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# *Urtica dioica* pollen allergy

## Clinical, biological, and allergomics analysis

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ABSTRACT

**Background:** The most emblematic members of Urticaceae at allergic risk level are wall pellitories (*Parietaria*), whereas nettle (*Urtica*) pollen is considered as poorly allergenic. No allergen from nettle pollen has yet been characterized, whereas 4 are listed for *Parietaria* pollen by the International Union of Immunological Societies. Clinical and biological profiles of 2 adult men who developed symptoms against nettle pollen and/or leaves were studied.

**Objective:** To characterize the allergic reaction and identify the potential nettle pollen sensitizing allergens.

**Methods:** IgE-mediated reaction to nettle pollen extract was evaluated by skin prick test, immunoassay, nasal provocation, and basophil activation test. To characterize specific nettle pollen allergens, an allergomic (IgE immunoproteomic) analysis was performed combining 1- and 2-dimensional electrophoresis, IgE immunoblots of nettle pollen extract, identification of allergens by mass spectrometry, and database queries.

**Results:** The results of biological and immunochemical analyses revealed that the allergic rhinitis was due to *Urtica dioica* pollen in both patients. The allergomic analysis of nettle pollen extract allowed the characterization of 4 basic protein allergens: a thaumatin-like protein (osmotin) with a relative molecular mass of 27 to 29 kDa, a pectinesterase (relative molecular mass, 40 kDa), and 2 other basic proteins with relative molecular masses of 14 to 16 kDa and 43 kDa. There is no or only very weak allergen associations between pellitory and nettle pollen.

**Conclusion:** Exposure to nettle pollen can be responsible of allergic symptoms, and several allergens were characterized. Unravelling the allergens of this underestimated allergy might help to improve diagnosis and care for patients, to predict cross-reactivities and design adapted specific immunotherapy.

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## Introduction

Urticaceae belong to the Herbaceous family, which includes nearly 2625 species divided into 60 genera. The most allergenic are pellitories (*Parietaria*), whereas nettle (*Urtica*) pollen is not considered very allergenic. A documented case of urticaria attributable to *Urtica dioica* in a neonate[1] and severe tongue edema induced by the stinging nettle[2] have been reported, whereas some cases of allergic rhinitis have been reported in the United States.[3–5] The prevalence of nettle pollen sensitization is therefore unknown in contrast to wall pellitory pollen, which has a reported prevalence of 35% in Spain[6] and up to 75% in Southern Italy.[7]

In Europe, 4 nettle species and 2 pellitory species with morphologically indistinguishable pollen grains have been described. Four allergens with several isoforms have been characterized for *Parietaria judaica* pollen, Par j 1 and 2, belonging to the lipid transfer protein (LTP) family, types 1 and 2, respectively.[8,9] Par

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j 3 belongs to the profilin pan allergen family (actin-binding protein),[10] and Par j 4 (polcalcin) is a 2 EF-hand calcium-binding protein.[11] No molecularly defined allergen from nettle (*U dioica*) pollen has yet been characterized, and no cross-reactivity has been observed between the pollen of *Parietaria* and *Urtica*[12] and *Boehmeria nivea* (ramie), another plant of the Urticaceae family.[13]

Nettles are ubiquitous in temperate areas, such as Northern Europe, North America, Western South America, Central Asia, and Southern Africa. The botanically related Urticaceae pellitories (especially *P judaica*) are found in Southern Europe and in some regions of the United States.[14,15] Nettles are found in open areas and especially in nitrate-rich soils. They are wind pollinated, and most nettle species flower from April to October. *U dioica* (stinging nettle) is perennial, whereas *Urtica urens* (dwarf nettle) is annual. Their leaves and stems are covered with stinging hairs (dwarf nettle leaves are smooth and more delicate). Besides a respiratory route for nettle pollen sensitization, ingestion of nettle proteins might also sensitize individuals because the leaves (*U dioica* and *U urens*) are consumed in salads, soups, tea, as a curdling agent, and as herbal remedies recommended notably for their anti-inflammatory properties, especially in the case of allergic rhinitis.[16–18]

In this study, we investigated the IgE response of 2 patients with clinical symptoms after exposure to nettle pollen. The clinical relevance of nettle pollen protein sensitization was assessed by the skin prick test (SPT), nasal provocation, and the basophil activation test, and an allergomic (IgE immunoproteomic) analysis was performed to characterize the specific nettle pollen allergens.

## Methods

### *Patients and Patient Sera*

Both patients (patients 1 and 2) experienced rhinoconjunctivitis symptoms from May to September. Written informed consent, in accord with an institutional board review by the Pneumology-Allergology Department, University Hospital, was obtained from the 2 patients.

Serum samples from 3 patients (patients 3, 4, and 5) were selected as positive controls for IgE reactivity to total protein extract from nettle and/or pellitory pollen and corresponded to residues from biological analysis performed to diagnose the allergy. Serum from a healthy individual (nonallergic, nonatopic) was used as a negative control. Serum samples were stored at −20°C until use.

### *Specific IgE Evaluations*

Serum IgE specific to nettle (*U dioica*) pollen extract were quantified by fluorescence enzyme immunoassay (Thermo Fisher Scientific, Villebon, France, and Phadia, Uppsala, Sweden) using an ImmunoCAP 250 apparatus. SPTs using pollen extracts provided by ALK-Abello (Vandeuil, France) and Stallergenes Laboratories (Antony, France), including *U dioica* pollen, were performed according to guidelines.[19] Skin prick to prick tests (SPPTs) were performed with fresh food provided by patient 2.

