Proteomic profile of extracellular vesicles in anaphylaxis and their role in vascular permeability

To the Editor,

Anaphylaxis is a life-threatening allergic disorder for which there is a need for improved diagnostic techniques and a deeper understanding of the molecular mechanisms involved. Extracellular vesicles (EVs) play a key role in cellular communication, offering new possibilities with which to analyze patient particularities.

We hypothesized that anaphylaxis-derived EVs could provide potential markers of anaphylaxis due to their participation in the underlying molecular mechanisms. Vesicles were purified from 86 plasma samples (collected from 43 patients) during the acute phase of anaphylaxis (AnEVs) and baseline (BEVs). Clinical description, demographic characteristics, and their experimental utilities are displayed in Supplementary Table S1.

AnEVs and BEVs were isolated and characterized. Electron microscopy and nanoparticle tracking analysis (NTA) showed particles that were consistent with the reported size range for EVs, Scale bar: 200 nm. Images depict representative circulating BEVs and AnEVs. Scale bar: 200 nm. Images depict representative EVs from both samples.

(A) Transmission electron microscopy images from representative circulating BEVs and AnEVs. Images depict representative EVs from both samples.

(B) Seven different EV preparations from each experimental condition were analyzed by NTA. Representative NTA histograms showed average particle sizes of 200 nm for BEVs and AnEVs. (C) AnEVs were characterized by Western blot. Panels show immunoblots from three representative patients. Bona fide EV markers, such as CD63, TSG101, Syntenin-1, and CD9 were detected.

(D) Venn diagram of brute data obtained by mass spectrometry-based quantitative proteomics representing the intersection between detected proteins in BEVs and AnEVs. (E) Volcano plot showing all proteins identified. Statistically significant differences (p > .05) in the AnEVs (right side) and in the BEVs (left side) appear in blue. Access number (UniProt) for proteins of interest (CDC42, Ficolin-2, S100A9) are shown. Principal component analysis (F) and unsupervised hierarchical clustering (G) of plasma-derived EVs.
LETTERS TO THE EDITOR

LETTERS TO THE EDITOR

(100–250 nm) (Figure 1A–B). These vesicles exhibited markers previously related to EVs (Figure 1C). Their proteome is increasingly considered a source of biomarkers for various disease states.3

To investigate anaphylaxis-derived EVs, we analyzed the protein pattern profiles in 10 acute and baseline paired samples using mass spectrometry. The label-free quantification method identified 1206 proteins, and by comparing brute values of AnEVs and BEVs, 526 proteins were exclusively detected in AnEVs (Figure 1D). The whole amount of proteins identified was depicted through a volcano plot (Figure 1E). Principal component analysis (PCA) was used to gather biological replicates, and separation of BEVs and AnEVs groups was performed by PC1 (Figure 1F). Statistical analysis revealed 99 differentially expressed EV proteins, of which 83 were increased in the acute phase, thus suggesting their potential as candidate biomarkers (Supplementary Table S2). The differential clustering of these proteins is shown in Figure 1G. Only a few proteins found in this panel have been previously related to human anaphylaxis, and 2% had not been included in comprehensive EV resources (Vesiclepedia).

Three proteins were selected to confirm our results in a larger cohort. Specific analysis of CDC42, Ficolin-2, and S100A9 demonstrated an increased abundance in AnEVs, thus supporting an anaphylactic EV-protein signature (Figure 2A). CDC42 is one of the most enriched proteins in AnEVs and its role in cytoskeletal reorganization is essential to EV secretion. Ficolin-2 is closely related to immune processes participating in signaling pathways which release inflammatory mediators such as IFNγ, IL-6, TNFα, and nitric oxide. S100-family proteins are activated under cell stress and contribute regulating inflammatory processes and endothelium activation. From the studies performed here, S100A7, S100A8, and S100A9 were found to be increased in AnEVs, supporting a possible role for alarmins in this event.4,5

A coordinated function displayed in the contents of anaphylaxis-derived EVs may provide information about the molecular bases of the reaction. Therefore, a comprehensive analysis based on the Uniprot database revealed a group of major immune proteins participating in the cellular structure (Figure 2B).
In addition, in silico Ingenuity pathway analysis (IPA) showed around 25% of the protein signature participating in leukocyte trans-endothelial migration and cell degranulation (Figure 2C and Supplementary Table S3).

