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

To the Editor,

Anaphylaxis is a life-threatening allergic disorder[1] for which there is a need for improved diagnostic techniques and a deeper understanding of the molecular mechanisms involved.[2] Extracellular vesicles (EVs) play a key role in cellular communication, offering new possibilities with which to analyze patient particularities.[3]

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

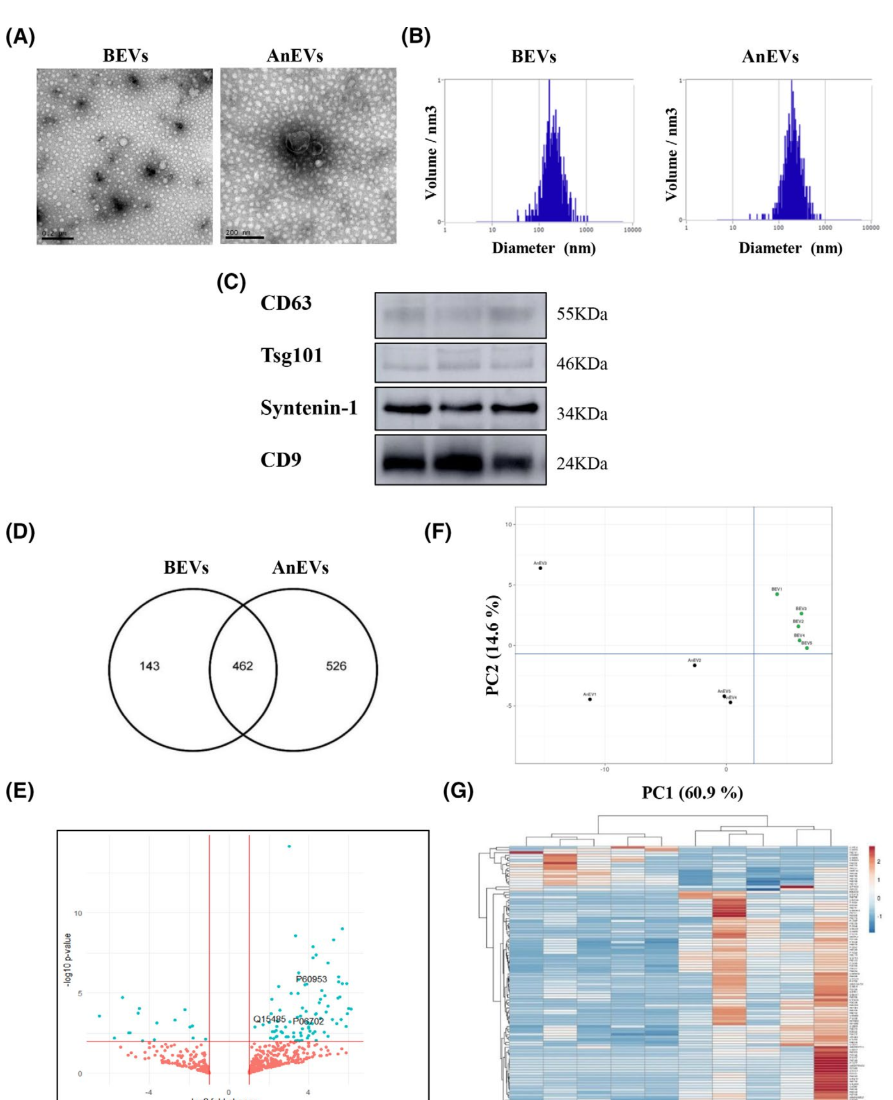

FIGURE 1 Characterization, proteomic profiling, and differential analysis of AnEVs and BEVs. (A) Transmission electron microscopy images from representative circulating BEVs and AnEVs. Scale bar: 200 nm. Images depict representative EVs from both samples. (B) Seven different EV preparations from each 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