Nasal provocation tests (NPTs) were performed with 1/100, 1/10, and 1/1 diluted nettle pollen extract solution (ALK-Abello) applied every 10 minutes to one side of the nose.[20] Total nasal symptom scores were assessed using a visual analog scale (VAS) for rhinitis symptom severity as recommended by the Allergic Rhinitis and its Impact on Asthma guidelines. A VAS score of less than 5 cm is associated with mild rhinitis and a VAS score more than 6 cm with moderate to severe rhinitis.[21] Endoscopic examination was recorded at a 1/1 dilution of nettle pollen extract. The basophil activation test was performed on the cells of the patient allergic to nettle pollen according to the manufacturer's instructions (Bühlmann, Schönenbuch, Switzerland) with the same nettle pollen extract solution (1/1) as the activating allergenic source. The negative control was performed on cells with no source of allergens.

### *Pollen Protein Extractions*

*Parietaria officinalis* pollen was supplied by Allergon AB (Angelholm, Sweden), and *U dioica* pollen was a generous gift from ALK Abello. Pollen (50 mg) was extracted with lysing matrix A or C (MP-Biomedicals, Illkirch, France) in 0.5 mL of phosphate buffer saline (PBS) (pH 7.4) using a multidirectional grinder (FastPrep-24; MP-Biomedicals) (6 m/s for 40 sec at 4°C) and centrifuged at 18,000*g* for 20 minutes at 4°C. Supernatants were collected and stored as aliquots at −20°C.[22]

### *Gel Electrophoresis Separation*

Proteins from the different pollen extracts were separated by 1-dimensional (1-D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and 2-dimensional (2-D) electrophoresis according to Shahali et al.[23] Molecular mass markers (GE Healthcare, Little Chalfont, United Kingdom) ranging from 14.4 to 94 kDa and isoelectric point (pI) markers (Bio-Rad, Hercules, California) ranging from 4.45 to 9.6 were used as comparative references. For protein detection, gels were stained with Coomassie blue or silver. The 1-D and 2-D electrophoresis gels used for mass spectrometry analysis were stained with colloidal Coomassie blue.

### *Immunoblotting*

Proteins separated by 1-D and 2-D electrophoresis were electrotransferred onto 0.2 $\mu$m cyanogen bromide–activated nitrocellulose as described[24] (Optitran BA-S 83, Schleicher and Schuell, Dassel, Germany). Membranes were then dried and stored at −20°C until use. For multiplexed 1-D electrophoresis immunoblot analysis, cyanogen bromide–activated nitrocellulose was cut into 2.5-mm-wide strips. For 2-D electrophoresis immunoblot, the whole cyanogen bromide–activated nitrocellulose was treated. Each strip or whole cyanogen bromide–activated nitrocellulose was then blocked with PBS that contained 0.3% (vol/vol) Tween 20 and incubated overnight with individual sera diluted 1:10 in PBS with 0.1% Tween. After washing, the membranes were incubated with alkaline phosphatase–conjugated goat anti-human IgE ($\varepsilon$ specific; Sigma-Aldrich, St Louis, Missouri), and alkaline phosphatase activity was detected using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate according to Shahali et al.[23]

### *IgE-Binding Inhibition Using Bromelain*

To assess the oligosaccharide or peptide nature of IgE epitopes, binding inhibition tests were performed on immunoblots by preincubation of the individual serum with 0.1% bromelain (from pineapple stem; Sigma-Aldrich) as previously described.[23,24]

### *Mass Spectrometry Analysis*

After matching of the coordinates (relative molecular mass and pI) between IgE-binding proteins revealed by immunoblotting and gel proteins, the spots were manually excised. Proteins were then treated according to Abou Chakra et al.[25] Mass spectrometry (MS) and MS/MS Orbitrap analyses were performed using an Ultimate 3000 rapid separation liquid chromatographic system (Thermo Fisher Scientific) online with a hybrid linear trap quadrupole–Orbitrap–Velos mass spectrometer (Thermo Fisher Scientific). Peptide treatment and mass spectrometer data acquisition were performed according to Immel et al.[26]

### *Database Search*

Proteome Discover software, version 1.4.0.288 (Thermo Fisher Scientific), was used to create search files and to interpret MS and

MS/MS spectra. The search engine Mascot Server 2.4 (MatrixScience, London, United Kingdom) was used on Viridiplantae taxonomy (Green Plants, 36,711 sequences) from either the National Center for Biotechnology Information nr databank, containing 67,337,701 sequences (May 2015), or SwissProt, containing 549,008 sequences (August 2015) (www.ncbi.nlm.nih.gov/). The database search was performed for tryptic peptides only, with cysteine carbamidomethylation and methionine oxidation as variable modifications. Precursor and fragment error tolerances were set to 5 ppm and 0.56 Da, respectively. Up to 1 missed cleavage was accepted. Protein identification was validated if the search yielded an identification score associated with a significance level better than 95% and if at least 2 peptides were sequenced with an ion score above a threshold of 25.