Another process closely associated with anaphylactic reactions is vascular permeability (v.p.).\(^6\) To evaluate the impact of EVs in v.p., we incubated them together with human microvascular endothelial cells-lung (HMVEC-Ls), revealing no differences in cellular uptake and showing a perinuclear localization (Figure 2D). However, AnEVs uptaked-ECs exhibited morphological contractile changes. In agreement, v.p. assays in 16 patients demonstrated that AnEVs induced a greater increase in the cellular permeability compared to BEVs (Figure 2E and Supplementary Figure S1).

Use of EVs as disease biomarkers is a matter of intense research\(^3\) that could improve knowledge and management of anaphylaxis. Using a challenging sample dataset, we identified an anaphylaxis EV signature for the first time and performing pilot biomarker studies. Functional involvement of EVs in anaphylaxis and v.p. needs further clarification. Future studies are necessary to determine their possible diagnostic utility. Though exploratory, our findings suggest the clinical potential of EVs, possibly leading to new therapeutic directions. Proteomic profiling of these plasma-derived AnEVs is a great resource for the allergy community.

ACKNOWLEDGMENTS
We thank Maria del Mar Gonzalez García-Parreño for technical support and Oliver Shaw for editing the manuscript and matters concerning English use.

FUNDING INFORMATION
This research was supported by the Spanish Council Ministry of Science and Innovation (Ramón y Cajal Program RyC-12880-2013), grants from the Instituto de Salud Carlos III (PI16/00888, PI16/01334 and PI18/00348) and FEDER Thematic Networks and Cooperative Research Centers RETICS ARADyAL (RD16/0006/0003, RD16/0006/0013, RD16/0006/0033). This work was also supported by the SEAIC (19_A08) and Alfonso X el Sabio University Foundations. ENB is the beneficiary of grant funding from the Community of Madrid included in the project FOOD-AL (CM_P2018/BAAQA-4574). Proteomic analysis was performed in the Proteomics Unit of UCM, a member of ProteoRed and is supported by Grant PRB3 (IPT17/0019-ISCIII-SGECI/ERDF).

CONFLICT OF INTEREST
There are no conflicts of interest.

Emilio Nuñez-Borque\(^1\)
Sergio Fernandez-Bravo\(^1\)
Carlos Pastor-Vargas\(^1,2,3\)
Gloria Alvarez-Llamas\(^4,5\)
Maria Dolores Gutierrez-Blazquez\(^6\)
Ebrahim Alwashali\(^6\)
Jose Julio Laguna\(^3,7,8\)
Javier Dionicio\(^3,7\)
Diana Betancor\(^1\)
Victoria Villalobos\(^1\)
Jaime Tome-Amat\(^3,9\)
Javier Cuesta-Herranz\(^1,3\)
Alberto Benito-Martin\(^10,11\)
Vanesa Esteban\(^13,8\)

\(^1\)Department of Allergy and Immunology, IIS-Fundación Jiménez Díaz, UAM, Madrid, Spain

\(^2\)Department of Biochemistry and Molecular Biology, Universidad Complutense de Madrid, Madrid, Spain

\(^3\)Red de asma, reacciones adversas y alérgicas (ARADyAL), Instituto de Salud Carlos III, Madrid, Spain

\(^4\)Immunology and Proteomics Laboratory, IIS-Fundación Jiménez Díaz, UAM, Madrid, Spain

\(^5\)Red de Investigación Renal (REDINREN), Instituto de Salud Carlos III, Madrid, Spain

\(^6\)CAI Genomics and Proteomics, Proteomic Unit, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain

\(^7\)Allergy Unit, Allergo-Anaesthesia Unit, Hospital Central de la Cruz Roja, Madrid, Spain

\(^8\)Faculty of Medicine and Biomedicine, Alfonso X el Sabio University, Madrid, Spain

\(^9\)Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Universidad Politécnica de Madrid, Madrid, Spain

\(^10\)Department of Medicine, Weill Cornell Medicine, New York, New York, USA

\(^11\)Instituto Biomedico de Nutrición y Salud, IBIONS, Spain

Correspondence
Vanesa Esteban, Department of Allergy and Immunology, IIS-Fundación Jiménez Díaz, Avda Reyes Católicos 2, Madrid 28040, Spain.
Email: vesteban@fjd.es
Alberto Benito-Martin, Department of Medicine, Weill Cornell Medicine, 1300 York Avenue, New York, NY 10065, USA.
Email: albertobenitomartin@gmail.com

Editor: Cezmi Akdis
Benito-Martin Alberto and Esteban Vanesa equal contribution.

