

Age-related sensitization profiles for hazelnut (*Corylus avellana*) in a birch-endemic region

K. J. De Knop¹, M. M. Verweij¹, M. Grimmelikhuijsen¹, E. Philipse¹, M. M. Hagendorens^{1,2}, C. H. Bridts¹, L. S. De Clerck¹, W. J. Stevens¹ & D. G. Ebo¹

¹Faculty of Medicine, Department of Immunology – Allergology – Rheumatology, University of Antwerp, Antwerp, Belgium; ²Department of Paediatrics, University of Antwerp Hospital, Antwerp, Belgium

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basophil activation; birch; Cor a 1.04; Cor a 8; Cor a 9; component resolved diagnosis; hazelnut; IgE; microarray

Correspondence

Prof. Dr. W. Stevens, Department of Immunology, University of Antwerp, Campus Drie Eiken T4, Universiteitsplein 1, 2610 Antwerpen, Belgium.
Tel.: ++ 32 (0) 3 2652595
Fax: ++ 32 (0) 3 2652655
E-mail: immuno@ua.ac.be

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Abstract

Background: Symptoms of hazelnut allergy seem related to geographic and possibly age variations in allergen recognition.

Objective: To investigate sensitization profiles of hazelnut allergy in different age groups in a birch-endemic region using component resolved diagnosis (CRD) by microarray.

Methods: Sixty-five patients with hazelnut allergy, 27 healthy control individuals tolerant to hazelnut, and 34 birch pollen allergic but hazelnut tolerant individuals were included. All blood samples were analyzed using ISAC microarray.

Results: Twenty-nine patients with hazelnut allergy suffered from a systemic reaction (17 preschool children with a median age of 2 years, six school children, and six adults), whereas 36 patients reported an oral allergy syndrome (OAS; three preschool and nine school children and 24 adults). In the hazelnut allergic preschool children with systemic reactions, 65% were sensitized to Cor a 9, 12% to Cor a 8, 18% to Cor a 1.04, 6% to Cor a 1.0101, and 29% to Bet v 1. Of the school-aged systemic reactors, 50% were sensitized to Cor a 9, 17% to Cor a 8, 50% to Cor a 1.04 and Cor a 1.0101, and 67% to Bet v 1. In adults with hazelnut allergy, 3.3% were sensitized to Cor a 9, 6.7% to Cor a 8, 90% to Cor a 1.04 and Bet v 1, and 87% to Cor a 1.0101. In regard to systemic reactors in this group, 17% were sensitized to Cor a 9, 33% to Cor a 8 and Cor a 1.0101, and 50% to Cor a 1.04 and Bet v 1. In the patients with OAS, irrespective the age group, all were sensitized to Bet v 1 and over 97% to Cor a 1.04 and Cor a 1.0101. No sensitization to Cor a 9 or Cor a 8 was found in patients with only an OAS. Of the patients with birch pollen allergy, tolerant to hazelnut, none were sensitized to Cor a 9 or Cor a 8, 56% to Cor a 1.0101, 82% to Cor a 1.04, and 92% to Bet v 1. In healthy controls, no sensitization to components of hazelnut, hazel pollen or birch pollen was demonstrable.

Conclusion: Hazelnut allergy in a birch-endemic region exhibits age-related sensitization profiles with distinct clinical outcomes that can be identified using CRD. The majority of hazelnut allergic preschool and school children in a birch-endemic region show systemic reactions on consumption of processed hazelnut, mostly being sensitized to the hazelnut legumin-like allergen Cor a 9 but unrelated to birch pollen allergy. In contrast, adults generally suffer from an OAS apparently as a result of cross-reactivity between Cor a 1.04 from hazelnut and Bet v 1 from birch pollen.

Abbreviations

Bet v, *Betula verrucosa*; Cor a, a, *Corylus avellana*; CRD, component resolved diagnosis; OAS, oral allergy syndrome; sIgE, specific IgE.

Hazelnut is an important cause of food allergy in Europe and the United States of America in children as well as in adults (1, 2). Authors have alluded to geographic and possibly also age-related variation regarding the severity of symptoms, depending on which allergen component(s) the patient is

sensitized to. Actually, it has been demonstrated that birch pollen-related hazelnut allergy is predominantly observed in adults from birch-endemic regions and is generally characterized by a mild oral allergy syndrome (OAS) because of extensive cross-reactivity between the labile Cor a 1.04 and Bet v 1 (3–7), the major allergen from birch (*Betula verrucosa*) pollen (8). In contrast, the more severe, systemic reactions are generally not associated with an underlying pollen allergy and are frequently related to sensitization to the hazelnut lipid transfer protein Cor a 8 or the 11S legumin-like seed storage protein Cor a 9. Sensitization to Cor a 8 was first described in Spanish adults (4, 9, 10) and more recently also in children in the Netherlands, a non-Mediterranean, birch-endemic region (11). Beyer et al. (12) have suggested that the 11S legumin-like seed storage protein Cor a 9 might be involved in severe hazelnut allergy in children, and recently, Hansen et al. (6) found sensitization to Cor a 9 in four of seven Spanish patients with severe hazelnut allergy. However, as acknowledged by the authors from this European epidemiologic survey, additional studies are mandatory to further elucidate the putative role of Cor a 8, Cor a 9, and other components in hazelnut allergy, particularly in young children without pollen allergy (6).

The distinct specific IgE (sIgE) sensitization patterns can be analyzed using purified natural or recombinant allergen molecules (13, 14). Recent advances in component resolved allergy diagnosis (CRD) using microarray technology create the opportunity to perform multiple sIgE quantifications simultaneously with a minimal use of serum, thus providing individual sensitization and risk profiles in a single run [for review: Ref. (15)].

In this study, we sought to investigate sensitization profiles in hazelnut allergy in different age groups starting with young children at preschool age in a birch-endemic region, using CRD by microarray.