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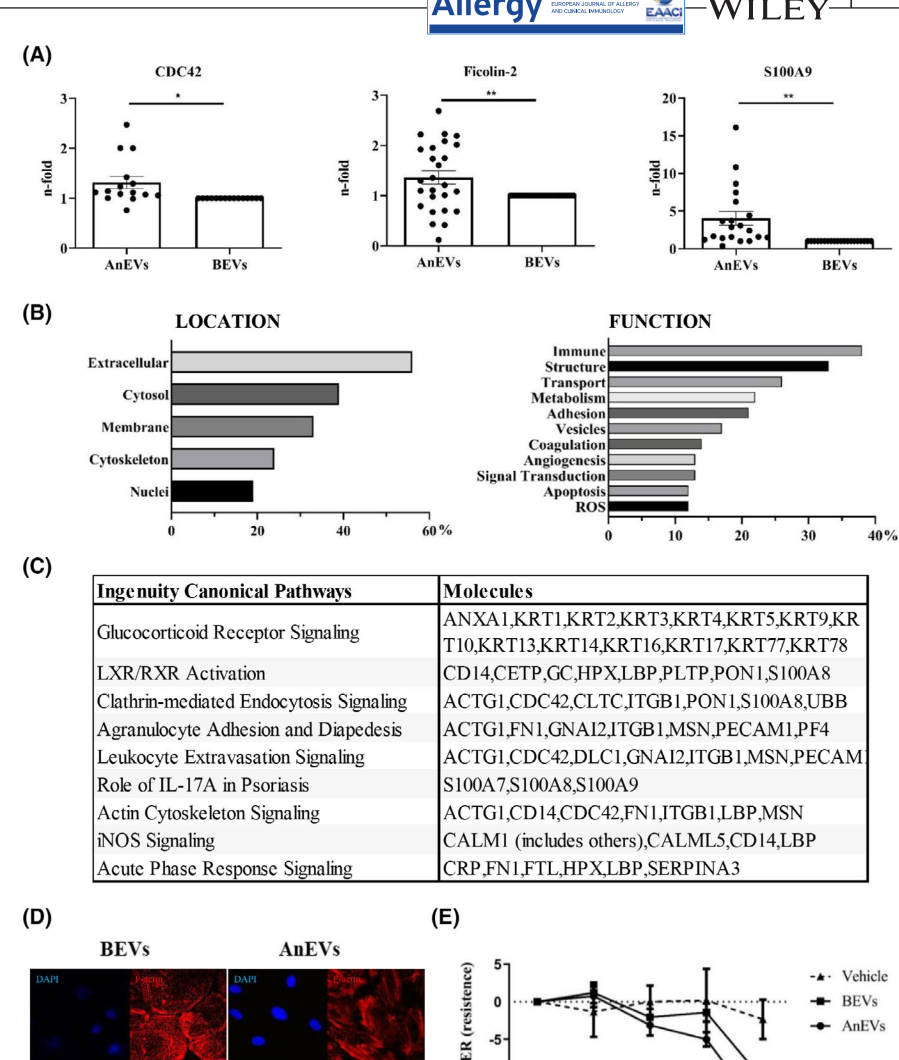

Ingenuity Canonical Pathways	Molecules
Glucocorticoid Receptor Signaling	ANXA1,KRT1,KRT2,KRT3,KRT4,KRT5,KRT9,KRT10,KRT13,KRT14,KRT16,KRT17,KRT77,KRT78
LXR/RXR Activation	CD14,CETP,GC,HPX,LBP,PLTP,PON1,S100A8
Clathrin-mediated Endocytosis Signaling	ACTG1,CDC42,CLTC,ITGB1,PON1,S100A8,UBB
Agranulocyte Adhesion and Diapedesis	ACTG1,FN1,GNAI2,ITGB1,MSN,PECAM1,PF4
Leukocyte Extravasation Signaling	ACTG1,CDC42,DLC1,GNAI2,ITGB1,MSN,PECAM1
Role of IL-17A in Psoriasis	S100A7,S100A8,S100A9
Actin Cytoskeleton Signaling	ACTG1,CD14,CDC42,FN1,ITGB1,LBP,MSN
iNOS Signaling	CALM1 (includes others),CALML5,CD14,LBP
Acute Phase Response Signaling	CRP,FN1,FTL,HPX,LBP,SERPINA3

FIGURE 2 Functional protein association networks in AnEVs. (A) CDC42, S100A9, and Ficolin-2 are increased in AnEVs. The graphics represent the abundance protein levels expressed as the quantified ratio (AnEVs/BEVs and BEVs/BEVs) of a larger group of anaphylactic plasma paired samples. CDC42 (*$p = .0137$, $n = 15$, MW=21 KDa), S100A9 (**$p = .0017$, $n = 20$, MW = 14 KDa), Ficolin-2 (**$p = .0092$, $n = 26$, MW = 34 KDa). (B) Classification of the main cellular localization and function of the protein panel based on the UniProt database. (C) Top Canonical Pathways obtained by IPA and symbols of the molecules. (D–E) HMVEC-Ls were incubated with 100 µg/ml of purified BEVs and AnEVs. The images reveal PKH67-EVs around the nuclei (DAPI) and cytoskeletal fibers (F-actin) from a representative paired EVs patient sample from 4 performed (D). (E) The graphic shows the change in TEER measurements after the addition of BEVs and AnEVs ($n = 16$ patients). Two-way ANOVA followed by the Bonferroni test was performed: **$p = .0033$, ****$p \le .0001$ versus Vehicle (EBM + PBS); #$p = .0285$ versus BEVs)

(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 BEV 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.

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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[1,3,8]

[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]*Immunoallergy 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

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SUPPORTING INFORMATION

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

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

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 acid[5]; 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 allergies[6,7];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

Mitsuru Yamagishi and Shohei Akagawa contributed equally to this manuscript.