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Dear Editor

Pine Nut Anaphylaxis: A Proteomic Study

Pine nuts are the edible seeds derived from several varieties of pine trees. They are able to induce severe allergic reactions, usually anaphylaxis, in sensitized patients. Commonly, other nuts also induce this clinical picture in the same patient. There have been identified several allergens related with pine nut-induced anaphylaxis by SDS-PAGE and Immunoblotting.[1-3] Nevertheless, to the best of our knowledge, a proteomic study on this entity has not been performed so far.

We studied a 19-year-old woman who presented two anaphylactic reactions after eating small amounts of pine nuts when she was 4 and 14 years old, respectively. Up to date, she completely tolerates all foods including peanut, and the rest of tree nuts. She had never suffered from atopic symptoms. Skin-prick-testing (SPT) was performed using a subset of both indoor and outdoor aeroallergens, including pollens from *Pinus pinea* and *Olea europea*, as well as, against the panallergens profilin and lipid transfer protein (LTP) (ALK-Abelló, Madrid, Spain). Skin prick-by-prick (SPP) tests were also performed against both fresh and roasted nuts, including peanut, hazelnut, chestnut, sunflower seed, pistachio, cashewnut, almond and pine nut, plus negative (50% glycerinated saline) and positive (histamine, 10 mg/mL) controls. The results were measured 15 minutes after application. Both SPT and SPP tests were considered positive if the mean weal diameter was ≥3 mm than the saline control. Data were excluded if the saline control was ≥3 mm, the histamine control was <3 mm, or the difference of histamine minus saline was <3 mm. A serum total IgE and a serum specific IgE against those allergens used in SPT and SPP, were measured by ImmunoCAP™ (Phadia, Uppsala, Sweden), following the manufacter's instructions. The calibration range for serum total IgE was from 2.0-5,000 kU/L, while for serum specific IgE it was 0.1-100 kU_A/L. A serum total IgE was considered normal if the data was up to 100 kU/L, and a serum specific IgE was considered negative if its value was <0.35 kU_A/L. Pine nuts were extracted (1 : 10 [wt/vol], 4℃) during 90 minutes with PBS by magnetic sterring. Subsequently, extract was centrifuged and supernatants were filtered in a 0.2 μm membrane and frozen at -20 ℃ until used. Samples were separated by means of SDS-PAGE (10-20% polyacrylamide) according to the method of Laemmli and electrotransferred onto nitrocellulose membranes as described by Towbin *et al.*.[4] After blocking with 1% casein in PBS buffer during 1 hour at room temperature, membranes were incubated during about 18 hours with the serum of the patient (1 : 5 dilution). Nitrocellulose membranes containing the same extract were incubated with 1% casein in PBS, and alternatively with a serum from a non-atopic individual, as negative controls. After washing by means of 0.1% Tween-20 in PBS, nitrocellulose membranes were incubated during 2 hours at room temperature with the monoclonal antibody anti-human IgE HE-2 at 1 : 3,000 dilution.[5] Washing of the membranes was performed subsequently and then, an anti-mouse IgG peroxidase-conjugate antibody (RAM-HRP, DAKO, Barcelona, Spain) (1 : 5,000 dilution) was added during 1 hour at room temperature. Detection of reactive bands was performed using enhanced chemiluminescence, following the manufacter's instructions (ECL, GE Healthcare, Buckinghamshire, UK). IgE-Immunoblotting against pollen extracts was performed to rule out evidence for cross-reactivity of pine nut with a series of pollens (*Olea europea*,[6] *Pinus pinea*,[7] *Artemisia vulgaris*,[8] and *Lolium perenne*)[9] as previously proved. SDS-PAGE associated with a MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization, Time-Of-Flight) Mass Spectrometry (MS) analysis was carried out to identify new allergen molecules. The main IgE-binding bands revealed by Immunoblotting were excised from gels, alkylated and digested with trypsin for identification by MALDI-TOF-MS and MALDI-TOF-MS/MS. MALDI-MS and MS/MS data were combined through the BioTools programme (Bruker Daltonics, Billerica, MA, USA) to search a non-redundant protein database (NCBInr; National Centre for Biotechnology Information, Bethesda, MD, USA) using the Mascot software (Matrix Science, London, UK). MALDI-MS (MS) spectra and database search results were manually inspected in detail using the above programmes.

SPT against commercial common aeroallergens, profilin and LTP were performed with positive result exclusively against *Olea europaea*. SPP testing to raw and cooked pine nuts were all positive, unlike the rest of the nuts tested. Serum total IgE was 73 UI/mL, specific IgE to pine nut of 16.20 kU/L, and specific IgE to n*Ole e* 1 of 0.73 kU/L. The rest of specific IgE to nuts was negative. A Coomassie blue staining of the pine nut extract, and a SDS-PAGE followed by IgE-Immunoblotting assays were performed revealing IgE-reactivity at 30 and 33 kDa (Fig. 1). Additional weaker bands were observed at 15, 20 and 60 kDa. No signal was obtained for the negative controls. We did not find cross-reactivity with *Olea europaea*, *Pinus pinea*, *Artemisia vulgaris*, and *Lolium perenne* pollens after performing IgE-Immunoblotting assays.

Mass Spectrometry and database searching demonstrated that the sequence of the two bands of 30 and 33 kDa matched with a short-chain dehydrogenase (where the following sequences were obtained:

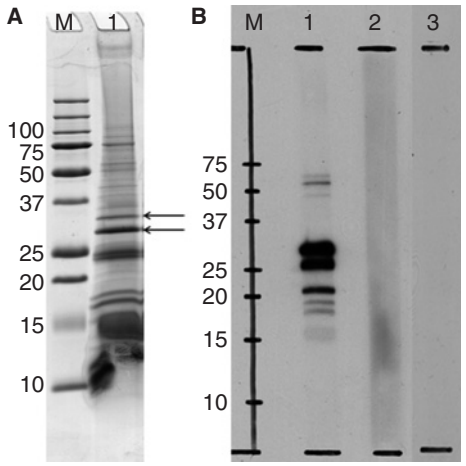

Fig. 1 **A**) Coomassie blue staining of the pine nut extract. M: molecular weight markers indicated in kDa. Lane 1: different bands obtained from the study. Excised bands for mass spectrometry analysis are shown by arrows. **B**) Pine nut IgE-Immunoblotting. M: molecular weight markers indicated in kDa. Lane 1: incubated with the patient's serum. Lane 2: negative control (serum from a non-atopic patient). Lane 3: negative control (in the absence of patient's serum).

VALVTGGDSGIGR; GLALQLVKR; FGSQVPMGR) and a pectin-methylesterase (where the following sequence was obtained KPMDLQK), respectively. Other peptides were measured for this last sample, but neither their masses nor their fragment ion masses matched with any significant protein or peptide candidate by database searching.

We present the first proteomic study in a rather unusual and interesting case of anaphylaxis due to allergy to pine nut, with actual tolerance to the rest of nuts or seeds. A short-chain dehydrogenase and a pectin-methylesterase seem to be involved in this exclusive sensitization. Therefore *Olea europaea* subclinical sensitization could be justified by the presence of pectin-methylesterase encountered in this patient, as it is known that *Ole e* 11 is classified as a pectin-methylesterase.[10] Taking this into account, it seems that the short-chain dehydrogenase of 30 kDa could be the genuine agent implicated in the anaphylaxis suffered by this patient with exclusive allergy to pine nut. Further investigation must be carried out in order to enlighten the cross-reactivity of this seed and to clarify its clinical relevance owing to the potential severity of reactions which pine nut may cause.

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