Materials and methods

Subjects

Sixty-five consecutive patients with immediate allergic symptoms upon consumption of raw and/or processed hazelnut were enrolled in the outpatient allergy and pediatric allergy clinic of the University Hospital of Antwerp and stratified into six groups according to the severity of the clinical reaction and age. All patients and control individuals were assessed through a standardized questionnaire regarding symptoms upon overt ingestion of hazelnut or hazelnut-containing foods. The diagnosis of systemic hazelnut allergy was based upon an evocative history of rhinoconjunctivitis, asthma, gastrointestinal symptoms, angioedema, hypotension and/or shock on ingestion of raw hazelnut, and in children predominantly processed hazelnut in chocolate, chocolate spread, or pastries. Owing to the severity of the systemic reactions after consumption of processed hazelnut, double-blind placebo-controlled food challenges were avoided because of the potential risk of serious reactions (6, 12, 16). The diagnosis of an OAS to hazelnut relied upon a compelling history of repetitive pruritus and/or angioedema of the

lips, tongue, throat, and/or palate on consumption of hazelnut. In OAS, provocations were considered unnecessary as clinical history was highly reliable and symptoms were easily recognized and described by the patient (17, 18). As it is known that the evaluation of a diagnostic test cannot be considered complete when it has failed to include the study of confounders that could influence the outcome, we included 34 patients with a birch pollen allergy but tolerant to raw hazelnut. This is to assess whether the microarray could reveal a difference in sensitization patterns between patients with genuine hazelnut allergy and individuals with mere sensitization to hazelnut (19). Diagnosis of birch pollen allergy was based upon a history of rhinitis, conjunctivitis, and/or asthma during the birch pollen season and documented by a positive sIgE and skin prick test for birch. Twenty-seven healthy control individuals without a history of inhalation or food allergy were also studied. Sera of nine Spanish hazelnut allergic adults with systemic reactions and positive sIgE to Cor a 8 using ImmunoCAP were separately studied to test the analytical sensitivity of the microarray.

Approval for this study was obtained from the local ethics committee. Patients or their representative were asked to approve an informed consent in accordance with the Declaration of Helsinki.

Total and specific IgE

Singleplexed assay

Quantification of total IgE and sIgE to birch, rBet v 1, Cor a 1.04-supplemented hazelnut extract, rCor a 1, and rCor a 8 was performed according to the manufacturer's instructions (FEIA ImmunoCAP, Phadia, Uppsala, Sweden). Values of allergen-specific IgE ≥ 0.35 kUa/l were regarded positive.

Multiplexed (CRD microarray)

We employed an allergen microarray immunoassay (ImmunoCAP ISAC containing 103 components, Phadia, Uppsala, Sweden) as recently described elsewhere (19) (see Table 1). Briefly, microarray reaction sites were incubated with 20 μ l of undiluted patients' serum for 2 h to capture allergen-specific IgE antibodies by their corresponding allergen. Subsequently, the microarray slides were rinsed and washed to remove unbound sIgE. After drying, complexes of allergen-bound sIgE were stained with a secondary, fluorescence-labeled anti-human IgE for 1 h at room temperature protected from light. After a second rinsing and washing procedure, the obtained fluorescence signals were scanned using a laser scanner (LuxScan 10K; CapitalBio, Beijing, China). Analysis of the corresponding digitized microarray images was performed using ImmunoCAP ISAC software, and image information was transformed into numerical data according to a reference serum of known IgE content. Results were expressed as ISAC Standardized Units (ISU/l), and the threshold was set at 0. Allergen components denoted by 'r' are recombinant proteins and those denoted by 'n' are natural purified proteins. We focused on sensitization to Cor a 1.04, Cor a 8, and Cor a 9 of hazelnut, Bet v 1 of birch pollen, and Cor a 1.0101 of hazel pollen. In retrospect, we

Table 1 IgE reactivity to all 103 purified and recombinant components tested in the microarray in healthy controls (controls), patients with a systemic reaction (Hazelnut+ SR+) on consumption of hazelnut, patients with an oral allergy syndrome (Hazelnut+ OAS+) on consumption of hazelnut, and patients allergic for birch pollen but tolerant to hazelnut (Birch pollen+/Hazelnut-)

