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

Emilio Nuñez-Borque[1], Sergio Fernandez-Bravo[1], Carlos Pastor-Vargas[1], Gloria Alvarez-Llama[1], Maria Gutierrez-Blazquez[2], Ebrahim Alwashali[2], José Laguna[3], Javier Dionicio[3], Diana Betancor[1], Victoria Villalobos[1], Jaime Tome-Amat[4], Javier Cuesta Herranz[1], Alberto Benito-Martin[5], and Vanesa Esteban[1]

[1]Instituto de Investigacion Sanitaria de la Fundacion Jimenez Diaz
[2]Universidad Complutense de Madrid Facultad de Farmacia
[3]Hospital Central de la Cruz Roja
[4]Centro de Biotecnologia y Genomica de Plantas
[5]Weill Cornell Medicine

March 30, 2022

Abstract

Background: Anaphylaxis is the most severe manifestation of allergic disorders. Diagnostic and understanding of molecular mechanisms need to improve. Extracellular vesicles (EVs) play a key role in cellular communication offering new possibilities to unravel patient-based particularities. The aim of this study is to analyze the protein profile of anaphylaxis-derived EVs providing a resource of potential markers for anaphylactic reactions, and to characterize their molecular mechanisms. Methods: EVs were isolated from 86 plasma samples (collected from 43 patients) during the acute phase of anaphylaxis (AnEVs) and at their baseline (BEVs). For comparison, EVs were characterized and their protein patterns were analyzed by mass spectrometry-based quantitative proteomics (LC-MS/MS). System Biology Analysis (SBA) was applied to identify main canonical pathways and molecules involved. In addition, in vitro permeability assays based on EVs-endothelial cells (ECs) were performed. Results: Differential proteomic analysis performed in 10 EVs paired patients' samples identified 1206 proteins of which 99 were modulated in the AnEVs signature. CDC42, Ficolin-2 and S100A9 enrichment was confirmed in a larger cohort of patients. SBA revealed diverse group of immune proteins as the main canonical pathways altered in AnEVs. Thus, leukocyte extravasation and granulocyte adhesion-diapedesis processes stand out. In addition, marked-EVs from anaphylactic patients were captured by ECs decreasing the resistance of human endothelial monolayers. Conclusion: Our findings identify for the first time a differential EVs pattern signature in anaphylaxis revealing a source of potential biomarkers. Furthermore, these vesicles could participate in altered immune molecular mechanisms and present a role increasing vascular permeability.

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

Nuñez-Borque Emilio, BSc[a], Fernandez-Bravo Sergio, BSc[a], Pastor-Vargas Carlos, PhD[a,b,c], Alvarez-Llamas Gloria, PhD[d,e], Gutierrez-Blazquez Maria Dolores, PhD[f], Alwashali Ebrahim, PhD[f], Laguna Jose Julio, MD[c,g,h] Javier Dionicio, MD[c,g], Betancor Diana, MD[a], Villalobos Victoria, MD[a], Tome-Amat Jaime, PhD[i], Cuesta-Herranz Javier, PhD, MD[a,c], Benito-Martin Alberto, PhD[j,*]and Esteban Vanesa, PhD[a,c,h,*]

[a]*Department of Allergy and Immunology, IIS-Fundación Jiménez Díaz, UAM, Madrid 28040, Spain.*

[b]*Department of Biochemistry and Molecular Biology, Universidad Complutense de Madrid, Spain.*

[c]*Red de asma, reacciones adversas y alérgicas (ARADyAL). Instituto de Salud Carlos III. Spain.*

[d]*Immunoallergy and Proteomics Laboratory, IIS-Fundación Jiménez Díaz, UAM, Madrid 28040, Spain.*

[e]*Red de Investigación Renal (REDINREN). Instituto de Salud Carlos III. Madrid.*

[f]*CAI Genomics and Proteomics, Proteomic Unit, Faculty of Pharmacy, Complutense University of Madrid, Spain.*

[g]*Allergy Unit, Allergo-Anaesthesia Unit, Hospital Central de la Cruz Roja. Madrid, Spain .*

[h]*Faculty of Medicine and Biomedicine, Alfonso X El Sabio University, Madrid, Spain .*

[i]*Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Universidad Politécnica de Madrid, Pozuelo de Alarcón, Madrid, Spain*

[j]*Department of Medicine, Weill Cornell Medicine, New York, USA*

Corresponding authors:

* Equal contribution of the last 2 senior authors.

Vanesa Esteban. Department of Allergy and Immunology, IIS-Fundación Jiménez Díaz, Avda Reyes Católicos 2, Madrid 28040, Spain. Phone: +34 91 550 48 00 Fax N.+34 91 544 82 46. Email: vesteban@fjd.es

Alberto Benito-Martin. Department of Medicine, Weill Cornell Medicine;1300 York Avenue, New York, NY 10065,

Email: albertobenitomartin@gmail.com

Disclosure of potential conflict of interest: In relation to this manuscript, the authors declare that they have no relevant conflicts of interest.

SHORT TITLE: Extracellular vesicle profile in anaphylaxis

ABSTRACT

Background : Anaphylaxis is the most severe manifestation of allergic disorders. Diagnostic and understanding of molecular mechanisms need to improve. Extracellular vesicles (EVs) play a key role in cellular communication offering new possibilities to unravel patient-based particularities. The aim of this study is to analyze the protein profile of anaphylaxis-derived EVs providing a resource of potential markers for anaphylactic reactions, and to characterize their molecular mechanisms.

Methods: EVs were isolated from 86 plasma samples (collected from 43 patients) during the acute phase of anaphylaxis (AnEVs) and at their baseline (BEVs). For comparison, EVs were characterized and their protein patterns were analyzed by mass spectrometry-based quantitative proteomics (LC-MS/MS). System Biology Analysis (SBA) was applied to identify main canonical pathways and molecules involved. In addition,*in vitro* permeability assays based on EVs-endothelial cells (ECs) were performed.

Results: Differential proteomic analysis performed in 10 EVs paired patients' samples identified 1206 proteins of which 99 were modulated in the AnEVs signature. CDC42, Ficolin-2 and S100A9 enrichment was confirmed in a larger cohort of patients. SBA revealed diverse group of immune proteins as the main canonical pathways altered in AnEVs. Thus, leukocyte extravasation and granulocyte adhesion-diapedesis processes stand out. In addition, marked-EVs from anaphylactic patients were captured by ECs decreasing the resistance of human endothelial monolayers.

Conclusion : Our findings identify for the first time a differential EVs pattern signature in anaphylaxis revealing a source of potential biomarkers. Furthermore, these vesicles could participate in altered immune molecular mechanisms and present a role increasing vascular permeability.

KEYWORDS

Anaphylaxis,

Endothelial cell,

Extracellular vesicles,

Immune mechanisms,

Protein profile,

Proteomics,

System biology analysis,

Vascular permeability.