## Results

### *Patients' Symptoms and Tests*

Both patients studied presented seasonal rhinoconjunctivitis from May to September during the nettle flowering and pollination period. Patient 1, a 48-year-old man from a nonatopic family, was monosensitized to nettle pollen. Ear, nose, and throat symptoms were very disabling despite treatment with antihistamines and topical steroids. The results of SPTs with 32 aeroallergens, including house dust mites, cat and dog dander, and several pollen extracts, were all negative except for nettle pollen extract (Fig 1A). IgE specific to this pollen extract was clearly detectable, and test results were slightly positive to rPar j 2 (Table 1). The NPT result was positive, with a total nasal symptom score progressively increasing from 0 at baseline to 5 after the instillation of 1 mL of 1/100 diluted solution to 7 after 1 mL of 1/10 diluted solution to 9 after 1 mL of undiluted solution. Endoscopic examination, performed before (Fig 1B) and after (Fig 1C) these nasal instillations, revealed a total obstruction. Results of the basophil activation test using nettle pollen extract, performed with patient cells, was strongly positive, with 93% of basophils expressing CD63 on activation (Fig 1D).

Patient 2, a 53-year-old man, is polysensitized to various pollens, horse, and dog dander and has rhinoconjunctivitis and asthma symptoms from May to June (Table 1). SPTs were performed with 11 aeroallergens, and results were positive for *Dermatophagoides pteronyssinus*, cat, dog and different pollen extracts. The strongest reaction was observed with the nettle pollen extract. Moreover, this patient had anaphylactic shock (hives, palpebral edema, and laryngeal dyspnea) in June 2015 after exercise (mountain climbing), 2 hours after ingesting a nettle leaf soup and drinking a beer. During the meal, roasted peanuts and dough wheat flour with pesto sauce were also eaten. The SPT results were slightly positive for the nettle soup and nettle leaves (Table 1). This result is likely to be related to a lower concentration of nettle leaf allergens and/or

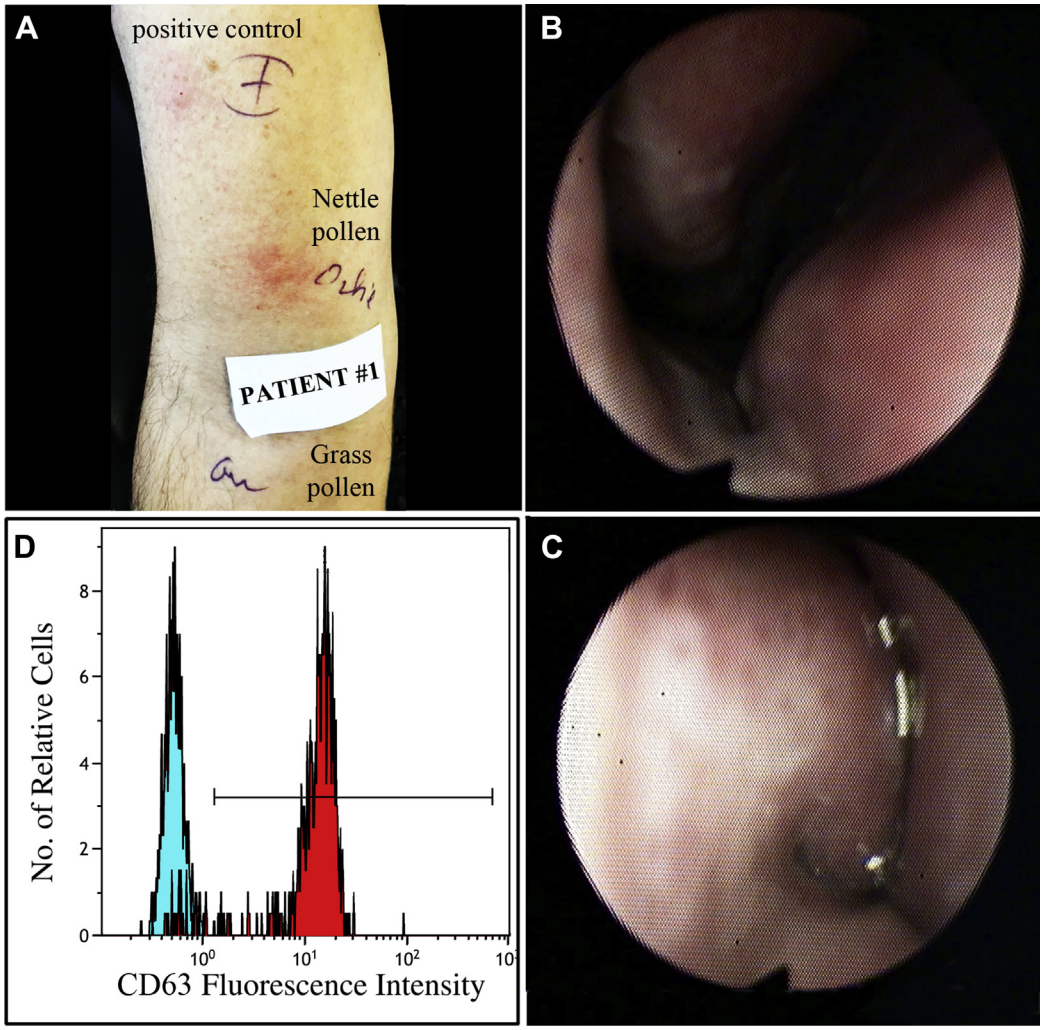


**Figure 1.** Clinical and biological test results for patient 1. A, Skin prick test to nettle and grass pollen extracts. B and C, Nasal provocation test with nettle pollen extract. Right nostril is shown before (B) and after (C) instillation of undiluted nettle pollen extract. D, Basophil activation test showing percentage of CD63-positive basophils after activation with nettle pollen extract (red histogram) or not activated (negative control, blue histogram).