	Controls (n = 27)		Hazelnut+ SR+ (n = 29)		Hazelnut+ OAS+ (n = 36)		Birch pollen+/Hazelnut - (n = 34)	
	N	Median (range)	N	Median (range)	N	Median (range)	N	Median (range)
<i>Plants</i>								
Primarily genuine markers								
nAct d 1	—	—	4	0.91 (0.54–5.21)	2	1.29 (0.58–1.99)	2	4.81 (0.52–9.09)
nAct d 2	—	—	3	0.91 (0.86–7.52)	4	3.70 (1.46–29.9)	2	1.85 (0.78–2.92)
nAct d 5	—	—	2	0.91 (0.38–1.43)	—	—	—	—
rBer e 1	—	—	2	0.32 (0.22–0.42)	—	—	—	—
rAna o 2	—	—	—	—	—	—	—	—
nCor a 9	—	—	15	1.13 (0.25–3.83)	—	—	—	—
nSes i 1	—	—	7	8.78 (1.11–28.69)	—	—	—	—
nAra h 1	—	—	6	1.37 (0.71–6.34)	2	3.94 (3.29–4.58)	4	1.56 (0.2–15.5)
nAra h 2	—	—	8	4.54 (2.04–21.7)	3	10.70 (3.33–21.8)	5	7.29 (1.52–16.2)
nAra h 3	—	—	6	0.80 (0.24–3.82)	2	3.48 (2.83–4.13)	4	1.03 (0.42–7.4)
nGly m β-conglycinin	—	—	5	0.43 (0.21–0.62)	—	—	—	—
nGly m 6	—	—	6	0.61 (0.29–0.88)	2	0.58 (0.31–0.85)	1	0.43 (0.43–0.43)
nTri a Gliadin	—	—	3	2.40 (0.13–4.11)	—	—	—	—
nTri a aA_T1	—	—	—	—	—	—	—	—
rTri a 19.0101	—	—	—	—	—	—	1	0.60 (0.6–0.6)
nTri a 18	—	—	—	—	1	0.83 (0.83–0.83)	—	—
nCyn d 1	2	0.75 (0.36–1.14)	8	3.31 (0.49–17.43)	23	11.00 (0.36–35.59)	21	6.55 (0.4–58.56)
rPhl p 1	3	0.44 (0.32–2.24)	11	6.09 (0.97–64.4)	27	22.04 (0.46–69.18)	27	11.90 (0.29–63.55)
rPhl p 2	—	—	3	11.42 (9.36–33.25)	18	6.57 (0.53–40.42)	13	4.58 (0.21–64.65)
rPhl p 4	2	0.25 (0.25–0.25)	7	0.50 (0.2–12.17)	24	3.05 (2.28–46.69)	23	2.02 (0.12–58.75)
rPhl p 5	1	0.31 (0.31–0.31)	7	10.58 (0.45–48.26)	20	13.34 (0.92–70.56)	16	11.40 (0.16–65.03)
rPhl p 6	—	—	4	0.98 (0.17–5.38)	17	3.45 (0.34–26.49)	11	4.54 (0.29–58.1)
rPhl p 11	—	—	1	9.60 (9.6–9.6)	7	4.08 (0.56–34.19)	7	5.72 (0.24–32.4)
nOle e 1	—	—	3	2.69 (0.72–8.21)	7	2.99 (0.45–8.64)	8	1.71 (0.2–11.57)
rPla a 1	—	—	1	0.15 (0.15–0.15)	—	—	—	—
nPla a 2	—	—	2	1.42 (0.11–2.73)	7	0.58 (0.38–2.75)	4	0.59 (0.25–3.64)
nCry j 1	—	—	1	0.57 (0.57–0.57)	6	0.92 (0.25–45.53)	2	14.88 (0.99–28.76)
nCup a 1	—	—	1	0.58 (0.58–0.58)	6	0.48 (0.26–10.1)	2	2.97 (0.98–4.95)
nAmb a 1	—	—	—	—	—	—	—	—
nArt v 1	—	—	1	1.12 (1.12–1.12)	—	—	2	24.43 (2.17–46.69)
nSal k 1	1	4.70 (4.7–4.7)	—	—	—	—	—	—
rHev b 1	—	—	2	0.23 (0.16–0.29)	1	0.20 (0.2–0.2)	—	—
rHev b 3	—	—	—	—	1	0.27 (0.27–0.27)	—	—
rHev b 5	1	0.37 (0.37–0.37)	—	—	—	0.30 (0.3–0.3)	2	1.02 (0.28–1.76)
rHev b 6	—	—	2	0.37 (0.16–0.58)	—	—	2	4.94 (1.06–8.81)
Cross-reactive markers								
PR 10/Bet v 1 homologues								
rBet v 1	—	—	12	8.24 (0.51–55.99)	36	39.35 (4.05–70.11)	31	9.12 (0.56–64.91)
rAln g 1	—	—	6	17.65 (3.08–45.97)	35	17.31 (0.61–64.71)	25	4.59 (0.19–39.49)
rCor a 10101	—	—	6	7.86 (2.79–49.88)	35	6.36 (0.28–40.49)	20	5.62 (0.47–44.09)
rCor a 10401	—	—	9	7.49 (0.21–41.85)	35	7.08 (0.56–54.43)	28	3.79 (0.19–47.92)
rMal d 1	—	—	8	4.13 (0.61–40.97)	35	7.63 (0.32–54.53)	22	3.36 (0.16–11.57)
rPru p 1	—	—	8	7.65 (0.72–31.6)	34	7.21 (0.28–56.38)	22	4.23 (0.13–58.44)
rGly m 4	—	—	5	1.42 (0.38–17.69)	21	3.80 (0.32–16.8)	16	2.29 (0.44–14.25)
rAra h 8	—	—	6	3.45 (1.28–20.27)	28	2.10 (0.5–18.7)	17	2.17 (0.23–18.16)
rApi g 1	—	—	2	0.67 (0.45–0.88)	5	0.47 (0.32–1.12)	7	1.77 (0.41–3.5)
rDau c 1	—	—	1	0.62 (0.62–0.62)	3	0.49 (0.39–1.7)	2	1.50 (0.62–2.37)
nAct d 8	—	—	5	6.96 (1.44–17.9)	8	0.70 (0.41–12.98)	6	1.61 (0.44–4.59)
Lipid transfer proteins								
nPru p 3	—	—	5	2.02 (0.44–23.87)	1	1.46 (1.46–1.46)	1	0.33 (0.33–0.33)

Table 1 (Continued)