ABBREVIATIONS

EVs : Extracellular vesicles

AnEVs : Acute anaphylaxis derived extracellular vesicles

BEVs : Baseline derived extracellular vesicles

SBA : System Biology Analysis

IPA : Ingenuity pathway analysis

NTA : Nanoparticle tracking analysis

RT : Room temperature

UCM : Universidad Complutense of Madrid

GAR : Goat anti-rabbit

RAM : Rabbit anti-mouse

RP-LC-ESI-MS/MS : Liquid chromatography and mass spectrometry in tandem

PL : Proline 2.0 software

HMVEC-L : Human microvascular endothelial cell-lung

EBM : EGT-2MV BulletKit Medium

TW : Transwell

TEER : Trans-endothelial-electrical-resistance

EC : Endothelial cell

SEM : Standard error of the mean

SC : Spectral Counting

PCA : Principal component analysis

MW : Molecular Weight

FC : Fold change

INTRODUCTION

Anaphylaxis is the most severe manifestation of allergic disorders being a systemic hypersensitivity life-threatening reaction that usually evolves rapidly [1;2]. Currently, 50-112 cases per 100.000 habitants are registered per year, but this incidence seems to be underestimated [3]. The plethora of features associated to anaphylaxis confers difficulties to its diagnosis, thus impairing the ability to treat adequately these severe reactions. The only marker currently used in clinical practice is the serum tryptase, however levels of this

molecule are not altered in all cases [4;5]. High-throughput and systems biology analysis (SBA) implementation allow gaining knowledge on novel clues of action, aimed to improve patients' management towards a personalized medicine for allergy treatment [6]. Different phenotypes and underlying anaphylactic endotypes have been described based on a few diagnostic biomarkers [7;8]. The current criteria to define anaphylaxis is clinical, however considering the pleiotropic features of the reactions, the approach to precision medicine evolves towards a larger knowledge of the etiopathogenesis. Mechanistically, anaphylactic reactions are established, but it is necessary to set up cellular and molecular processes beyond IgE, mast cells and tryptase [4;9;10]. Among the affected systems, cutaneous symptoms are the most frequent, but the respiratory and circulatory ones are relevant in the onset of severe anaphylactic reactions. The vessels stand out due to the increased vascular permeability and vasodilation that occurs during this pathological response, which promotes the development of anaphylactic shock [11]. The systemic nature of the anaphylactic reaction demands new approaches to improve diagnosis and molecular characterization.

Extracellular vesicles (EVs) is the generic term for a heterogeneous group of particles naturally released by most cell types, delimited by a lipid bilayer and that cannot replicate [12;13]. A growing body of evidence points to EVs as an efficient system to transfer targeted information to recipient cells playing an active role in cell-to-cell communication [14]. They carry molecular determinants from their origin including nucleic acids, proteins, lipids and others [15]. EVs biological functions are wide and include antigen presentation, angiogenesis, inflammation and coagulation. Importantly for the allergy field, recent findings identify exosomes as important in the progression of asthma [16;17]. Exosomes are the most known sub-family of EVs and have been deeply studied in immunity and cancer [18;19]. Mast cells, basophils and other members of the immune system secrete EVs creating a dynamic network that could be crucial for the anaphylactic response [20]. The soluble nature of EVs, their presence in all the biological fluids and their early detection in many different pathologies propose them as an economic and promising tool for diagnosis [21;22]. Even more, proteomic analysis of EVs has been recently proposed as a *bona fide* diagnosis method for some cancers [23]. EVs profiling and their role in human anaphylaxis has not been reported.

In this study, we have performed a quantitative proteomic signature of plasma circulating anaphylaxis-derived EVs and point to clarify their biological and functional properties. In addition, an *in vitro*evaluation of the endothelial barrier function has been carried out in response to these particles.

METHODS

An expanded Materials and Methods Section is available in the Online Repository Material.

Patients' selection

The studied population included 86 paired peripheral blood samples (plasma) from 43 patients presenting anaphylaxis and recruited at Fundacion Jimenez Diaz and Hospital Central de la Cruz Roja, Madrid, Spain. From every subject, EVs were purified from plasma collected in the acute phase of the episode (AnEVs) and at baseline (BEVs) at least 14 days after reaction Patients who fulfilled the definition of anaphylaxis according to the 2006 National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network criteria were included [2]. The protocol was approved by the Ethics Committee (PIC38/2016_FJD and PIC142/2016_FJD).

EVs purification

EVs purification was carried out from plasma samples by centrifugation at 12,000g for 20 minutes at 10degC. Supernatants were transferred to ultracentrifuge tubes (Beckman, Coulter), supplemented with PBS and ultracentrifuged (XL-80, rotor 70Ti; Beckman, Coulter) twice at 100,000g for 1 hour and 10 minutes at 10oC. The pellet obtained was resuspended in PBS and stored at 4oC until its use in electron microscopy, *in vitro* vascular permeability assays and proteomic analysis or -20oC for nanoparticle tracking analysis (NTA) and Western blot determination.

Electron microscopy

Electron microscopy was carried out from 2μg of EVs that were fixed in PBS-formaldehyde 2% (v/v) for 16 hours at 4°C and shaking. Particles were adsorbed on copper-carbon grids (copper-formwar/carbon-coated grids 200 mesh, Fedelco) for 3 minutes, at room temperature (RT), and were contrasted with uranyl acetate at 1% (v/v) for 30 seconds. Samples were observed in a JEOL 1010 transmission electron microscope (JEOL), using a working voltage of 80kV. The analysis was carried out at the Electron Microscopy Center in the Universidad Complutense of Madrid (UCM), using the Soft Imaging Viewer program.

Nanoparticle Tracking Analysis

NTA was carried out with nanoparticle analyzer ZetaView from Particle Metrix (Germany) and Solmeglas Biotechnology Company (Spain).

Western blot analysis

Total protein concentration of EVs was determined with the Bradford Protein Assay Kit (ThermoFisher) [24]. Samples were lysed in reducing conditions with β-mercaptoethanol and 30μl of EVs were loaded per lane. EVs were analyzed by Western Blot with primary antibodys anti-CD9 (Invitrogen), anti-CD63 (Invitrogen), anti-Syntenin-1 (Novus Biologicals), anti-TSG101 (Abcam), anti-S100A9 (Cell signaling technology), anti-CDC42 (Cell signaling technology) and anti-Ficolin-2 (Abcam). Goat anti-rabbit (GAR) or rabbit anti-mouse (RAM) HRP-conjugated secondary antibodies were used (Jackson Laboratory, Bar Harbor, Me).

Proteins digestion and peptides fractionation

For proteins identification and relative quantification by label free approach, 10 paired samples of human EVs (AnEVs *versus* BEVs) were used, each constituted by pools of 2 individual samples. The same amount of total protein from each pool was tryptic digested and the resulting peptides were pre-fractionated in 7 fractions by High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific).

Protein quantification by liquid chromatography and mass spectrometry in tandem

Peptides fractions were analyzed by reversed phase liquid chromatography and mass spectrometry in tandem (RP-LC-MS/MS) in an EASY-nLC 1000 System coupled to a Q-Exactive HF mass spectrometer through a Nano-Easy spray source (Thermo Scientific, Bremen, Germany). All data were acquired in data-dependent acquisition (DDA) mode with Xcalibur 4.0 software. Peptide identification was carried out using Mascot v. 2.6.1 search engine through Protein Discoverer 2.3 Software (Thermo Scientific). Protein quantitation was performed by spectral counting with Proline 2.0 software (PL).

System biology Analysis

We used Ingenuity pathway analysis (IPA) software (Qiagen) to interpret the differentially expressed data including biological processes, canonical pathways and gene networks. Gene list contained in the main canonical pathways observed by IPA were analyzed in STRING (https://string-db.org/) and functional protein associated networks were visualized. The principal component analysis (PCA) and the heat map were realized in the website ClustVis (https://biit.cs.ut.ee/clustvis/).

Cellular culture and *in vitro* vascular permeability assays

Human Microvascular Endothelial Cells-Lung (HMVEC-L) were acquired from Lonza CC-2527 and maintained in EGM-2MV BulletKit. The effect of EVs in the endothelial barrier integrity was evaluated using Transwell 24-well cell culture inserts (TWs), as previously described [24]. HMVEC-L *in vitro* TW systems were incubated with 100μg/ml of AnEVs or BEVs in EBM-2 medium (EBM)+PBS or mediators of interest. The trans-endothelial-electrical-resistance (TEER) value of the monolayer was measured at different times (10 minutes, 1, 2 and 5 hours) through an EndOhm chamber (WPI) in individual cups.