**Table 1**
Clinical Data of the 2 Studied Nettle Pollen Allergic Patients

| Characteristic | Patient 1 | Patient 2 |
|---|---|---|
| Sex | M | M |
| Date of birth | July 2, 1968 | April 15, 1963 |
| Age at symptom onset, y | 44 | 51 |
| Months of ENT symptoms | July–September | May–June |
| Symptoms | | |
| Nettle soup | Unknown | AS |
| Nettle pollen | RC | RC |
| Specific IgE, kU/L | | |
| *Urtica dioïca* pollen extract | 9.84 | 20 |
| *Parietaria judaïca* pollen | | |
| Extract | 0.10 | ND |
| rPar j 2 | 0.63 | ND |
| *Artemisia vulgaris* pollen | | |
| Extract | ND | 0.45 |
| nArt v 1 | <0.1 | <.01 |
| nArt v 3 | <0.1 | <.01 |
| *Ambrosia artemisiifolia* pollen | | |
| nAmb a 1 | <0.1 | ND |
| *Phleum pratense* pollen | | |
| rPhl p 1+5 | ND | 14 |
| rPhl p 7 | ND | <0.1 |
| *Betula verrucosa* pollen | | |
| rBet v 1 | ND | 0.7 |
| rBet v 2 | <0.1 | <0.1 |
| rBet v 4 | <0.1 | ND |
| *Olea europea* pollen nOle e 7 | <0.1 | ND |
| *Prunus persica* fruit rPru p 3 | <0.1 | <0.1 |
| Skin prick test or prick to prick test wheal diameter, mm | | |
| Nettle leave | 0 | 4 |
| Cooked nettle soup | 0 | 2 |
| Dough wheat flour | 0 | 0 |
| Peanut | 0 | 0 |
| Pesto sauce | 0 | 0 |
| Beer | 0 | 0 |
| Nettle pollen | 9 | 10 |
| Grass pollen | 0 | 6 |
| Birch pollen | 0 | 5.5 |
| Ash pollen | 0 | 5 |
| Plantain pollen | 0 | 5 |
| NPT result for nettle pollen | Positive | ND |
| BAT result for nettle pollen | Strongly positive (93% of basophils $CD63^+$) | ND |

Abbreviations: AS, anaphylactic shock; BAT, basophil activation test; ENT, ear, nose, and throat; ND, not determined; NPT, nasal provocation test; RC, rhinoconjunctivitis.

modified allergens by cooking in nettle soup. The SPPT results were negative for peanut, wheat, pasta, tomato, and beer. Specific IgE test results were positive for nettle pollen and mugwort pollen extracts. All other components of the meal were reintroduced without any reaction, except for the nettle leaf soup.

### *Immunoblot 1-D Electrophoresis: IgE Sensitization to Nettle Pollen Extract of Allergic Patients*

The specificity of serum IgE was evaluated by immunoblot after 1-D electrophoresis separation (SDS-PAGE) of proteins extracted from *U dioica* (Fig 2A) and *P officinalis* pollen (Fig 2B). Although no IgE reactivity was observed with control PBS (lane C1) or control serum from nonallergic individuals (lane C2), patients' serum samples revealed IgE reactivity against nettle pollen proteins (Fig 2A, lanes 1 and 2). IgE from patient 1 had strong reactivity against a 14-kDa protein and weak reactivity against 2 proteins with relative molecular masses of 27 and 43 kDa. IgE reactivity against the latter protein (43 kDa) was mainly directed against carbohydrate moieties because it was inhibited by bromelain. Conversely, IgE binding was to peptide epitopes for the 14- and 27-kDa proteins (Fig 2A, lane 1, bromelain inhibition). The serum sample of patient 1 did not reveal IgE reactivity against pellitory pollen extract (Fig 2B, lane 1). Patient 2 had a heterogeneous IgE reactivity profile, including the profile of patient 1 with an additional band of approximately 30 kDa (Fig 2A, lane 2). Simultaneous incubation of serum 2 with bromelain resulted in inhibition of binding to all proteins larger than 25 kDa. Therefore, as for patient 1, anti–14-kDa IgE of patient 2 was directed against peptide epitopes. Interestingly, serum from patient 2 had some IgE reactivity against proteins larger than 30 kDa from pellitory pollen extract (Fig 2B, lane 2), which are likely to be anti–cross-reactive carbohydrate determinants.

The use of additional patient sera with IgE specific to nettle pollen confirmed the specificity of the IgE reactivities of patients 1 and 2 to nettle pollen. Patient 5, who was sensitized to nettle pollen, had IgE reactivity to proteins larger than 30 kDa in pellitory and nettle pollen extracts, all inhibited by bromelain, and reactivity to proteins of approximately 14 kDa in pellitory pollen only (Fig 2A and B, lane 5). Patient 4, who was sensitized to pellitory pollen, exhibited IgE binding to 2 proteins with low relative molecular masses (10 and 14 kDa) in pellitory pollen extract, but the only IgE reactivity to nettle pollen extract was to proteins smaller than 10 kDa (Fig 2A and B, lane 4), and reactivity against proteins with high relative molecular masses was inhibited by bromelain for the 2 pollen extracts. Patient 3, who was sensitized to pellitory pollen, had very strong IgE reactivity to proteins with low relative molecular masses (<30 kDa) (Fig 2B, lane 3), but no IgE binding to nettle pollen was observed (Fig 2A, lane 3).