	Controls (n = 27)		Hazelnut+ SR+ (n = 29)		Hazelnut+ OAS+ (n = 36)		Birch pollen+/Hazelnut – (n = 34)	
	N	Median (range)	N	Median (range)	N	Median (range)	N	Median (range)
rCor a 8	–	–	5	1.01 (0.5–1.43)	–	–	–	–
nArt v 3	–	–	2	2.55 (0.46–4.64)	–	–	2	3.81 (0.9–6.71)
rPar j 2	–	–	–	–	–	–	–	–
Profilins								
rBet v 2	–	–	1	4.89 (4.89–4.89)	7	2.47 (1.18–13.68)	8	4.10 (0.8–32.76)
nOle e 2	–	–	1	4.73 (4.73–4.73)	7	2.54 (1–19.99)	8	3.80 (0.55–32.77)
rHev b 8	–	–	1	5.94 (5.94–5.94)	7	2.82 (1.02–13.27)	8	4.47 (0.99–32.97)
rMer a 1	–	–	1	5.87 (5.87–5.87)	7	3.34 (1.69–28.34)	8	4.24 (1.06–41.29)
rPhl p 12	–	–	1	3.83 (3.83–3.83)	7	1.46 (0.76–16.7)	8	2.97 (0.62–24.06)
Calcium-binding 2-EF-hand proteins								
rBet v 4	–	–	–	–	1	13.65 (13.65–13.65)	2	11.16 (6.54–15.77)
rPhl p 7	–	–	–	–	1	17.74 (17.74–17.74)	2	11.38 (10.55–12.21)
CCD marker								
nAna c 2	–	–	–	–	4	1.20 (0.34–9.36)	–	–
Non-plants								
Primarily genuine markers								
nGal d 1	–	–	10	0.70 (0.28–9.94)	–	–	–	–
nGal d 2	–	–	5	1.33 (0.9–2.45)	1	0.62 (0.62–0.62)	–	–
nGal d 3	–	–	3	0.81 (0.26–1.81)	2	4.01 (1.25–6.76)	–	–
nBos d 4	1	0.34 (0.34–0.34)	5	0.41 (0.2–0.68)	–	–	–	–
nBos d 5	–	–	2	1.10 (0.64–1.56)	–	–	–	–
nBos d 8	1	0.55 (0.55–0.55)	4	0.44 (0.16–1.13)	1	0.77 (0.77–0.77)	2	0.37 (0.13–0.6)
nBos d lactoferrin	–	–	6	1.07 (0.3–11.44)	–	–	–	–
rBla g 1 (*)	4	0.55 (0.2–0.84)	–	–	1	0.23 (0.23–0.23)	–	–
rBla g 2	1	0.69 (0.69–0.69)	–	–	–	–	–	–
rBla g 4	1	0.58 (0.58–0.58)	4	0.71 (0.55–0.73)	6	0.61 (0.39–0.73)	6	0.59 (0.29–0.78)
rBla g 5	–	–	–	–	–	–	–	–
rFeld 1	–	–	16	8.30 (0.5–60.1)	17	10.15 (0.25–40.53)	15	8.46 (1.17–60.8)
rFeld 4	–	–	7	2.35 (0.43–49.14)	5	0.40 (0.23–5.61)	6	1.65 (0.23–8.29)
rCan f 1	1	0.37 (0.37–0.37)	8	6.11 (4.23–28.9)	6	4.89 (0.94–10.42)	7	38.79 (0.37–51.36)
rCan f 2	–	–	4	13.39 (1.23–40.46)	1	1.11 (1.11–1.11)	4	7.20 (0.64–32.16)
nMus m 1	–	–	2	14.13 (5.42–22.83)	2	8.35 (4.49–12.2)	2	22.24 (1.01–43.47)
rAlt a 1	–	–	6	8.19 (0.66–59.13)	7	3.34 (0.46–56.55)	4	4.94 (0.34–29.46)
rAlt a 6	1	0.23 (0.23–0.23)	1	3.15 (3.15–3.15)	3	1.14 (0.46–8.03)	1	0.42 (0.42–0.42)
rAsp f 1	1	0.43 (0.43–0.43)	1	0.21 (0.21–0.21)	3	0.40 (0.36–0.46)	3	0.47 (0.3–1.47)
rAsp f 2	–	–	1	0.12 (0.12–0.12)	–	–	–	–
rAsp f 3	–	–	1	1.49 (1.49–1.49)	1	6.42 (6.42–6.42)	1	1.01 (1.01–1.01)
rAsp f 4	–	–	–	–	–	–	–	–
rAsp f 6	1	0.84 (0.84–0.84)	1	0.67 (0.67–0.67)	2	1.50 (1.04–1.95)	–	–
iCla h 8	–	–	–	–	3	0.61 (0.34–1.58)	2	0.55 (0.32–0.78)
rAni s 1	–	–	4	0.79 (0.65–2.11)	–	–	–	–
nApi m 1	–	–	4	1.98 (0.29–2.71)	14	1.69 (0.65–13.26)	1	1.36 (1.36–1.36)
nApi m 4 (*)	5	0.47 (0.44–1.13)	–	–	2	0.51 (0.38–0.64)	3	0.28 (0.19–0.28)
Cross-reactive markers								
Cysteine protease								
nDer p 1	2	0.54 (0.42–0.65)	11	25.15 (0.42–59.63)	17	17.82 (0.29–58.04)	14	16.77 (0.35–57.37)
nDer f 1	1	0.25 (0.25–0.25)	10	21.49 (4.18–58.9)	15	19.11 (0.34–49.73)	14	6.24 (0.23–48.09)
NPC2 family								
rDer f 2	1	0.47 (0.47–0.47)	11	32.63 (0.34–63.49)	15	43.76 (0.63–72.14)	13	12.61 (0.2–64.02)
nDer p 2	1	1.13 (1.13–1.13)	10	34.73 (0.34–61.87)	15	41.63 (0.76–71.34)	13	13.32 (0.19–64.74)
rEur m 2	–	–	9	5.82 (0.7–14.01)	14	8.67 (0.32–19.07)	11	1.25 (0.19–13.06)
Parvalbumin								
rCyp c 1	–	–	3	13.73 (7.77–30.68)	4	0.74 (0.18–0.94)	1	0.67 (0.67–0.67)
rGad c 1	1	0.36 (0.36–0.36)	3	9.76 (0.47–17.66)	–	–	–	–

Table 1 (Continued)

	Controls (n = 27)		Hazelnut+ SR+ (n = 29)		Hazelnut+ OAS+ (n = 36)		Birch pollen+/Hazelnut – (n = 34)	
	N	Median (range)	N	Median (range)	N	Median (range)	N	Median (range)
Tropomyosin								
rPen a 1	1	0.37 (0.37–0.37)	2	0.28 (0.16–0.39)	2	5.11 (0.34–9.88)	–	–
nPen i 1	1	0.32 (0.32–0.32)	–	–	1	9.77 (9.77–9.77)	1	0.24 (0.24–0.24)
nPen m 1	2	0.41 (0.39–0.42)	1	0.42 (0.42–0.42)	1	11.80 (11.8–11.8)	1	0.34 (0.34–0.34)
rDer p 10	–	–	1	0.73 (0.73–0.73)	3	0.36 (0.12–10.22)	1	0.10 (0.1–0.1)
nBla g 7	–	–	–	–	1	11.50 (11.5–11.5)	1	0.35 (0.35–0.35)
rAni s 3	–	–	1	0.90 (0.9–0.9)	1	9.17 (9.17–9.17)	–	–
Serum albumin								
nBos d 6	–	–	11	0.53 (0.35–7.13)	1	0.25 (0.25–0.25)	2	0.16 (0.13–0.18)
nFel d 2	–	–	3	0.48 (0.44–0.8)	1	0.43 (0.43–0.43)	–	–
nCan f 3	–	–	1	0.52 (0.52–0.52)	–	–	–	–
nEqu c 3 (*)	9	0.97 (0.38–3.77)	5	0.95 (0.37–1.64)	2	0.29 (0.26–0.31)	7	0.41 (0.23–0.54)
nGal d 5	–	–	2	0.44 (0.19–0.68)	2	5.79 (0.26–11.32)	–	–

also studied the other 11S legumin-like seed storage proteins spotted on the microarray to elucidate on possible routes of sensitization.

Basophil activation test

The principles of the basophil activation test (BAT) are detailed elsewhere (20). Briefly, pre-warmed blood samples were stimulated with four different concentrations (0.001,

0.01, 0.1, and 1 µg/ml) of the same purified Cor a 9 and recombinant Cor a 8 (kindly provided by Phadia AB, Uppsala, Sweden) as spotted onto the microarray, recombinant Cor a 1.04 (Biomay AG, Vienna, Austria) and a positive control (anti-human IgE, 10 µg/ml, BD Biosciences, Erembodegem, Belgium), or HBSS buffer (Gibco, InVitrogen, Ghent, Belgium) to measure spontaneous CD63 expression (negative control). To estimate activated basophils, cells were triple stained with CD123, HLA-DR, and CD63 (BD

Table 2 Characteristics of the children and adults included in the hazelnut component resolved diagnosis microarray study, divided into three different age (0–6 years, 7–18 years and >18 years) and four clinical groups: hazelnut allergic patients with systemic reaction (HZN+SR+), hazelnut allergic patients with an oral allergy syndrome (HZN+OAS+), healthy control individuals (HC), and patients allergic to birch pollen but tolerant to hazelnut (HZN–BP+)