PKH67 EVs staining and Immunofluorescence

AnEVs were stained with the green fluorescent membrane dye PKH67 accordingly to manufacture instructions (Sigma). To evaluate the interaction between EVs from anaphylactic patients and endothelial cells (EC)

in vitro confocal microscopy was performed. HMVEC-L were seeded in P8 chambers (IBIDI) and incubated with PKH67-EVs during 5h. Next, cells were fixed and stained with Texas Red-X phalloidin and DAPI. Images were acquired in an inverted confocal microscope (LSM700; Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

Statistical analysis was carried out with the Graph Pad Prism 8 program (La Jolla, CA, USA) using Student's t analysis to determine the significant differences between the 2 groups and the analysis of variances 2-way ANOVA and Bonferroni's multiple comparisons test when compared more than 2. All values are represented with their mean ± standard error of the mean (SEM) and the differences were considered significant at a level of p value <0.05.

RESULTS

Characteristics of the cohort

The studied population included a sum of 86 plasma samples from 43 anaphylactic patients ranged in age from 20-76 years old (45.2±2.3) and 65% were female. Triggers suspected to provoke reactions were drugs (67%), food (19%) and others (14%). The details of each individual case (criteria of severity) were applied based on clinical symptoms and according to the grading system established in Brown classification [25]. The mild group was grade 1 (12% of the population), moderate was grade 2 (56%), and severe was grade 3 (32%). This last classifies severe anaphylaxis with cardiovascular involvement and hypotension. The percentage of administered drugs was: epinephrine (53%), β2-adrenergic agonist (14%), histamine receptor antagonist type 1 (65%) and 2 (21%) and steroids (67%). In addition, 39% of patients presented elevated tryptase (>11.4µg/l) [26] in the acute phase with a medium value of 11.8±1.8µg/l in our group. A fulfilled clinical description and demographic characteristics of the anaphylactic population are showed in **Table 1.**

Characterization of EVs from anaphylactic patients

AnEVs and BEVs were both isolated and characterized using different techniques. Electron microscopy showed 100-250nm particles consistent with EVs reported size range [27] (**Figure 1A**). These results were confirmed by NTA, revealing heterogeneous particle populations (**Figure 1B**). The analysis showed a 200nm peak, confirming EVs enrichment in our preparation. Furthermore, no differences were observed in particle number or concentration between BEVs and AnEVs (**Figure 1C-D**). In addition, we confirmed EV purification by immunodetection of TSG101, CD9, CD63 and Syntenin-1, all of them considered *bona fide* EVs protein markers (**Figure 1E**).

Proteomic profiling of AnEVs and BEVs

To investigate the protein pattern profile of plasma EVs in anaphylaxis, 10 acute and baseline paired samples were analyzed by RP-LC-MS/MS and the PL relative quantification method identified 1206 proteins. When comparing brute values of AnEVs and BEVs, we found 526 proteins exclusively detected in EVs released during the acute phase of the reaction (**Figure 2A**). Volcano plot depicts the whole amount of proteins identified, in which we observed different abundance of some markers of interest (**Figure 2B**). Principal component analysis (PCA) revealed grouping of biological replicates and separation of BEV and AnEVs groups by PC1 (**Figure 2C**). Statistical analysis revealed 99 differentially expressed EVs proteins. 83 were enriched in the acute phase, and 16 were more abundant in basal conditions (**Supplemental Table 1**). The profile of these proteins shows how different AnEv samples cluster together and clearly differentiated from BEVs (**Figure 2D**).

CDC42, S100A9 and Ficolin-2 are increased in AnEVs

Three proteins (CDC42, S100A9 and Ficolin-2) were selected in order to mainly confirmation of the massive previous identification. By differential proteomic analysis, we observed that the abundance of CDC42, S100A9 and Ficolin-2 was increased in AnEVs. Therefore, we extended the isolation of plasma circulating EVs to a larger cohort of samples obtained in basal conditions and during the acute phase of the anaphylactic

reaction. The specific analysis for CDC42, S100A9 and Ficolin-2 demonstrated an increase of these proteins in AnEVs, which agreed with previous observed data (**Figure 3A-B**).

Canonical pathways altered by the EVs signature

The coordinated function displayed by the proteins contained in the anaphylactic panel would support information about the molecular bases of the reaction, especially of those exclusively found in the acute condition. Therefore, it was performed two comprehensive analyses: a "manual" based in the Uniprot database and an *in silico*approach through IPA. The first revealed a major group of immune proteins and those participating of the cellular structure, supporting a role of EVs in anaphylaxis. A summary of the major cell location as well as the main biological processes of identified EV markers is illustrated in **Figures 4A-B** . In agreement, the *in silico* IPA revealed different canonical pathways, candidates to underlie signaling in anaphylaxis. A list including those with higher significance is showed in **Figure 4C** . This analysis showed that these proteins altered involve mainly immunological processes such as degranulation of cells (mainly neutrophils) and adhesion-binding of leukocytes (**Sup Table 2**). A major interest was aroused by the appearance/repetition of same molecules participating in the different top canonical pathways; like CDC42. Therefore, to better visualize associations between the key proteins and their multiple participation in the functional networks described previously, a graphical representation based on STRING analysis was performed. As expected, this demonstrated that some AnEVs proteins are grouped in a unique cluster, such is the case of the keratinocytes include in the glucocorticoid receptor signaling pathway. In addition, Clathrin-mediated endocytosis, a process related with the own biology of EVs, was also observed.

AnEVs induce vascular permeability

Vascular permeability is one of the processes tightly associated to the anaphylactic reactions [28]. To analyze a plausible role of AnEVs in endothelial permeability, *in vitro* assays combined with immunofluorescence staining were carried. Images showed that an interaction between the PKH67-stained EVs and ECs exists. A clear perinuclear EVs localization revealed that HMVEC-Ls engulf and internalize these particles (**Figure 5A-B**). Once confirmed that AnEVs and HMVEC-Ls interact with each other, the purpose was to evaluate the functional effect of these vesicles on the endothelial barrier. Vascular permeability assays showed that the incubation of EVs extracted from anaphylactic patients produced a decrease in the resistance of the endothelial monolayer (**Figure 5C-D**). Importantly, this analysis including 16 patients demonstrated that AnEVs induced a greater increase of the cellular permeability compared to the BEVs (**Figure 5D**). As control of the cellular system created, vasoactive agents were also evaluated showing the effect of thrombin and platelet-activating factor (PAF) as destabilizers of the monolayer and the AMPc as an endothelial stabilizer. In all cases the expected effect was observed (**Supplemental Figure 1**).

DISCUSSION

Anaphylaxis includes a variety of hypersensitivity reactions mainly classified accordingly to the clinical features. However, their underlying cellular and molecular mechanisms required to be fully deciphered. The fact why some reactions spread from local to systemic might be based on the importance of intercellular communication taking place in anaphylactic microenvironments and established between different cellular compartments. Therefore, circulating EVs might participate in the development of these pathophysiological processes [29]. Our studies show a different composition of plasma derived EVs in patients undergoing an anaphylactic reaction. This unique material has allowed us to identify a signature protein panel, which to our knowledge has not been previously characterized. Furthermore, our research reveals a close enrichment of immune canonical pathways related to leukocyte extravasation, agranulocytes adhesion and diapedesis. In addition, our data support a role of EVs from anaphylactic patients in vascular permeability.

The EVs proteome is increasingly considered as a rich source of biomarkers for various disease states [23]. We have identified 1206 plasma EVs-associated proteins using mass spectrometry-based quantification. A total of 83 proteins were exclusively detected in the acute phase, favoring their potential role as diagnostic markers and proposing them as candidates' biomarkers. However, future studies are necessary to determine their possible diagnostic utility since only a few proteins found in this panel have been previously related

with human anaphylaxis. In addition, 2% of the proteins contained in the panel were not already included in comprehensive EVs resources (Vesiclepedia).