### *Characterization of Nettle Pollen Allergens by MS Analysis After 2-D Electrophoresis Separation*

Approximately sixty proteins from nettle pollen extract were silver stained after 2-D electrophoresis separation (Fig 3A), with relative molecular masses between 14 and 94 kDa and pIs ranging from 3.0 to 9.5. Some proteins had a very basic pI (from 9 to 10).

The cyanogen bromide–activated nitrocellulose membranes obtained after transfer of proteins from 2-D electrophoresis gels were incubated with the allergic patients' sera. IgE from the serum of patient 1 revealed 3 groups of basic allergens, each one with putative isoforms, with relative molecular masses of 14, 27, and 43 kDa (Fig 3B). The most basic allergen, with a relative molecular mass of 14 kDa, presented strong IgE reactivity. IgE responses of the serum from patient 2 were more heterogeneous (Fig 3C) than those of the serum from patient 1. Serum from patient 2 had strong IgE reactivity to 14-, 27-, and 35-kDa proteins and weaker binding to a 43-kDa basic allergen and to several other allergens between 35 to 70 kDa, with pIs from 5 to 9. In agreement with the 1-D electrophoresis experiments, coincubation of the serum from patient 2 with bromelain inhibited IgE binding to proteins with high relative molecular masses (>30 kDa) and resulted in an IgE reactivity profile very close to that of patient 1 (Fig 3D).

After matching the coordinates (relative molecular mass and pI) between IgE-binding proteins from studied sera and stained proteins, 8 IgE-binding protein spots were excised from the 2-D electrophoresis gel (spots 5, 8, 9, 10, 11, 16, 17, and 18) (Fig 3A). Ten other non–IgE-binding proteins were also excised from the gel (spots 1, 2, 3, 4, 6, 7, 12, 13, 14, and 15) (Fig 3A). Then, these proteins and IgE-binding proteins were submitted to MS and MS/MS analysis, and the results of databank queries are given in Table 2. The list of identified proteins in the Viridiplantae database is presented together with data related to protein names, the plant species in which the optimal homology was found, the total ion scores, the number of peptides identified and also the coordinates as relative molecular mass and pI.

With regard to relevant allergenic spots, hypothetical proteins were found in spot 5, with a theoretical relative molecular mass of 46 to 47 kDa, and in spot 8 a pectinesterase or pectin

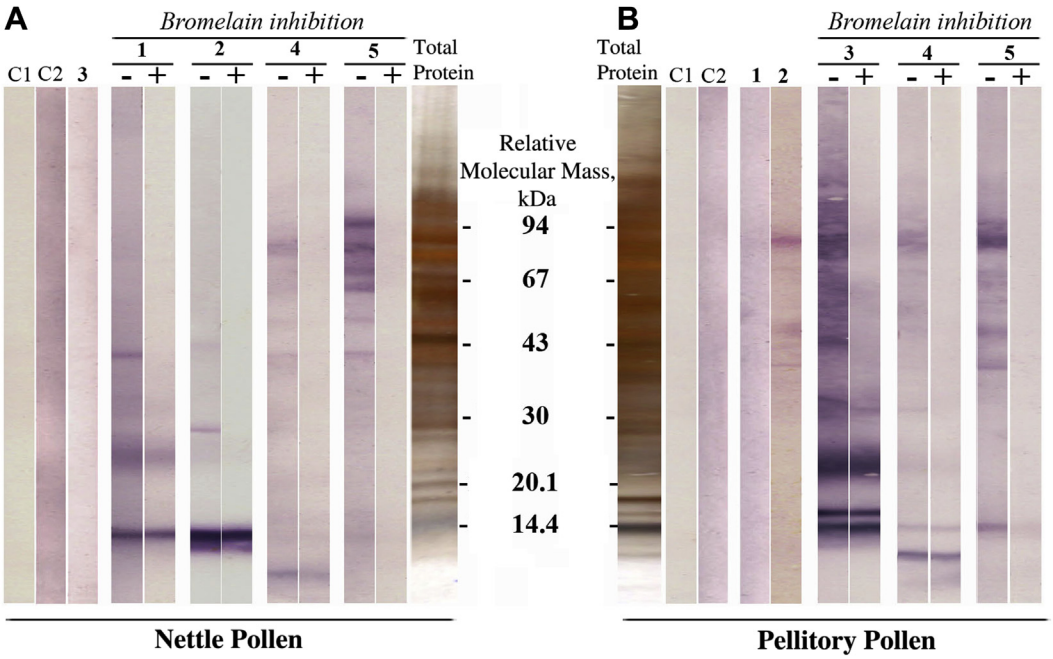


**Figure 2.** IgE immunoreactivity of selected patients' serum samples studied by immunoblots after sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation of nettle (*Urtica dioica*) (A) or pellitory (*Parietaria officinalis*) (B) pollen extracts. Strips were incubated with selected patients' sera diagnosed allergic to pellitory and/or nettle pollen (1 to 5) without (−) or with (+) bromelain. Specific IgE (kU/L) for additional patients were as follows: patient 3, *P officinalis* pollen 3.50; patient 4, *Parietaria judaica* pollen 15.6 and grass pollen 33.7; patient 5, *U dioica* pollen 1.93, grass pollen 30.6, and cypress pollen 0.52. Relative molecular masses are indicated for both gels. Total protein indicates total protein pattern stained with silver nitrate; C1, negative control 1 (no serum); C2, negative control 2 (serum from a nonallergic individual).

methylesterase homologous to that of *Eucalyptus grandis* was found, with a theoretical relative molecular mass of 52 kDa. In spots 10 and 11, a member of the thaumatin-like family of proteins was identified: an osmotin, similar to that of *Piper colubrinum*. In spot 17 a cytochrome C–like protein was revealed together with an unknown protein also found in spot 16.