	N	Age (year)	Tot IgE	IgE HZN	IgE BP
		Median (range)	Median (range)	Median (range)	Median (range)
HZN+SR+					
Preschool	17	2 (0–5)*	423 (20–2007)*	25 (0.93–>100)*	0.17 (0.05–>100)*
School	6	10 (8–16)	1414 (86–5045)*	37.45 (1.72–>100)*	9.29 (0.05–>100)*
Adults	6	27.5 (24–32)	333 (64–1747)*	9.2 (0.05–37)*	2.72 (0.05–26.70)*
HZN+OAS+					
Preschool	3	4 (2–6)	347 (25–4751)	61 (10–97.70)*	85.1 (13.40–>100)*
School	9	11 (7–16)	439 (43–2168)*	49 (1.49–>100)*	60.7 (3.44–>100)*
Adults	24	34 (19–63)	197 (9–679)*	11.75 (0.03–>100)*	28.15 (2.30–>100)*
HC					
Preschool	5	5 (3–6)	16.1 (16–278)	<0.10 (all <0.10)	<0.10 (all <0.10)
School	13	9 (8–13)	154 (6–436)	<0.10 (<0.10–0.14)	<0.10 (all <0.10)
Adults	9	35 (21–56)	32 (2–73)	<0.10 (all <0.10)	<0.10 (all <0.10)
HZN–BP+					
Preschool	10	4 (2–6)	196 (16–1294)*	12.27 (0.39–>100)*	35.2 (0.79–>100)*
School	12	12 (7–16)	373 (88–1758)*	8.31 (0.34–86.70)*	17.05 (1.31–>100)*
Adults	12	28 (18–53)	135 (45–2408)*	3.8 (0.14–56)*	10.36 (0.50–>100)*

*p < 0.01 when compared to age-matched controls (HC).

**p < 0.01 HZN+ vs. HZN–BP+.

Biosciences). Flow cytometric analysis of basophil activation was performed. Side scatter, CD123⁺, and HLA-DR⁺ were used to gate out the basophils. Subsequently, within this gate, the percentage of activated basophils, i.e., co-expressing CD63, was measured. Results were expressed as percentages of CD63⁺ basophils after subtraction of the negative control.

Statistical analysis

Results were expressed as median (range). Non-parametric tests were used where appropriate. IBM SPSS 17 (IBM, Chicago, IL, USA) software was used for data analysis. Chi-squared analysis was performed to compare frequencies. A *p* value of <0.05 was regarded as statistically significant.

Results

Demographics and clinics

Table 1 displays the results of all 103 purified and recombinant components spotted on the microarray. Demographics and results (median and range) of total IgE and singleplexed sIgE to hazelnut and birch pollen of patients and control

individuals are summarized in Table 2. Twenty-nine patients in our cohort suffered from a systemic reaction [17 preschool children (median age of 2 years), six school children, and six adults] and 36 patients reported an oral allergy syndrome (three preschool children, nine school children, and 24 adults).

Sensitization to hazelnut components

The percentages of sensitization to the different purified and recombinant hazelnut components (rCor a 1.04, rCor a 8, and nCor a 9) and rBet v 1 from birch pollen are displayed in Fig. 1.

The major observation is that in our birch-endemic region, 65% of preschool children and 50% of the school children with systemic reactions on ingestion of processed hazelnut (chocolate or chocolate spread) are sensitized to Cor a 9. In hazelnut allergic adults with systemic reactions, sensitization to Cor a 9 was demonstrable in 17%. The clinical data and sensitization profiles of the Cor a 9 sensitized patients are displayed in Table 3. All our patients sensitized to Cor a 9 reported systemic reactions on ingestion of hazelnut in its

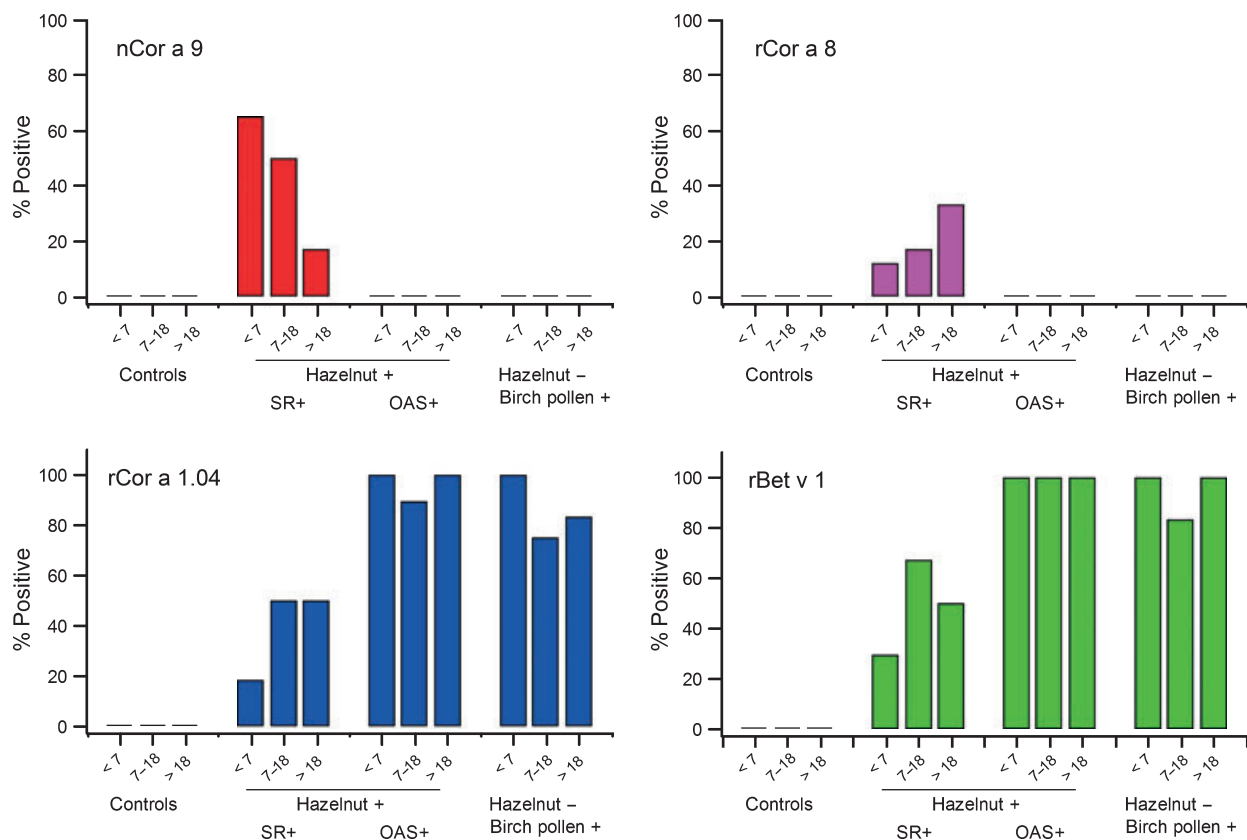


Figure 1 The percentages of sensitization to the three different hazelnut components (nCor a 9, rCor a 8, and rCor a 1.04) and rBet v 1 from birch pollen spotted on the microarray, in healthy control individuals, patients with systemic reaction on consumption of hazelnut (Hazelnut+, SR+), hazelnut allergic patients with merely an