CDC42, Ficolin-2 and S100A9 were selected in our study to confirm the AnEVs profile in a bigger cohort of sample patients, supporting a robust anaphylactic EV-protein signature identified. CDC42 is one of the most enriched proteins in AnEVs. It belongs to small GTPases Rho family, and it is one of the main endothelial barrier-stabilizer proteins [30], playing a crucial role in vascular injury recovery [31]. The cytoskeletal reorganization is essential for the secretion of EVs and CDC42 has been previously involved in this process [32]. Alterations of this molecule have been reported in chronic asthma in mice [33]. Ficolin-2 is a protein involved in the activation of the lectin complement pathway and it is closely related to immune processes. It induces opsonization by stimulating M1 polarization through the TLR4/MyD88/MAPK/NF-$\varkappa\beta$ signaling pathway in macrophages and the consequent release of several inflammatory mediators such as IFNγ, IL-6, TNFα and nitric oxide [34;35]. Interestingly, some of these molecules have been remarked as biomarkers in anaphylaxis [24;36]. Moreover, Ficolin-2 deficiency associates with allergic disorders in children [37]. Phagocytic S100 family proteins are activated under cell stress situations and participate in the regulation of inflammatory processes. From the proteomic studies performed here, it has been found S100A7, S100A8 and S100A9 increased in AnEVs. S100A8 and S100A9 form a heterodimer named calprotectin, modulating different functions such as leukocyte traffic, rearrangement of the cytoskeleton and neutrophil activation. Surprisingly, these are the main biological processes significantly altered in AnEVs, pointing to a calprotectin role in anaphylaxis. Even more, alarmins participate in endothelium activation increasing permeability and facilitating immune cell recruitment [38;39]. Specifically, the S100A8/A9 heterodimer complexes bind to human ECs [40]. In the allergy field, controversial roles have been pointed for calprotectin in asthma [41], however, it is not here the first time that increased levels of S100A9 in human anaphylaxis has been observed as well as the consequent activation of neutrophils [42;43]. These facts point to a possible role for alarmins in AnEVs.

The signaling pathways involved in anaphylaxis are multiple and the*picture* including phenotypes, endotypes and mediators (potential biomarkers for diagnosis) is enormous [8]. Granulocytes and phagocytic cells (mainly mast cells and basophils) have been described as the key cell players, which release multiple mediators to the extracellular space during the acute phase of the anaphylactic reactions [44;45]. However, increasing evidences point to a role for other immune cells like macrophages and neutrophils [46-48]. Accordingly, our results show that around 25% of the AnEVs protein signature participates in leukocyte trans-endothelial migration and neutrophil degranulation. Some of these proteins (Anexin-1, CD14, S100A7/8/9 and Platelet Factor-4) are not only dysregulated, but they are also considered part of canonical pathways supporting the activation of monocytes and neutrophils. In addition, we have detected an important group of keratinocytes-derived proteins, which are addressed as related with glucocorticoid receptor pathway representing around of 12% of the AnVEs profile. The multisystem nature of anaphylaxis and in particular its relationship with epithelial and skin damage points to an interesting role for keratinocytes, considered contaminants in extracellular proteomics for a long time but supported as a systemically source of interleukins even in the allergy field [49].

Our knowledge regarding circulating EVs has increased exponentially in the last decade, and we know they can modify cellular functions due to the action of molecules they contain [50]. EVs participate in the destabilization of the endothelial barrier in inflammatory diseases such as sepsis or cancer [23]. This is related to a key pathophysiological characteristic of anaphylactic reactions, such as the increase in vascular permeability, which give rise to breakdown of the endothelial monolayer [51]. Our data show how ECs uptake AnEVs that are preferentially located in the perinuclear area. These results are consistent with previous publications [52], supporting a potential role in altering intracellular trafficking or even gene expression of the recipient cell, in this case the endothelial niche. In addition, AnEVs induce a greater leakage than BEVs by measuring the resistance values of the endothelial monolayer through an EndOhm device. However, further research needs to be conducted in order to clarify EVs role in endothelial damage and the differential effect of AnEVs.

We show here a challenging study of samples from human anaphylactic patients classified by allergology's experts in the field. The EVs usage as disease biomarker is a matter of intense research [23] that could improve our knowledge and management of anaphylaxis. We have identified for the first time an anaphylactic EVs signature, and we performed pilot studies on the validity of some of these proteins as potential biomarkers. Nevertheless, based on the results of our work, analysis of EV-associated proteins in larger patient cohorts is well-warranted. Even more, our study supports a functional role for the AnEV signature in key processes related with the disease onset and progression. The functional involvement of EVs in activation of neutrophils and endothelial cells needs further clarification, but our results suggest a relevant underlying mechanism connecting these cell types with the bases of the anaphylactic reactions. Even considering our findings exploratory in nature, their potential diagnostic and prognostic value might be crucial for new therapeutic directions and the proteomic profiling of these plasma derived AnEV is a great resource for the allergy community.

Acknowledgements

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 was granted by funding from the Community of Madrid included in the project FOOD-AL (CM_P2018/BAAA-4574).

The proteomic analysis was performed in the Proteomics Unit of UCM, a member of ProteoRed and is supported by Grant PRB3 (IPT17/0019-ISCIII-SGEFI/ERDF)

Author Contribution Statement

ENB and SFB contributed to the performance of the experimental work with participations of CPV and GAL. VE coordinated the work. JJL, JD DB, VV, and JCH provided the clinical support and recruitment of human samples. EA, MDGB participated in the proteomic and statistical analysis. VE, ABM and ENB designed the experiments and interpreted the results. VE ABM and JTA wrote the manuscript. All the authors reviewed the manuscript.

Reference List

[1] Reber LL, Hernandez JD, Galli SJ: The pathophysiology of anaphylaxis. J Allergy Clin Immunol 2017;140:335-348.

[2] Sampson HA, Munoz-Furlong A, Campbell RL, Adkinson NF, Jr., Bock SA, Branum A, Brown SG, Camargo CA, Jr., Cydulka R, Galli SJ, Gidudu J, Gruchalla RS, Harlor AD, Jr., Hepner DL, Lewis LM, Lieberman PL, Metcalfe DD, O'Connor R, Muraro A, Rudman A, Schmitt C, Scherrer D, Simons FE, Thomas S, Wood JP, Decker WW: Second symposium on the definition and management of anaphylaxis: summary report–Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. J Allergy Clin Immunol 2006;117:391-397.

[3] Tejedor Alonso MA, Moro MM, MÃºgica GarcÃa MV: Epidemiology of anaphylaxis. Clin Exp Allergy 2015;45:1027-1039.

[4] Khan BQ, Kemp SF: Pathophysiology of anaphylaxis. Curr Opin Allergy Clin Immunol 2011;11:319-325.

[5] Sala-Cunill A, Cardona V, Labrador-Horrillo M, Luengo O, Esteso O, Garriga T, Vicario M, Guilarte M: Usefulness and limitations of sequential serum tryptase for the diagnosis of anaphylaxis in 102 patients. Int Arch Allergy Immunol 2013;160:192-199.

[6] Agache I, Akdis CA: Precision medicine and phenotypes, endotypes, genotypes, regiotypes, and theratypes of allergic diseases. J Clin Invest 11-3-2019;130:1493-1503.

[7] Muraro A, Lemanske RF, Jr., Castells M, Torres MJ, Khan D, Simon HU, Bindslev-Jensen C, Burks W, Poulsen LK, Sampson HA, Worm M, Nadeau KC: Precision medicine in allergic disease-food allergy, drug allergy, and anaphylaxis-PRACTALL document of the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma and Immunology. Allergy 2017;72:1006-1021.