ORCID
Carlos Pastor-Vargas https://orcid.org/0000-0002-4678-7967
Gloria Alvarez-Llamas https://orcid.org/0000-0002-3313-721X
Jaime Tome-Amat https://orcid.org/0000-0003-4442-3649
Alberto Benito-Martin https://orcid.org/0000-0002-0700-3891
Vanesa Esteban https://orcid.org/0000-0002-7500-6277
To the Editor,

Recent research advances on human gut microbiota have revealed that imbalanced gut microbiota (also known as dysbiosis) can contribute to the development of various chronic diseases.

Butyric acid, a short-chain fatty acid produced by butyric acid-producing bacteria (BAPB) residing in the intestine, induces the maturation of regulatory T cells (Tregs) from naïve T cells. Tregs are critical for suppressing excessive immune responses and are believed to suppress the onset of allergic diseases by secreting inhibitory cytokines.1

This study aimed to determine whether children with chicken egg allergy have dysbiosis characterized by a reduced number of BAPB. Stool samples were collected from 18 children with egg allergy (median age: 3.1 years, interquartile range [IQR]: 1.5–5.5) and 22 healthy controls (median age: 4.0 years, IQR: 2.9–6.1). Blood samples were also collected from allergy patients, who were diagnosed with egg allergy based on either a positive result in oral food challenge or a convincing reaction and sensitization with an egg-specific immunoglobulin E level (>0.35 kU A/L). The proportion of BAPB, alpha and beta diversity, and relative abundance of gut microbiota were assessed using 16S rRNA sequencing. The percentage of Tregs in CD4+ cells was assessed via flow cytometry.

No significant differences in age and sex between the groups were observed (Table S1). The allergy group was found to have less diversity with significantly lower values for observed species, Shannon index, and Simpson index (Figure 1A, Table S2). The visible and apparent clustering distances observed in a principal coordinate analysis plot of Bray-Curtis dissimilarity revealed distinct gut microbiota structures in the two groups (Figure 1B). In the allergy group, the percentage of Enterobacteriales was significantly higher (17.0% (9.5–22.3) vs. 1.8% (0.9–10.9), p = 0.029), whereas the percentage of Lactobacillales was significantly lower (7.1% (3.6–10.1) vs. 11.5% (7.5–18.5), p = 0.012) (Figure 2A,B, Table S3). The proportion of BAPB in the gut microbiota was significantly lower in the allergy group than in the control group [2.3% (1.0–5.2) vs. 6.9% (2.5–9.6), p = 0.013] (Figure 2C). The median percentage of Tregs in the allergy group was 2.7%, which was lower in six of nine patients compared with the reference range (Figure 2D).2

In summary, this study revealed that children with egg allergies have less BAPB and tendentially fewer circulating Tregs than the reported reference range. Several studies have reported dysbiosis in patients with allergy. Hua et al. analyzed 1879 patient samples and found that those with allergy had reduced abundance of Clostridiales.3 Concerning children, Savage et al. analyzed 216 samples from infants aged 3 to 6 months with and without food sensitization at 3 years of age and found that Clostridium were underrepresented in those with food sensitization.4 Clostridium (belonging to the order Clostridiales) clusters IV, XIVa, and XVIII are known to produce butyric acid5; therefore, decreased butyric acid in the gut may have contributed to the allergy or food sensitization. Although we cannot conclude if decreased BAPB abundance is the cause or result of food allergy, studies in birth cohorts have shown that changes in gut microbiota and organic acid concentrations occur before the onset of allergies6,7; rendering dysbiosis as the possible cause for allergies.

To our knowledge, this is the first report focusing on BAPB. Although fecal butyric acid concentrations were not measured, a study by Sandin et al. reported that allergy pediatric patients had lower fecal butyric acid levels compared with non-allergic children, which supports the possibility that fecal butyric acid is lower in children with egg allergies due to reduced BAPB abundance.8

In conclusion, children with egg allergies can experience dysbiosis characterized by the decreased abundance of BAPB. Administration

REFERENCES
1. Reber LL, Hernandez JD, Galli SJ. The pathophysiology of anaphylaxis. J Allergy Clin Immunol 2017;140:335–348.
2. Castells M. Diagnosis and management of anaphylaxis in precision medicine. J Allergy Clin. 2017;140(2):321–333.
3. Hoshino A, Kim HS, Bojmar L, et al. Extracellular vesicle and particle globulin E level (>0.35 kU A/L). The proportion of BAPB, alpha and beta diversity, and relative abundance of gut microbiota were assessed using 16S rRNA sequencing. The percentage of Tregs in CD4+ cells was assessed via flow cytometry.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

DOI: 10.1111/all.14795

Decreased butyric acid-producing bacteria in gut microbiota of children with egg allergy

Mitsuru Yamagishi and Shohei Akagawa contributed equally to this manuscript.