Among proteins not recognized by IgE from the 2 studied patients, some have been described as allergens in other plants, fungi, or fruits: a methionine synthase, an endopeptidase-like enzyme, a $\beta$-xylosidase, an enolase, an aldolase, and a $\beta$-glucosidase. Fourteen additional proteins, not reported as allergens to our knowledge, were identified (Table 2).

## Discussion

The allergenic potential of a specific pollen is difficult to define because it depends on the degree of exposure, a value related to the total amount of pollen in the atmosphere (from intact or fragmented grains), the phenologic conditions in the area considered, and other cofactors, such as temperature, hygrometry, and air pollution. However, pollen sources are highly variable in their allergenicity. The pollen of nettle (*U dioica*) is considered less allergenic than its botanically related species wall pellitory (*Parietaria*), the pollen of which is one of the most common causes of pollinosis currently known in the Mediterranean area.

Two cases of rare respiratory allergy to nettle pollen are reported herein. Both patients have rhinitis. One (patient 2) also experienced an anaphylactic shock after consumption of a nettle soup. Besides the SPT performed to confirm the diagnosis of allergic rhinitis caused by nettle pollen, an NPT result in the other patient (patient 1) was positive, as was the basophil activation test result with total extract. Therefore, nettle pollen allergens should be considered as clinically relevant, and the prevalence of nettle pollen allergy is probably underestimated because its pollination period overlaps with that of grasses.

To gain insight into the specific allergens involved in allergy to nettle pollen, Urticaceae sensitized allergic patients were tested in parallel using *Urtica* and *Parietaria* pollen extracts and immunoprinting methods. Besides anti–cross-reactive carbohydrate determinants, no cross-reactivities were observed, suggesting that despite the botanical association between these 2 plants, protein structures are different, at least at the level of peptide IgE epitopes. These results are in accordance with an immunocytochemical study also showing no cross-reactivity between *Parietaria* and *Urtica* pollen.[27]

Ours is the first study, to our knowledge, to investigate the allergens from *U dioica* pollen extract by means of an allergomic approach. Nettle pollen proteins were PBS extracted using a multidirectional grinder, which is qualitatively and quantitatively very efficient.

Using 2-D analysis, 8 IgE-binding proteins from *U dioica* pollen were characterized. Four of them were hypothetical or unidentified proteins. Two of them were isoforms of the pathogenesis-related protein 5 osmotin, with a relative molecular mass of 27 to 29 kDa and a pI of 8.35 to 8.9, close to the relative molecular mass and pI of osmotin from *Piper colubrinum* (wild pepper). An osmotin from the leaves of *Nicotania tabacum* (Nict Osmotin) has been reported to be allergenic,[28] and Cap a 1, an osmotin-like protein, is the major allergen of *Capsicum annuum*, the bell pepper.[29] Osmotin proteins belong to the family of thaumatin-like protein allergens, which are physiologically related to plant stress responses. They constitute the third group of cypress pollen allergens that are upregulated in polluted environment.[30,31] It is not known whether this last characteristic also applies to nettle osmotin and might be relevant because nettles are found in cities where atmospheric pollution is sometimes high. Thaumatin-like proteins are also reported to be involved in pollen food–associated syndromes.[32] Patient 2 underwent an anaphylactic shock 2 hours after consumption of a nettle soup. It is not known whether osmotin or other nettle allergens are involved in food-dependent exercise-induced anaphylaxis, and this should be investigated further. A pectin methylesterase was also