[8] Castells M: Diagnosis and management of anaphylaxis in precision medicine. J Allergy Clin Immunol 2017;140:321-333.

[9] Brown SG, Stone SF, Fatovich DM, Burrows SA, Holdgate A, Celenza A, Coulson A, Hartnett L, Nagree Y, Cotterell C, Isbister GK: Anaphylaxis: clinical patterns, mediator release, and severity. J Allergy Clin Immunol 2013;132:1141-1149.

[10] Pastor-Vargas C, Esteban V: Editorial: New Insights In Anaphylaxis. Front Immunol 2018;9:506.

[11] Poulsen LK, Jensen BM, Esteban V, Garvey LH: Beyond IgE-When Do IgE-Crosslinking and Effector Cell Activation Lead to Clinical Anaphylaxis? Front Immunol 2017;8:871.

[12] Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D, Tkach M, ThÃ©ry C: Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proc Natl Acad Sci U S A 23-2-2016;113:E968-E977.

[13] Zhang H, Freitas D, Kim HS, Fabijanic K, Li Z, Chen H, Mark MT, Molina H, Martin AB, Bojmar L, Fang J, Rampersaud S, Hoshino A, Matei I, Kenific CM, Nakajima M, Mutvei AP, Sansone P, Buehring W, Wang H, Jimenez JP, Cohen-Gould L, Paknejad N, Brendel M, Manova-Todorova K, MagalhÃ£es A, Ferreira JA, OsÃ³rio H, Silva AM, Massey A, Cubillos-Ruiz JR, Galletti G, Giannakakou P, Cuervo AM, Blenis J, Schwartz R, Brady MS, Peinado H, Bromberg J, Matsui H, Reis CA, Lyden D: Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. Nat Cell Biol 2018;20:332-343.

[14] Tkach M, ThÃ©ry C: Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. Cell 10-3-2016;164:1226-1232.

[15] Colombo M, Raposo G, Thery C: Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol 2014;30:255-289.

[16] Mazzeo C, Canas JA, Zafra MP, Rojas MA, Fernandez-Nieto M, Sanz V, Mittelbrunn M, Izquierdo M, Baixaulli F, Sastre J, Del P, V: Exosome secretion by eosinophils: A possible role in asthma pathogenesis. J Allergy Clin Immunol 2015;135:1603-1613.

[17] Canas JA, Sastre B, Rodrigo-Munoz JM, Del P, V: Exosomes: A new approach to asthma pathology. Clin Chim Acta 2019;495:139-147.

[18] van NG, Porto-Carreiro I, Simoes S, Raposo G: Exosomes: a common pathway for a specialized function. J Biochem 2006;140:13-21.

[19] Zubiri I, Posada-Ayala M, Benito-Martin A, Maroto AS, Martin-Lorenzo M, Cannata-Ortiz P, de la Cuesta F, Gonzalez-Calero L, Barderas MG, Fernandez-Fernandez B, Ortiz A, Vivanco F, Alvarez-Llamas G: Kidney tissue proteomics reveals regucalcin downregulation in response to diabetic nephropathy with reflection in urinary exosomes. Transl Res 2015;166:474-484.

[20] Benito-Martin A, Di GA, Ceder S, Peinado H: The new deal: a potential role for secreted vesicles in innate immunity and tumor progression. Front Immunol 2015;6:66.

[21] Madrigal-Matute J, Lindholt JS, Fernandez-Garcia CE, Benito-Martin A, Burillo E, Zalba G, Beloqui O, Llamas-Granda P, Ortiz A, Egido J, Blanco-Colio LM, Martin-Ventura JL: Galectin-3, a biomarker linking oxidative stress and inflammation with the clinical outcomes of patients with atherothrombosis. J Am Heart Assoc 5-8-2014;3.

[22] Benito-Martin A, Ucero AC, Zubiri I, Posada-Ayala M, Fernandez-Fernandez B, Cannata-Ortiz P, Sanchez-Nino MD, Ruiz-Ortega M, Egido J, Alvarez-Llamas G, Ortiz A: Osteoprotegerin in exosome-like vesicles from human cultured tubular cells and urine. PLoS One 2013;8:e72387.

[23] Hoshino A, Kim HS, Bojmar L, Gyan KE, Cioffi M, Hernandez J, Zambirinis CP, Rodrigues G, Molina H, Heissel S, Mark MT, Steiner L, Benito-Martin A, Lucotti S, Di GA, Offer K, Nakajima M, Williams C, NoguÃ©s L, Pelissier Vatter FA, Hashimoto A, Davies AE, Freitas D, Kenific CM, Ararso Y, Buehring W, Lauritzen P, Ogitani Y, Sugiura K, Takahashi N, AleÄ?koviÄ++ M, Bailey KA, Jolissant JS, Wang H, Harris A, Schaeffer LM, GarcAa-Santos G, Posner Z, Balachandran VP, Khakoo Y, Raju GP, Scherz A, Sagi I, Scherz-Shouval R, Yarden Y, Oren M, Malladi M, Petriccione M, De Braganca KC, Donzelli M, Fischer C, Vitolano S, Wright GP, Ganshaw L, Marrano M, Ahmed A, DeStefano J, Danzer E, Roehrl MHA, Lacayo NJ, Vincent TC, Weiser MR, Brady MS, Meyers PA, Wexler LH, Ambati SR, Chou AJ, Slotkin EK, Modak S, Roberts SS, Basu EM, Diolaiti D, Krantz BA, Cardoso F, Simpson AL, Berger M, Rudin CM, Simeone DM, Jain M, Ghajar CM, Batra SK, Stanger BZ, Bui J, Brown KA, Rajasekhar VK, Healey JH, de SM, Kramer K, Sheth S, Baisch J, Pascual V, Heaton TE, La Quaglia MP, Pisapia DJ, Schwartz R, Zhang H, Liu Y, Shukla A, Blavier L, DeClerck YA, LaBarge M, Bissell MJ, Caffrey TC, Grandgenett PM, Hollingsworth MA, Bromberg J, Costa-Silva B, Peinado H, Kang Y, Garcia BA, O'Reilly EM, Kelsen D, Trippett TM, Jones DR, Matei IR, Jarnagin WR, Lyden D: Extracellular Vesicle and Particle Biomarkers Define Multiple Human Cancers. Cell 20-8-2020;182:1044-1061.

[24] Mendez-Barbero N, Yuste-Montalvo A, Nunez-Borque E, Jensen BM, Gutierrez-Munoz C, Tome-Amat J, Garrido-Arandia M, Diaz-Perales A, Ballesteros-Martinez C, Laguna JJ, Beitia JM, Poulsen LK, Cuesta-Herranz J, Blanco-Colio LM, Esteban V: The TNF-like weak inducer of the apoptosis/fibroblast growth factor-inducible molecule 14 axis mediates histamine and platelet-activating factor-induced subcutaneous vascular leakage and anaphylactic shock. J Allergy Clin Immunol 31-10-2019.

[25] Brown SG: Clinical features and severity grading of anaphylaxis. J Allergy Clin Immunol 2004;114:371-376.

[26] Fellinger C, Hemmer W, WAPhrl S, Sesztak-Greinecker G, Jarisch R, Wantke F: Clinical characteristics and risk profile of patients with elevated baseline serum tryptase. Allergol Immunopathol (Madr) 2014;42:544-552.