oral allergy syndrome (Hazelnut+, OAS+), and patients tolerant to hazelnut but allergic to birch pollen (Hazelnut-, Birch pollen+). These groups were divided into three different age categories. For exact numbers, see Table 2.

Table 3 Characteristics of patients allergic to hazelnut and sensitized to Cor a 9. The clinical symptoms on consumption of hazelnut (HZN), soy, and peanut and the sensitization profile to birch pollen (BP), (recombinant) Bet v 1, Cor a 1.04-spiked hazelnut (HZN*), Cor a 1.04 and Cor a 8 using ImmunoCAP, and Cor a 1.01, Cor a 1.04, Cor a 8, and Cor a 9 using ISAC microarray

				Symptoms			IgE ImmunoCAP (kUa/L)					ISAC (ISU/L)			
	Age						rBet		Cor a	Cor a		Cor a	Cor a	Cor a	Cor a
Patient	(years)	Sex	Dermatitis	HZN	Soy	Peanut	BP	v 1	HZN*	1.04	8	1.01	1.04	8	9
1	1	M	+	C	NOE	A, C	<0.1	ND	10	<0.1	<0.1	0	0	0	0.25
2	7	M	+	A, C	NS	NOE	0.57	0.26	3.92	ND	<0.1	0	0	0	0.30
3	10	M	–	GI	NS	NS	18	ND	66.5	ND	ND	3.82	7.49	1.01	3.02
4	3	M	+	R	NS	NOE	5.9	3.3	46.6	ND	<0.1	0	1.91	0	1.13
5	1	M	+	C	NS	NOE	<0.1	ND	25	ND	<0.1	0	0	0	0.50
6	2	F	+	C	NS	NOE	<0.35	ND	3.68	ND	ND	0	0	0	0.61
7	13	M	+	A	NOE	A	>100	ND	>100	ND	ND	49.88	33.1	0	1.30
8	2	M	+	A	NS	NOE	0.16	<0.35	>100	<0.1	2.12	0	0	1.01	0.86
9	1	M	+	A, R	NS	NOE	<0.35	<0.35	70.7	ND	<0.35	0	0	0.50	1.73
10	3	F	+	A, C	NS	NOE	<0.1	ND	44.3	<0.1	<0.1	0	0	0	0.56
11	2	M	+	GI	NS	NOE	0.42	ND	37	<0.1	0.22	0	0	0	2.55
12	2	F	+	A, C, R	NOE	NOE	4.73	ND	24.1	ND	<0.1	0	0.21	0	3.83
13	1	M	+	GI	NS	NOE	>100	85.2	72.1	55.8	<0.1	0	0	0	0.43
14	3	F	+	C	NS	NOE	<0.35	<0.35	3.04	<0.1	<0.35	0	0	0	1.15
15	27	M	+	C, R	NOE	C, R	1.15	ND	30	ND	0.48	0	0	0	2.15

M, Male; F, Female; +, atopic dermatitis in past or present; –, no history of atopic dermatitis; A, angioedema; C, cutaneous symptoms; GI, gastrointestinal symptoms; R, respiratory symptoms; NS, no symptoms on consumption; NOE, no overt exposure.

processed form in spreads or chocolate. In our series, no cases of Cor a 9-related asymptomatic sensitization or mere OAS were observed in older children and adults.

In contrast to Cor a 9, monosensitization to Cor a 1.04 was generally associated with an OAS in the context of cross-reactivity with birch pollen. However, 14% (4/29) of the systemic reactors were solely sensitized to Cor a 1.04 and Cor a 1.0101 and not to Cor a 8 or Cor a 9. In one patient with an OAS, no sensitization to Cor a 1.04 was seen, although this patient was sensitized to Cor a 1.0101. Similarly, another patient was sensitized to Cor a 1.04 but not to Cor a 1.0101. Alternatively, an immune response to Cor 1.04 was also observed in 82% of birch allergic individuals tolerant to hazelnut, indicating that patients with birch-related genuine hazelnut allergy and birch pollen allergic individuals without hazelnut allergy demonstrate a comparable sensitization pattern for this component.

Of our 29 hazelnut allergic patients with systemic reactions, only 5 (17%) were sensitized to Cor a 8 of whom two were preschool age, one school age and two adults. To assess whether this unexpected low prevalence of Cor a 8 sensitization (11) could result from the impossibility of IgE-binding to recombinant Cor a 8 (expressed in *Pichia pastoris*) on the microarray (21), sera of nine Spanish adults with a positive IgE result for rCor a 8 in the ImmunoCAP were separately analyzed using the microarray. Of these nine patients, seven demonstrated IgE seroreactivity to rCor a 8 on the microarray. No sensitization to Cor a 8 was found in patients with merely an OAS on consumption of hazelnut and in birch pollen allergic patients without hazelnut allergy. In healthy control individuals, no IgE reactivity to Cor a 1.04, Cor a 8, or Cor a 9 was demonstrable.

Of note is that of the 20 patients with hazelnut allergy unrelated to birch pollen, eight showed no IgE reactivity to any of the three hazelnut components, Cor a 1.0101 of hazelnut pollen, or profilins spotted onto the microarray. Only one of these patients was sensitized to Ara h 2 of peanut and another patient to Ber e 1 of brazil nut (*Bertholletia excelsa*). No sensitization to peanut or other nuts was demonstrated on the microarray in the other six patients.