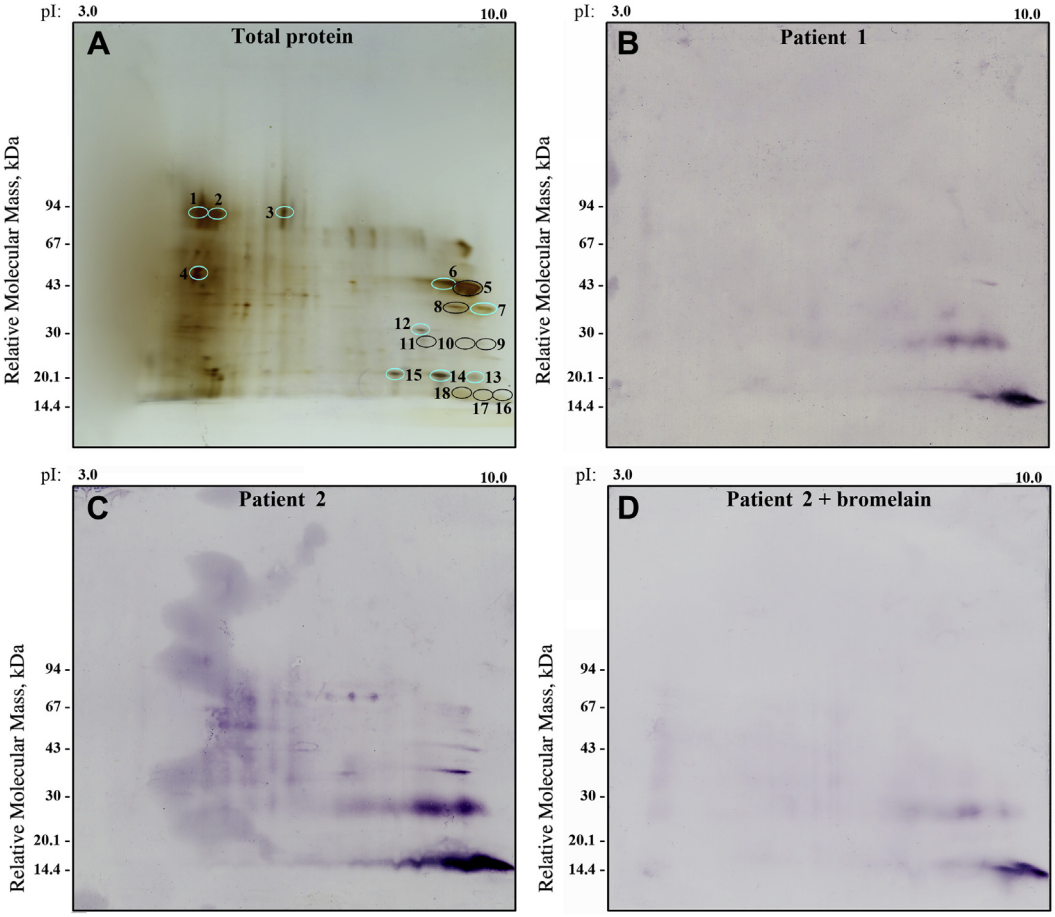


**Figure 3.** IgE immunoreactivity of the serum samples from patients 1 and 2 studied by immunoblots after 2-dimensional (2-D) electrophoresis separation of nettle (*Urtica dioica*) pollen extract. A, Total proteins of nettle pollen extract silver stained with indications as ellipses and numbers of excised spots for mass spectrometry analysis. IgE-binding proteins are circled with black ellipses and additional proteins with blue ellipses. B-D, 2-D electrophoresis immunoblots obtained after incubation with serum from patient 1 (B), serum from patient 2 (C), and serum from patients 2 in the presence of bromelain 0.1% (D). Isoelectric point (pI) values (at the top) and relative molecular mass on the left side are indicated for each gel.

identified in the nettle pollen extract. This protein has been described as a pollen allergen in several trees or shrubs, such as Bet v 8 in birch[33] and Fra e 11 in ash.[34] Pectin methylesterases are also food allergens in *Actinidia deliciosa*, kiwi fruit (Act d 7),[35] and *Solanum lycopersicum* (Sola l PME).[36]

We found strong IgE reactivity, likely against 3 isoforms of the same protein, in both our patients at basic pH in a low relative molecular mass zone (approximately 14–17 kDa). The allergens were not satisfactorily identified by MS analysis, although cytochrome c (a well-known allergen in grass pollen for instance[37]) and an unknown protein were listed after data bank searches. Because of their low relative molecular mass and high pI, they might correspond to *Urtica* LTPs, homologues of the major allergens *Parietaria* LTPs. However, anti-LTP antibodies specifically directed against Pru p 3, the LTP from peach, or against Tri a 14, the LTP from wheat, did not bind to these proteins in immunochemical experiments. More experiments are needed to unravel the physical-chemical nature of this highly IgE-reactive allergen reminiscent of what was found for *Cupressus sempervirens* pollen.[23]

We identified further proteins in nettle pollen extract. A methionine synthase and an endopeptidase could be considered as putative allergens in nettle pollen because they were found to be allergenic in *Salsola kali* pollen (Sal k 3)[38] and in the fungus *Rhizopus orizae* (Rhi o 1). In addition, a $\beta$-xylosidase similar to the one found in *Malus domestica* has been described as an allergen in *Aspergillus awamori* and *Aspergillus niger* (Asp aw 14 and Asp n 14). An enolase and an aldolase characterized in spots 3 and 4 have been described as allergens in *Cynodon dactylon* (Cyn d 22), *Plantago lanceolata* (Pla l 22 and Pla l PFA), *Manihot esculenta* in the case of the aldolase (Man e FPA) (http://www.allergome.org/), *Zea mays* (Zea m 22)[39] pollen, and *Hevea brasiliensis* in the case of the enolase (Hev b 9).[40] A $\beta$-glucosidase was also found in spot 7: $\beta$-glucosidase has been described as an allergen in different fungi (Asp f glucosidase)[41] and in fruit such as *Lycium barbarum* (Lyc ba glucosidase).[42]

To conclude, we found, with documented clinical and biological data from 2 patients, that *U dioica* pollen, which is not considered very allergenic despite its abundance in some regions[15] (www.pollens.fr/toustaxons2015.pdf, www.worc.ac.uk/discover/pollenforecast.html), sensitizes individuals and induces symptoms. Biochemical studies were conducted with the patients' serum samples to characterize the allergens involved. These nettle pollen allergens do not cross-react with wall pellitory allergens despite the common botanical origin of these 2 plants. The main IgE reactivities were found against a thaumatin-like protein and an unidentified basic allergen with low relative molecular mass. More patients and further research are needed to identify all the proteins responsible for this allergy and to establish the genuine allergenic potential of this pollen.