[27] ThA(c)ry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, Ayre DC, Bach JM, Bachurski D, Baharvand H, Balaj L, Baldacchino S, Bauer NN, Baxter AA, Bebawy M, Beckham C, Bedina ZA, Benmoussa A, Berardi AC, Bergese P, Bielska E, Blenkiron C, Bobis-Wozowicz S, Boilard E, Boireau W, Bongiovanni A, BorrA s FE, Bosch S, Boulanger CM, Breakefield X, Breglio AM, Brennan MA, Brigstock DR, Brisson A, Broekman ML, Bromberg JF, Bryl-GA3recka P, Buch S, Buck AH, Burger D, Busatto S, Buschmann D, Bussolati B, BuzAs EI, Byrd JB, Camussi G, Carter DR, Caruso S, Chamley LW, Chang YT, Chen C, Chen S, Cheng L, Chin AR, Clayton A, Clerici SP, Cocks A, Cocucci E, Coffey RJ, Cordeiro-da-Silva A, Couch Y, Coumans FA, Coyle B, Crescitelli R, Criado MF, D'Souza-Schorey C, Das S, Datta CA, de CP, De Santana EF, De WO, Del Portillo HA, Demaret T, Deville S, Devitt A, Dhondt B, Di VD, Dieterich LC, Dolo V, Dominguez Rubio AP, Dominici M, Dourado MR, Driedonks TA, Duarte FV, Duncan HM, Eichenberger RM, EkstrAPm K, El AS, Elie-Caille C, ErdbrA1/4gger U, FalcA3n-PA(c)rez JM, Fatima F, Fish JE, Flores-Bellver M, FAPrsAPnits A, Frelet-Barrand A, Fricke F, Fuhrmann G, Gabrielsson S, GAmez-Valero A, Gardiner C, $GArtnerK, GaudinR, GhoYS, GiebelB, GilbertC, GimonaM, GiustiI, GoberdhanDC, GAPrgensA, GorskiSM, GreeningD$ $TalismanT, JungS, KalluriR, KanoSI, KaurS, KawamuraY, KellerET, KhamariD, KhomyakovaE, KhvorovaA, Kierul$ $+MM, KovAcsAF, KrA$mer-Albers EM, Krasemann S, Krause M, Kurochkin IV, Kusuma GD, Kuypers S, Laitinen S, Langevin SM, Languino LR, Lannigan J, $LAsserC, LaurentLC, LavieuG, LAzaro - IbAA + -ezE, LeLS, LeeMS, LeeYXF, LemosDS, LenassiM, LeszczynskaA, LiIT, LiaoK, LibregtsSF, LigetiE, LimR, LimSK, L$ $UzunovaES, Martin-JaularL, MartinezMC, MartinsVR, MathieuM, MathivananS, MaugeriM, McGinnisLK, McVey$ $KastresanaA, MorhayimJ, MullierF, MuracaM, MusanteL, MussackV, MuthDC, MyburghKH, NajranaT, NawazM, N$ $tHoenEN, NorenHN, O'DriscollL, O'GradyT, O'LoghlenA, OchiyaT, OlivierM, OrtizA, OrtizLA, OsteikoetxeaX, A sterg$

$Minimalinformationforstudiesofextracellularvesicles2018(MISEV2018)$: $apositionstatementoftheInternationalSocietyforExtracellularVesiclesandupdateoftheMISEV2014guidelines.JExtrace$ 1535750.

[28] Nakamura T, Murata T: Regulation of vascular permeability in anaphylaxis. Br J Pharmacol 2018;175:2538-2542.

[29] Maia J, Caja S, Strano Moraes MC, Couto N, Costa-Silva B: Exosome-Based Cell-Cell Communication in the Tumor Microenvironment. Front Cell Dev Biol 2018;6:18.

[30] Broman MT, Mehta D, Malik AB: Cdc42 regulates the restoration of endothelial adherens junctions and permeability. Trends Cardiovasc Med 2007;17:151-156.

[31] Lv J, Zeng J, Guo F, Li Y, Xu M, Cheng Y, Zhang L, Cai S, Chen Y, Zheng Y, Hu G: Endothelial Cdc42 deficiency impairs endothelial regeneration and vascular repair after inflammatory vascular injury. Respir Res 8-2-2018;19:27.

[32] EkstrÃ¶m EJ, Bergenfelz C, von Bl, V, Serifler F, Carlemalm E, JÃ¶nsson G, Andersson T, Leandersson K: WNT5A induces release of exosomes containing pro-angiogenic and immunosuppressive factors from malignant melanoma cells. Mol Cancer 26-4-2014;13:88.

[33] Zhuang TT, Pan C, Chen JJ, Han F, Zhu XL, Xu H, Lu YP: Chronic asthma-induced behavioral and hippocampal neuronal morphological changes are concurrent with BDNF, cofilin1 and Cdc42/RhoA alterations in immature mice. Brain Res Bull 2018;143:194-206.

[34] Luo F, Sun X, Wang Y, Wang Q, Wu Y, Pan Q, Fang C, Zhang XL: Ficolin-2 defends against virulent Mycobacteria tuberculosis infection in vivo, and its insufficiency is associated with infection in humans. PLoS One 2013;8:e73859.

[35] Yang YF, Zhou YD, Hu JC, Luo FL, Xie Y, Shen YY, Bian WX, Yin ZN, Li HL, Zhang XL: Ficolin-A/2, acting as a new regulator of macrophage polarization, mediates the inflammatory response in experimental mouse colitis. Immunology 2017;151:433-450.

[36] Lin RY, Trivino MR, Curry A, Pesola GR, Knight RJ, Lee HS, Bakalchuk L, Tenenbaum C, Westfal RE: Interleukin 6 and C-reactive protein levels in patients with acute allergic reactions: an emergency department-based study. Ann Allergy Asthma Immunol 2001;87:412-416.

[37] Cedzynski M, Atkinson AP, St SA, MacDonald SL, Szala A, Zeman K, Buczylko K, Bak-Romaniszyn L, Wiszniewska M, Matsushita M, Szemraj J, Banasik M, Turner ML, Kilpatrick DC: L-ficolin (ficolin-2) insufficiency is associated with combined allergic and infectious respiratory disease in children. Mol Immunol 2009;47:415-419.

[38] Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA: Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. J Immunol 15-3-2003;170:3233-3242.

[39] Foell D, Wittkowski H, Vogl T, Roth J: S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. J Leukoc Biol 2007;81:28-37.

[40] Eue I, KÃ¶nig S, Pior J, Sorg C: S100A8, S100A9 and the S100A8/A9 heterodimer complex specifically bind to human endothelial cells: identification and characterization of ligands for the myeloid-related proteins S100A9 and S100A8/A9 on human dermal microvascular endothelial cell line-1 cells. Int Immunol 2002;14:287-297.

[41] Palmer LD, Maloney KN, Boyd KL, Goleniewska AK, Toki S, Maxwell CN, Chazin WJ, Peebles RS, Jr., Newcomb DC, Skaar EP: The Innate Immune Protein S100A9 Protects from T-Helper Cell Type 2-mediated Allergic Airway Inflammation. Am J Respir Cell Mol Biol 2019;61:459-468.

[42] JÃ¶nsson F, de CL, Granger V, Gouel-ChÃ©ron A, Gillis CM, Zhu Q, Dib F, Nicaise-Roland P, Ganneau C, Hurtado-Nedelec M, Paugam-Burtz C, Necib S, Keita-Meyer H, Le DM, Cholley B, Langeron O, Jacob L, Plaud B, Fischler M, Sauvan C, Guinnepain MT, Montravers P, Aubier M, Bay S, Neukirch C, Tubach F, Longrois D, Chollet-Martin S, Bruhns P: An IgG-induced neutrophil activation pathway contributes to human drug-induced anaphylaxis. Sci Transl Med 10-7-2019;11.

[43] Francis A, Bosio E, Stone SF, Fatovich DM, Arendts G, MacDonald SPJ, Burrows S, Brown SGA: Markers Involved in Innate Immunity and Neutrophil Activation are Elevated during Acute Human Anaphylaxis: Validation of a Microarray Study. J Innate Immun 2019;11:63-73.

