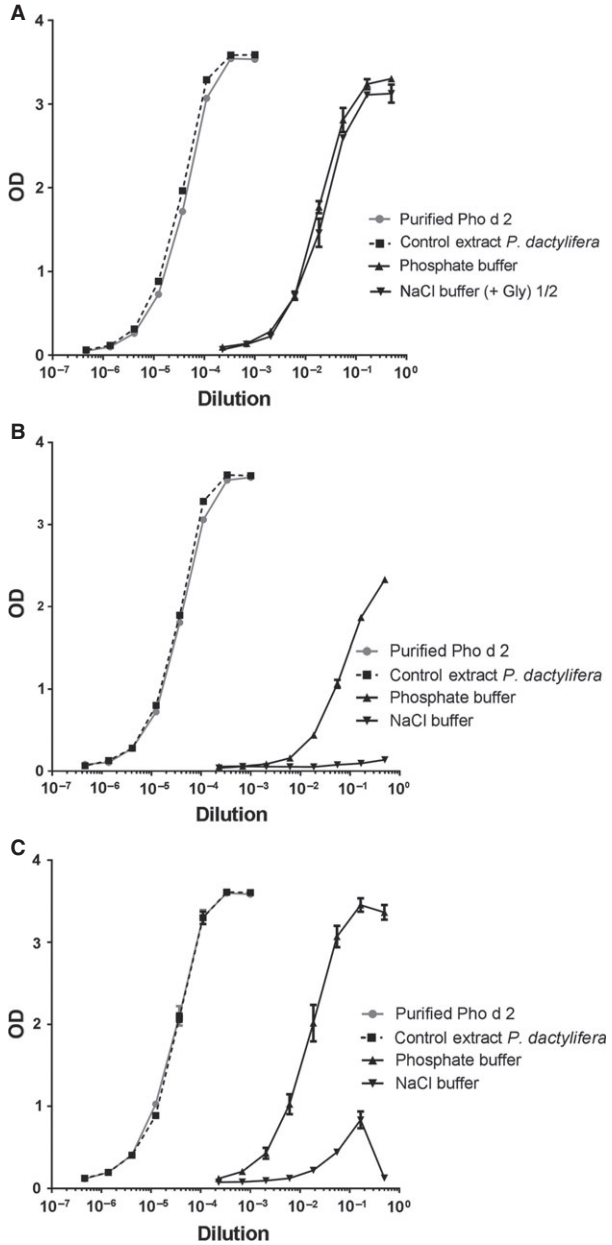

Figure 1 Dose–response curves obtained in the profilin ELISA for food extracts with different buffers, (A) Melon, (B) Tomato, (C) Orange.

Oral allergen challenges

Ten patients in Group 1 and 8 in Group 2 were provoked with profilin. Only one of the patients that reported severe reactions refused the procedure.

The results of the DBPCFC tests are summarized in Table 2. The nature of elicited reactions was in general in agreement with those self-reported by the patients.

Seven patients from Group 1 refused provocation (excluded in Table 2), while, as can be seen in the table, four had Grade III or IV reactions.

In Group 2, severe food-allergic patients, only one patient refused provocation. All provoked patients, but one, suffered objective reactions that were mostly classified as Grade III or IV. Uvular edema and FEV1 decline were the most frequently observed reactions.

As regards the dose of profilin that elicited the observed reactions, 11% of the patients reacted to doses as low as 0.074 μg, 22% to 7.2 μg, 50% to 74 μg, and 17% to 740 μg.

In the Table 2 is also shown the rescue medication. In five patients, it was necessary to use adrenaline, including patient 24 that reacted to 0.074 μg of profilin.

In vitro tests: sIgE and sIgG4

All of the patients were positive to at least one grass allergen (Fig. 2) in addition to profilin. Olive sensitization as determined by Ole e 1 positivity was frequent (40%). Other sensitization rates were lower than 25%. All patients but one were sensitized to Phl p 1 and profilin, and almost 70% reacted to four additional grass allergens (Phl p 5, 2, 4, and 6). The prevalence of Phl p 11 was lower than 25%.

Table 3 shows the serological statistical differences between both groups. There was a statistical significant difference ($P < 0.05$) in the sIgG4/sIgE ratio to Phl p 2, Phl p 5, Phl p 6, but not to profilin. Patients with severe reactions had lower IgG4/IgE ratios to the above-mentioned grass allergens. Group 1 patients showed higher sIgE levels to Phl p 1 that were compensated by an increased sIgG4 level. As a consequence, sIgG4/sIgE ratio to Phl p 1 was not statistically different between both groups.