**Table 2**
MS and MS/MS Analysis of Proteins and Allergens From Nettle Pollen Extracts After 2-Dimensional Electrophoresis Separation

| Spot No. | Experimental relative molecular mass, kDa | Experimental pI | Proteins | Species | Theoretical relative molecular mass, kDa | Theoretical pI | Total Ion Score | No. of peptides identified | Coverage, % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 94 | 3.3 | Methionine synthase | *Orobanche ramosa* | 85 | 6.0 | 758 | 13 | 21 |
| | | | Phosphatidylcholine-hydrolyzing phospholipase D1 | *Cynara cardunculus* | 92 | 5.2 | 212 | 4 | 5 |
| | | | Prolyl endopeptidase-like | *Elaeis guineensis* | 85 | 5.3 | 171 | 3 | 4 |
| | | | Cytosolic aconitase | *Nicotiana tabacum* | 98 | 5.9 | 119 | 3 | 3 |
| | | | $\beta$-xylosidase/$\alpha$-L-arabinofuranosidase 1-like | *Malus domestica* | 83 | 6.2 | 102 | 2 | 3 |
| 2 | 94 | 3.5 | Methionine synthase | *Orobanche ramosa* | 85 | 6,0 | 826 | 14 | 23 |
| | | | Protease 2 | *Morus notabilis* | 89 | 5.8 | 332 | 8 | 7 |
| | | | Puromycin-sensitive aminopeptidase-like isoform | *Glycine max* | 111 | 6.3 | 281 | 5 | 5 |
| | | | HIPL1 protein precursor, putative | *Ricinus communis* | 76 | 5.0 | 110 | 2 | 3 |
| | | | $\beta$-xylosidase/$\alpha$-L-arabinofuranosidase 1-like | *Malus domestica* | 83 | 6.2 | 91 | 2 | 3 |
| 3 | 94 | 5.5 | 5-methyltetrahydropteroyltriglutamate-homocysteine-methyltransferase | *Sesamum indicum* | 85 | 6.2 | 380 | 6 | 10 |
| | | | $\beta$-xylosidase/$\alpha$-L -arabinofuranosidase 1-like | *Malus domestica* | 83 | 6.2 | 96 | 2 | 3 |
| 4 | 54 | 3.3 | Enolase-like | *Pyrus x bretschneideri* | 48 | 6.1 | 500 | 8 | 23 |
| | | | Uncharacterized protein LOC101205302 | *Cucumis sativus* | 48 | 5.6 | 363 | 6 | 17 |
| | | | Hypothetical protein PRUPE_ppa005968mg | *Prunus persica* | 47 | 6.5 | 349 | 7 | 16 |
| | | | Monodehydroascorbate reductase | *Solanum lycopersicum* | 47 | 5.7 | 456 | 4 | 12 |
| | | | Cytosolic aldolase | *Fragaria x ananassa* | 38 | 6.9 | 145 | 2 | 8 |
| 5[a] | 43 | 8.9 | Hypothetical protein ARALYDRAFT_487142 | *Arabidopsis lyrata* | 47 | 5.5 | 162 | 3 | 9 |
| | | | Hypothetical protein POPTR0019s09470g, partial | *Populus trichocarpa* | 46 | 9.3 | 75 | 1 | 3 |
| | | | Monodehydroascorbate reductase | *Solanum lycopersicum* | 47 | 5.8 | 94 | 1 | 3 |
| | | | Glucan endo-1,3-$\beta$-glucosidase, basic isoform | *Morus notabilis* | 38 | 9.3 | 116 | 2 | 6 |
| 8[a] | 40 | 8.6 | Pectinesterase 2-like | *Eucalyptus grandis* | 52 | 8.0 | 157 | 2 | 4 |
| | | | Hypothetical protein POPTR_0011s13830g | *Populus trichocarpa* | 60 | 6.1 | 102 | 2 | 3 |
| 9[a] | 27 | 9.1 | No protein identified | NA | NA | NA | NA | NA | NA |
| 10[a] | 29 | 8.9 | Osmotin | *Piper colubrinum* | 25 | 8.1 | 70 | 1 | 6 |
| 11[a] | 29 | 8.35 | Osmotin | *Piper colubrinum* | 25 | 8.1 | 95 | 2 | 12 |
| 12 | 32 | 8.2 | No protein identified | NA | NA | NA | NA | NA | NA |
| 13 | 21 | 9.1 | Cytochrome c—like | *Brachypodium distachyon* | 12 | 9.5 | 173 | 4 | 33 |
| | | | Osmotin | *Piper colubrinum* | 25 | 8.1 | 78 | 1 | 6 |
| 14 | 22 | 8.3 | Cytochrome c-like | *Brachypodium distachyon* | 12 | 9.5 | 139 | 3 | 25 |
| 15 | 22 | 7.4 | Perchloric acid soluble translation inhibitor protein | *Arachis hypogaea* | 20 | 8.9 | 91 | 2 | 12 |
| 16[a] | 16 | 9.4 | Unknown | *Lotus japonicus* | 13 | 9.7 | 73 | 1 | 6 |
| 17[a] | 16 | 9.0 | Cytochrome c-like | *Brachypodium distachyon* | 12 | 9.5 | 147 | 3 | 25 |
| | | | Unknown | *Lotus japonicus* | 13 | 9.7 | 70 | 1 | 6 |
| 18[a] | 17 | 8.6 | No protein identified | NA | NA | NA | NA | NA | NA |

Abbreviations: NA, not applicable; pI, isoelectric point.
[a]Proteins recognized by IgE from patient serum samples that were studied.

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