Sensitization to birch and hazelnut pollen spotted on the microarray

Of our 65 patients with hazelnut allergy, 45 (69%) demonstrated a birch pollen allergy. As expected, birch pollen allergy was significantly less prevalent in young hazelnut allergic preschool children (30%), when compared to the older hazelnut allergic patients (87%) ($p < 0.001$). In all patients, sIgE to Cor a 1.04 was highly related to sIgE to Bet v 1 ($r = 0.922$, $p < 0.001$) and to sIgE to Cor a 1.0101 ($r = 0.891$, $p < 0.001$). In these patients, sIgE to Bet v 1 was also highly related to sIgE to Cor a 1.0101 ($r = 0.913$, $p < 0.001$) (Fig. 2).

In healthy control individuals, no sensitization to Bet v 1 or its homologues Cor a 1.0101 in hazelnut pollen and Cor a 1.04 in hazelnut was demonstrated.

Sensitization to the other 11S legumin-like seed storage proteins spotted on the microarray

As shown in Table 1, eight of 65 patients with hazelnut allergy were sensitized to Ara h 3 from peanut (*Arachis hypogaea*), of whom six were Cor a 9 positive. The same holds true

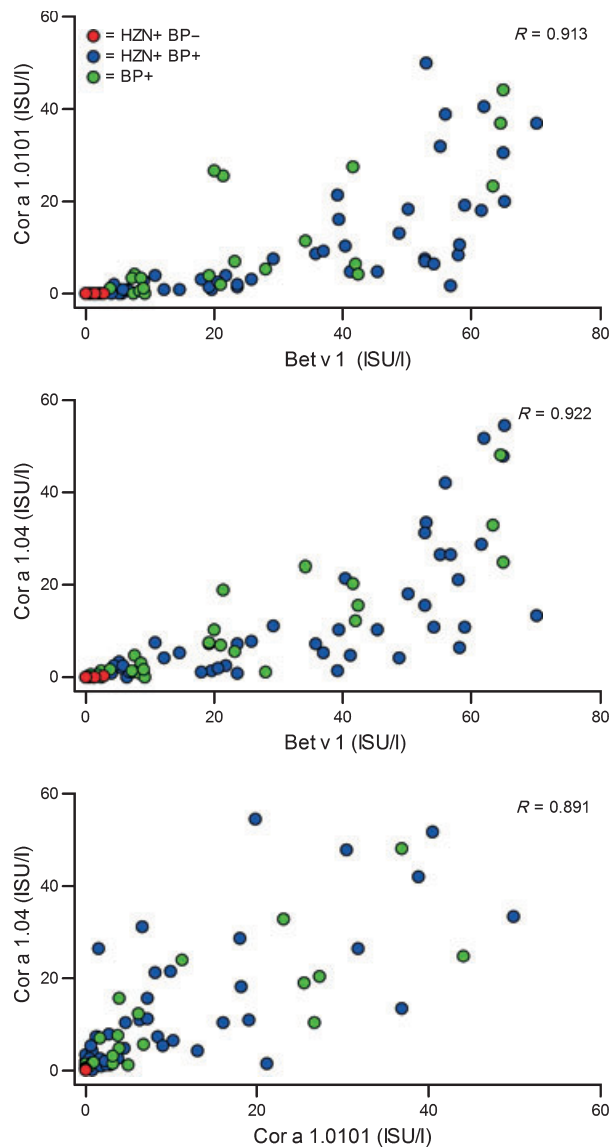


Figure 2 Correlation between Cor a 1.04, Cor a 1.0101, and Bet v 1 in the different patient groups.

for Gly m 6 from soy (*Glycin max*). Of the hazelnut tolerant patients with a birch pollen allergy, four were sensitized to Ara h 3 and one to Gly m 6. None of the included individuals reacted to Ana o 2 from cashew (*Anacardium occidentale*). No sensitization to any of the 11S legumin-like seed storage proteins was found in the healthy control individuals.

BAT with recombinant Cor a 1.04 and Cor a 8 and purified natural Cor a 9

From Fig. 3, it emerges that hazelnut allergic children sensitized to Cor a 9 demonstrate a dose-dependent up-regulation of CD63 upon stimulation of their basophils with Cor a 9 purified from hazelnut. In contrast, in five hazelnut allergic

patients without sensitization to Cor a 9 but sensitized to Cor a 1.04, in one patient sensitized to Cor a 8, and in three hazelnut tolerant healthy controls, the expression of CD63 by basophils remained absent after stimulation with Cor a 9. Adults with an OAS to hazelnut demonstrated a dose-dependent up-regulation of CD63 upon stimulation of their basophils with rCor a 1.04.

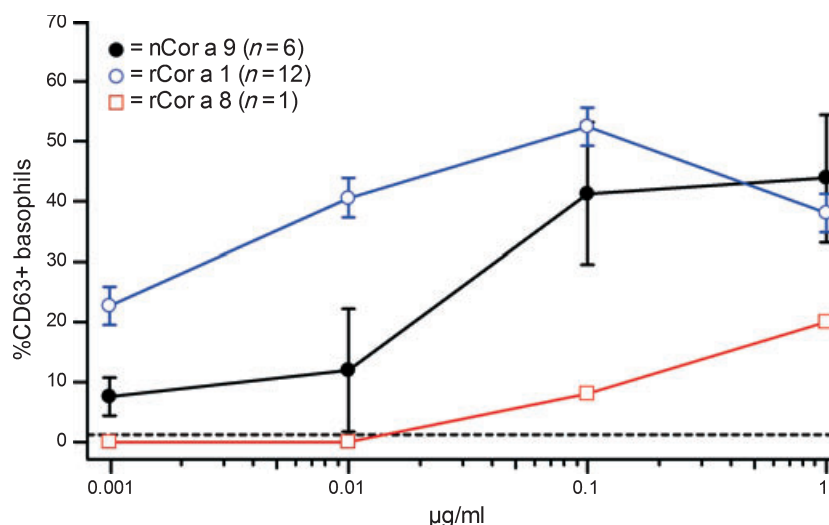
Discussion

The most relevant and original observation of our study is that hazelnut allergy in a birch-endemic region exhibits distinct age-related sensitization profiles with different clinical outcomes. Although in a birch-endemic region hazelnut allergy is most frequently associated with an OAS secondary to cross-reactivity to birch pollen, we demonstrate that young preschool children (median age of 2 years) generally demonstrate a more severe hazelnut allergy with systemic reactions, without being allergic to birch pollen. Using a recently developed CRD microarray technique, it seems that almost two-thirds of these preschool hazelnut allergic children are sensitized to Cor a 9, an 11S legumin-like seed storage protein. In a lesser extent, these findings also apply to our school-aged children, where half of the children with systemic reactions to hazelnut are sensitized to Cor a 9. Although, in the absence of objective symptoms, we might have failed to identify (very) young children with an OAS to Cor a 9, these results indicate that sensitization to this component is generally associated with systemic reactions and are in accordance with the observations made by Beyer et al. (12) who also reported severe reactions in Cor a 9-sensitized children after ingestion of hazelnut.