Discussion

In the present study, we wanted to assess the role of high grass pollen exposure in food allergies mediated by profilin sensitivity. Profilin-mediated food allergies are relatively common in Spain (16); however, there was no evidence to suggest that they could be involved in severe food allergy genesis, a role that from an epidemiological point of view is played mostly by LTPs in the studied territory (17).

First, we had to establish the adequate profilin levels to be assessed in a provocation test study design. We developed specific quantification methods and could verify that normal profilin intake is in the range of 0.3–1 mg. Thus, we established a target maximum dose of 0.74 mg for the provocation study.

Second, we had to be sure that profilin was the only relevant food allergen in the studied sample. We screened the patients with profilin and used an SPT to test for an LTP reaction. Finally, selected patients were evaluated with a full allergen panel *in vitro*, and those that proved to be negative to all tested food allergens except profilin were selected for the provocation test.

Provocations tests were performed in a DBPCFC setup with pure profilin. These results unequivocally proved that profilin can induce severe food-allergic reactions in these patients. In all the cases, the dose able to elicit systemic

Table 2 Results by patient on double-blind placebo-controlled food challenge with pure profilin (Pho d 2)

		Oral provocation outcome			
Inclusion criteria		Patient no.	Profilin dose μg	Reaction	Rescue medication
Group 1	Grade 1	1	74	OAS, DS, LE	AH
		2	0.074	OAS, FEV, DS	BD
		3	74	OAS, AE, LE	AH, BD
		4	74	OAS	NO
		6	740	OAS	NO
		7	7.4	OAS	AH
		8	74	OAS	AH
		9	740	OAS, FEV, LE, UE, CO	AH, CO, EP, BD
	Grade 2	13	7.4	OAS, FEV, AE	AH, BD
		17	7.4	OAS, FEV, AE, LE, NR	AH
Group 2	Grade 3	18	74	OAS, DS, AE	AH, CO
		20	74	OAS, FEV, UE	AH, BD, CO, EP
		21	740	OAS, UE	AH, CO, EP
	Grade 4	22	74	OAS, DS	NO
		23	74	OAS, DS, LE, UE	AH, CO, EP
		24	0.074	OAS, LE, UE	AH, CO, EP
		25	74	OAS, FEV	AH, BD
		26	7.4	OAS, FEV, AE	AH, BD

Reactions: OAS, oral allergy syndrome; DS, digestive symptoms; LE, lip edema; FEV, FEV1 decline by more than 15%; AE, aphthous stomatitis; CO, conjunctivitis; UE, uvula edema; NR, neck rush. Rescue medication: AH, antihistamines; BD, bronchodilators; CO, corticoids; EP, epinephrine.

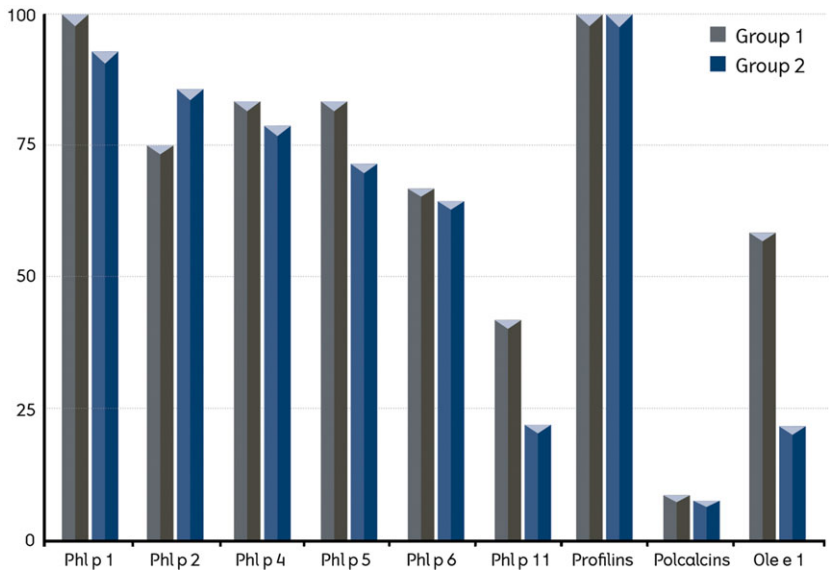

Figure 2 sIgE prevalence to the relevant allergens in base to sIgE (CAP-ISAC). Only prevalence above 25% is included. Group 1, mild reactors. Group 2, severe reactors.