[44] Galli SJ, Tsai M: IgE and mast cells in allergic disease. Nat Med 4-5-2012;18:693-704.

[45] Lieberman P, Garvey LH: Mast Cells and Anaphylaxis. Curr Allergy Asthma Rep 2016;16:20.

[46] Borregaard N, SÃ¸rensen OE, Theilgaard-MÃ¶nch K: Neutrophil granules: a library of innate immunity proteins. Trends Immunol 2007;28:340-345.

[47] RÃ¸rvig S, Ã stergaard O, Heegaard NH, Borregaard N: Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. J Leukoc Biol 2013;94:711-721.

[48] Wright HL, Moots RJ, Bucknall RC, Edwards SW: Neutrophil function in inflammation and inflammatory diseases. Rheumatology (Oxford) 2010;49:1618-1631.

[49] Leyva-Castillo JM, Galand C, Kam C, Burton O, Gurish M, Musser MA, Goldsmith JD, Hait E, Nurko S, Brombacher F, Dong C, Finkelman FD, Lee RT, Ziegler S, Chiu I, Austen KF, Geha RS: Mechanical Skin Injury Promotes Food Anaphylaxis by Driving Intestinal Mast Cell Expansion. Immunity 21-5-2019;50:1262-1275.

[50] Kourembanas S: Exosomes: vehicles of intercellular signaling, biomarkers, and vectors of cell therapy. Annu Rev Physiol 2015;77:13-27.

[51] Radeva MY, Waschke J: Mind the gap: mechanisms regulating the endothelial barrier. Acta Physiol (Oxf) 2018;222.

[52] Rappa G, Santos MF, Green TM, KarbanovÃ¡ J, Hassler J, Bai Y, Barsky SH, Corbeil D, Lorico A: Nuclear transport of cancer extracellular vesicle-derived biomaterials through nuclear envelope invagination-associated late endosomes. Oncotarget 28-2-2017;8:14443-14461.

FIGURE LEGENDS

FIGURE 1. Anaphylactic patients EVs characterization. Seven different EV preparations from each experimental condition were analyzed by NTA. (**A**) Transmission Electron Microscopy characterization of circulating EVs. Scale bar: 200 nm. Image depicts three representatives AnEVs. (**B**) Representative NTA showed an average size particle of 200 nm, (**C**) without significant differences between BEVs and AnEVs. (**D**) The particle number per ml of AnEVs is slightly increased compared with BEVs. (**E**) AnEVs were characterized by Western Blot. Panels show immunoblots from three representative patients. *Bona fide* EVs markers, such as CD63, TSG101, Syntenin-1 and CD9, were detected.

FIGURE 2. Proteomic profiling and differential analysis of AnEVs and BEVs . (**A**) Venn diagram of brute data obtained by MS/MS representing the intersection between detected proteins in BEVs and AnEVs. (**B**) Volcano plot showing all proteins identified. In blue color are showed these statistically significant ($p>0.05$) in the AnEVs (right side) and in the BEVs (left side). Access number (UniProt) for proteins of interest (CDC42, Ficolin-2, S100A9) are shown. Principal component analysis (**C**) and unsupervised hierarchical clustering (**D**) of plasma derived EVs.

FIGURE 3. CDC42, S100A9 and Ficolin-2 are increased in AnEVs.(**A**) Upper panels 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=0.0137, n=15, MW=21KDa), S100A9 (**p=0.0017, n=20, MW=14KDa), Ficolin-2 (**p=0.0092, n=26, MW=34KDa). (**B**) Bottom panels show three representative immunoblots from patients for each protein of interest. MW: Molecular weight

FIGURE 4. Functional protein association networks in AnEVs. Classification of the main cellular localization (**A**) and function (**B**) of the protein panel based on the UniProt database. (**C**) Top Canonical Pathways obtained by IPA and symbols of the molecules. (**D**) Panel illustrate the network established between the dataset of proteins contained in the Ingenuity Canonical pathways. Each color indicates a different canonical pathway. Variations in thickness of the connective lines show the evidence of interactions among proteins.

FIGURE 5. Plasma derived EVs from anaphylactic patients induce loss of the endothelial monolayer resistance. HMVEC-Ls were incubated with 100µg/ml of purified EVs from anaphylactic patients. (**A**) Images show a green PKH67 labeling of AnEVs after 5 hours of cell incubation (magnification 20X). (**B**) Orthogonal image reveals PKH67-EVs around the nuclei. (**C**) Graphic shows the change in TEER measurements after addition of BEVs and AnEVs (n=16 patients). 2 way ANOVA followed by the Bonferroni test was performed: **p= 0.0033, ****p[?] 0.0001 vs Vehicle (EBM + PBS); #p= 0.0285 vs BEVs). (**D**) Individual representation of change in TEER measurements at 5 hours; Unpaired t test; #p= 0.0302 vs BEVs