In contrast, adults in a birch-endemic region, as expected, are mostly sensitized to Cor a 1.04, the Bet v 1 homologue of hazelnut, and report an OAS on consumption of hazelnut. Only a minority of the adults reported systemic reactions, being sensitized to Cor a 1.04, Cor a 9, or Cor a 8. The observation that Cor a 9 sensitization was hardly found in adult hazelnut allergic patients (1/30) probably results from a diluting effect because of an increase in hazelnut allergy secondary to birch pollen allergy. However, to differentiate with possible outgrow, longitudinal follow-up of the hazelnut allergic (pre)school children is planned.

At this moment, it is not clear through which route(s) these children become sensitized to Cor a 9 of hazelnut. Although this study was not designed to clarify the potential sensitization routes, it is the authors impression that sensitization to Cor a 9 generally precedes birch pollen allergy and/or sensitization to Bet v 1. This is explained by the fact that sensitization to pollen takes several years and the prevalence of sensitization to pollen, including birch pollen, increases significantly in children during preschool age (11, 22). It is noteworthy that all of the Cor a 9-sensitized children had a history of physician-diagnosed atopic dermatitis, an established risk factor for food allergy (23). However, atopic dermatitis was also observed in the majority of hazelnut allergic children not sensitized to Cor a 9. Moreover, by applying the same microarray technique, we recently demonstrated that 12

Figure 3 Percentages of CD63-positive basophils after stimulation with four different concentrations of Cor a 9 in six patients with hazelnut allergy sensitized to Cor a 9 (●), five hazelnut allergic patients without sensitization to Cor a 9 but sensitized to Cor a 1 and/or Cor a 8, and three healthy controls (dashed line); after stimulation with Cor a 8 in one hazelnut allergic patient sensitized to Cor a 8 (□) and after stimulation with Cor a 1 in twelve patients sensitized to Cor a 1 (○). Results are expressed as the percentage CD63-positive basophils (means $\pm$ SEM).



of 20 infants with atopic dermatitis under the age of 12 months, sensitized to hazelnut, display sIgE reactivity to Cor a 9, independently of birch pollen allergy and before known overt consumption of nut-containing foods (including hazelnut; 24).

Only a few of our hazelnut allergic children were sensitized to Cor a 8 and all presented with severe reactions. This clearly contrasts to earlier observations where sensitization to Cor a 8 was found in a small series of eight Dutch children (4–12 years) with systemic reactions during a double-blind placebo-controlled challenge with hazelnut and even in about one-third of the hazelnut-sensitized pediatric population that lives in the same birch-endemic region as our children (11). Although the exact reason(s) for this discrepancy remain(s) elusive, we suppose that differences in study population might be a plausible explanation. First, we had the opportunity to systematically investigate 20 young preschool children (median age of 2 years). Secondly, most of our preschool children did not demonstrate sensitization or allergy to peanut or other tree nut varieties probably mirroring different nutritional habits compared to the Dutch population (25). It is of note, however, that the majority of the hazelnut allergic children in the series of Flinterman et al. (11) also demonstrated IgE reactivity to several other hazelnut components possibly including Cor a 9. Alternatively, as already addressed by others (6), this discrepancy might relate to impurities in the purified Cor a 8 used in the Dutch series.

In the absence of double-blind placebo-controlled challenges with hazelnut, a potential criticism on our study could be that our approach does not directly assess the clinical relevance of the *in vitro* sIgE observations. However, it is obvious that even a positive challenge with hazelnut would not provide an absolute proof of Cor a 9 being the culprit allergen. Therefore, to further and safely investigate the clinical relevance of anti-Cor a 9 IgE antibodies, flow-assisted analysis of *in vitro* Cor a 9-activated basophils was performed using the BAT (20). From these data, it emerges that anti-

Cor a 9 sIgE antibodies can trigger activation of basophils in an assay that closely resembles the *in vivo* pathway leading to the symptoms of an allergic reaction.

Another important finding in our study is that using the microarray, 40% of the patients with pollen-unrelated hazelnut allergy failed to demonstrate sIgE reactivity to Cor a 1.01 from hazel pollen, Cor a 1.04, Cor a 8, Cor a 9, or profilins, and 14% of the systemic reactors seem solely sensitized to Cor a 1.04. This probably reflects the fact that the panel of purified and recombinant proteins currently spotted on the microarray does not cover the entire hazelnut IgE response and that additional components such as the vicillin Cor a 11 (4, 7) and the 2S albumin (4, 26) might be necessary to establish correct diagnosis in some cases. Moreover, it seems that adding other components and cross-reactive structures might help to disentangle the underlying sensitization route(s). Alternatively, it cannot be excluded that low levels of anti-Cor a 1.04, Cor a 8, or Cor a 9 IgE antibodies could be missed by the microarray as it was recently reported that the analytical sensitivity of this assay could be less than for the traditional singleplexed IgE ImmunoCAP assay (24, 27).

As already exemplified above, hazelnut-mediated OAS in a birch-endemic region almost invariably results from cross-reactivity between the labile protein Cor a 1.04 and Bet v 1, both members of the PR10-family. Alternatively, it is confirmed that patients with an apparent monosensitization to Bet v 1 homologues using the microarray can suffer from more severe systemic reactions (28).

Finally, our results indicate that birch pollen allergic patients with and without OAS demonstrate comparable IgE immune responses to Cor a 1.04 and Cor a 1.0101. Actually, in this context, quantification of Cor a 1.04 and Cor a 1.0101 by microarray offers no advantage over the commonly used singleplexed Cor a 1.04-spiked hazelnut sIgE assay (6, 29, 30), and it will remain up to the clinician to determine case by case whether a particular immune response is linked to disease.

In conclusion, hazelnut allergy in a birch-endemic region exhibits age-related sensitization profiles with distinct clinical outcomes that can be identified using CRD. The most important observation of our study is that the majority of hazelnut allergic children in a birch-endemic region show systemic reactions on consumption of processed hazelnut, mostly being sensitized to the hazelnut legumin-like allergen Cor a 9 and not to Cor a 1.04, the hazelnut homologue of the major birch pollen allergen. In contrast, adults generally suffer from an OAS related to cross-reactivity between Cor a 1.04 from hazelnut and Bet v 1 from birch pollen. Large epidemiologic studies should not only take into account geographic differences but also confounders such as age.

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