reactions was lower than the planned maximum dose. On average, a dose as low as 7.4 μg was the threshold for systemic reactions. The low clinical relevance of profilin has been explained as due to the low stability of the protein and easy enzymatic degradation. Interestingly, melon extract showed a higher pH that most of the other vegetable extracts that favor stability and accessibility of the allergen in the oral cavity. Moreover, melon extract showed a very low total protein content, which would facilitate the access of profilin to effectors cells in the mucosa by decreasing matrix effect. Consequently, it is not strange that melon is the food most frequently associated with profilin reactions.

Table 3 Serological statistical differences between mild (Group 1) and severe (Group 2) profilin allergic patients

n	Group 1 (I + II) 17	Group 2 (III + IV) 9	*P*
IgE (ISU)			
Phl p 1	49	31	0.029
IgG4 (ISU-G4)			
Phl p 1	1.49	0.31	0.001
Phl p 2	1.96	0.37	0.015
Phl p 5	2.23	0.45	0.021
Phl p 6	0.31	0.17	0.019
IgG4/IgE			
Phl p 2	0.22	0.046	0.035
Phl p 5	0.082	0.021	0.007
Phl p 6	0.077	0.002	0.007

Recently, a link between allergic inflammation and epithelial barrier impairment has been demonstrated in rhinitis and asthma (18–22) and could be a plausible explanation for facilitated allergen uptake in patients with a more severe respiratory allergy phenotype.

There are other potential causes that could explain the enhanced clinical reactivity as increased sIgE complexity and levels, decreased sIgG4, or both. Mast cell sensitivity increases along with a rise in sIgE and relative sIgE to total IgE ratio (23), and thus, higher mast cell sensitivity to profilin on these patients might also be a feasible explanation.

Interestingly, patients prone to suffer severe reactions had lower sIgG4/sIgE ratios to Phl p 2 and Phl p 5, but not to profilin, suggesting that a more severe grass pollen-allergic phenotype is on the basis of severe profilin-mediated allergy and that profilin causes the reactions as is the only grass allergen that is present in foods.

Sublingual immunotherapy is a form of allergen-specific immunotherapy that is seeing increasing use. Severe reactions are infrequent, and it is generally not possible to analyze the underlying causes for such reactions due to the difficulty in finding adequate numbers of suitable patients. We have identified a group of patients who suffer similar reactions frequently.

Profilin is not only a diagnosis confounding factor and a minor respiratory allergen, but is also a potentially severe food allergen and a marker for severe grass allergies. To date, no immunotherapy clinical trials have been performed in these patients, so we do not know whether a grass allergy intervention will have a positive effect on associated food allergies. The association between profilin sensitivity and grass allergies should be taken into account when evaluating relevant allergen sensitivity. Consequently, a high profilin prevalence in a particular area could predict a higher risk of severe SIT-related adverse reactions.

The study was performed during two consecutive grass pollen seasons, the first being intense and the second rather mild. Interestingly, patients suffering from severe profilin-related allergic reactions that were very common during the first year were uncommon during the second, suggesting a natural variability of responses mediated by seasonal exposure and that high inhaled exposure to causal grass allergens promotes severe profilin food-mediated reactions.

Allergy is a complex disease model, where interactions between populations and allergens lead to multiple allergy phenotypes. This fact should be always considered when extrapolating clinical experience from one particular region to another. From a daily clinical perspective, regions with high grass pollen counts, apparent pollen poly-sensitization, and frequent food-allergic reactions mediated by melon or watermelon might identify similar clusters of allergic patients and that, in those areas, might be necessary to adapt clinical practice accordingly.

Author contributions

DB conceived the study, analyzed data, and wrote the article. MIA designed the clinical study, included patients, and supervised all ethical procedures; BR and MJL performed the clinical part of the study. LJ and PB performed all *in vitro* determinations, developed provocation kits and ELISA-based method for profilin quantization.

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Conflicts of interest

Lucia Jimeno, Fernando de la Torre and Patrice Boissy are employed by ALK-Abello.

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