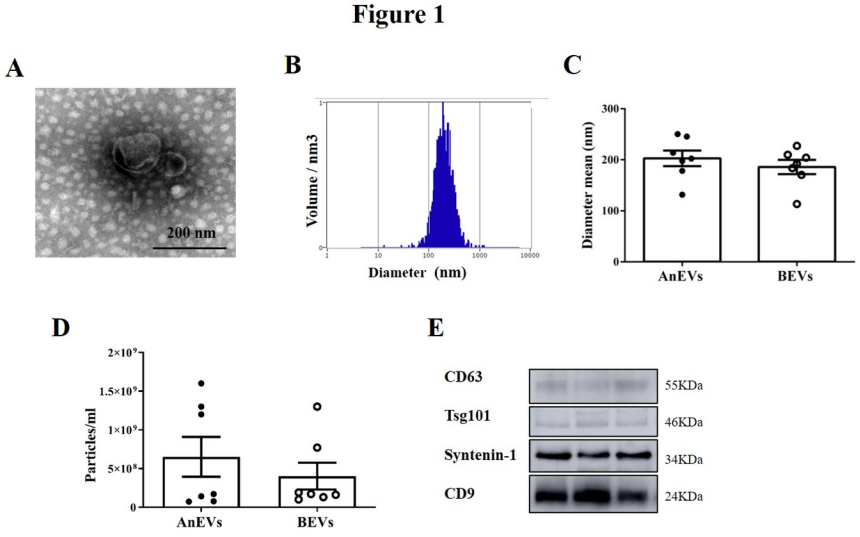


Figure 2

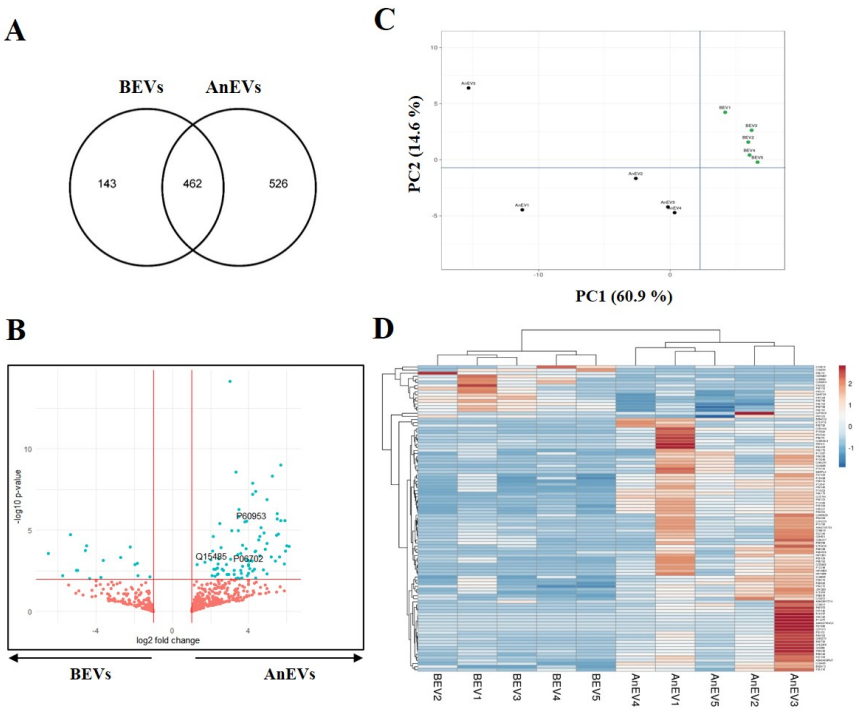


Figure 3

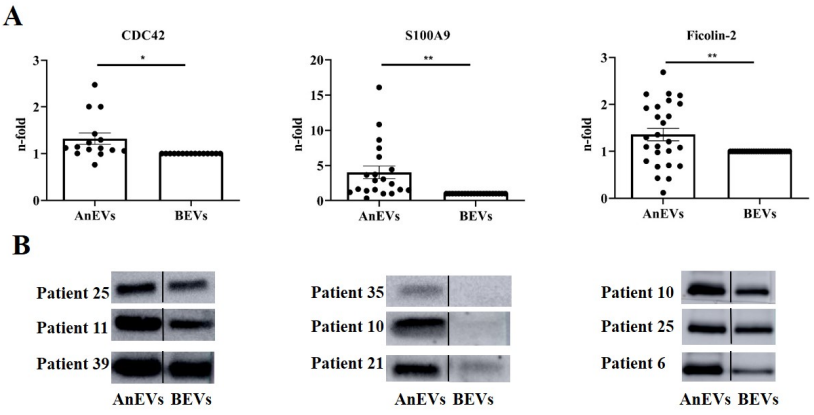


Figure 4

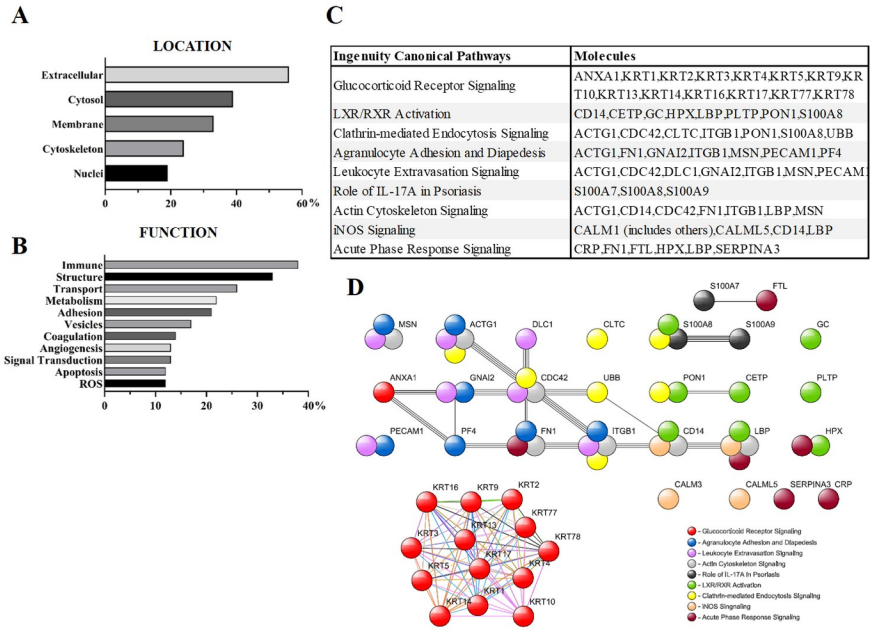
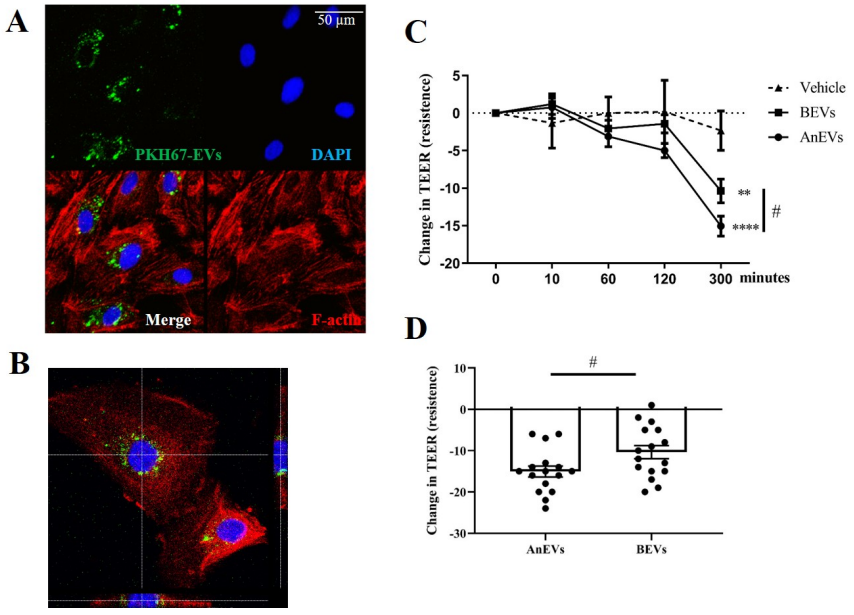


Figure 5



TABLES

Table 1. Clinical features of anaphylactic reactions included in this study. Gender: M (Male), F (Female). ICU: Intensive care unit. Symptoms: Sk (Skin), Mu (Mucous), Di (Digestive), Re (Respiratory), Cv (Cardiovascular), Ne (Neurological). Signs: SBP (Systolic Blood Pressure), DBP (Diastolic Blood Pressure), Hr (Hearth rate), $SatO_2$ (Oxygen Saturation). Treatments: Ep (Epinephrine), β2A (β2-adrenergic

agonist), H1AR (Histamine receptor type 1 antagonist), H2AR (Histamine receptor type 2 antagonist), St (Steroids).

Patients Gender	**Patients Age**	**Patients Etiology**	**Signs and symptoms Sk**	**Signs and symptoms Mu**	**Signs and symptoms Di**	**Signs a Re**
F	44	Drug	x		x	x
M	56	Drug	x	x	x	x
M	42	Food	x		x	x
F	31	Drug	x			
F	81	Drug	x	x		
F	65	Drug	x		x	x
F	51	Drug	x			
F	38	Drug	x			
M	44	Drug	x			
F	31	Drug	x		x	x
F	40	Drug	x	x		
F	49	Drug	x	x	x	x
F	46	Drug	x			x
F	20	Food			x	x
M	57	Others	x	x		x
F	57	Drug	x		x	
F	29	Drug	x			x
M	47	Drug	x	x		x
M	50	Drug	x	x	x	x
F	25	Drug	x			x
M	47	Drug	x			
M	42	Others	x	x		x
F	51	Food	x	x	x	x
F	56	Drug	x			x
F	49	Drug	x			x
M	51	Drug	x		x	
M	73	Others	x			x
M	22	Others	x	x		x
F	25	Others	x	x		x
F	39	Food	x		x	
F	22	Drug	x		x	x
F	33	Food	x	x		x
M	62	Drug		x	x	x
F	47	Drug	x		x	x
F	76	Drug	x		x	x
M	45	Food	x	x	x	x
F	36	Food	x	x	x	x
F	25	Food	x			x
M	33	Drug			x	x
F	37	Drug	x		x	x
F	49	Others	x	x	x	x
M	69	Drug	x	x		x
F	70	Drug	x			