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

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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 1**.

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 (100-250 nm) (**Figure 1A-B**). These vesicles exhibited markers previously related to EVs

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(**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 2**). 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 IFN γ , IL-6, TNF α , 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 (**Figures 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 (**Figures 2C and Supplementary Table 3**).

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 (**Figures 2D**). However, AnEVs uptaken-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 1**).

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 AnEV is a great resource for the allergy community.

Conflict of interest statement:

There are no conflicts of interest.

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Conflicts of interest

Dr. Nuñez-Borque has nothing to disclose.

Dr. Fernandez-Bravo has nothing to disclose.

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Legend to figures

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: 200nm. 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 ($p > 0.05$) 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.

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 (* $p=0.0137$, $n=15$, MW=21KDa), S100A9 (** $p=0.0017$, $n=20$, MW=14KDa), Ficolin-2 (** $p=0.0092$, $n=26$, MW=34KDa). (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 $\mu\text{g/ml}$ 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: ** $p=0.0033$, **** $p \leq 0.0001$ vs Vehicle (EBM + PBS); # $p=0.0285$ vs BEVs).

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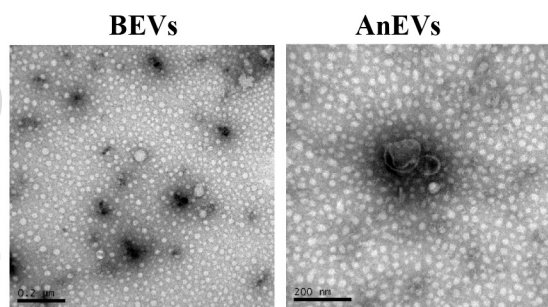
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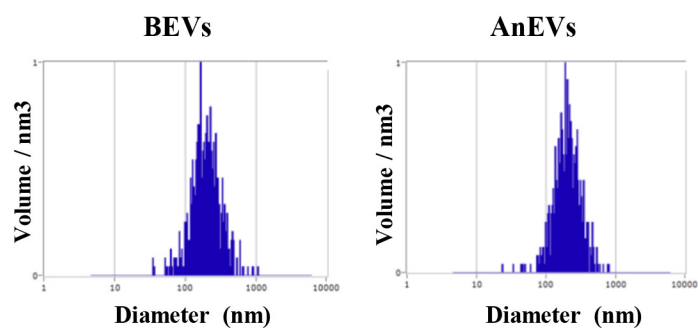
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Figure 1

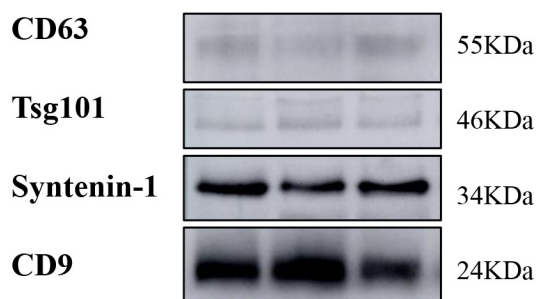
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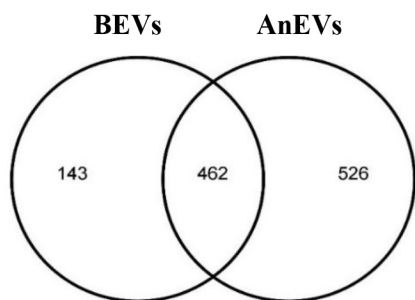
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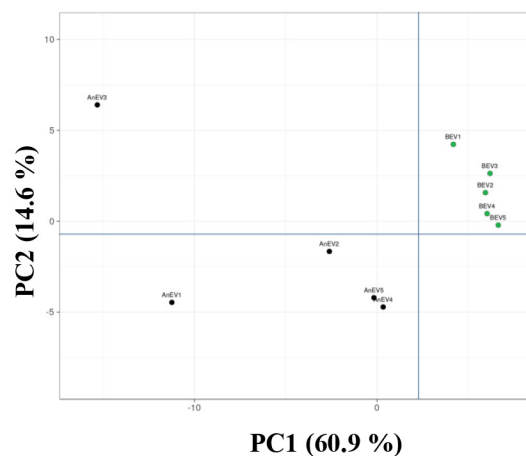
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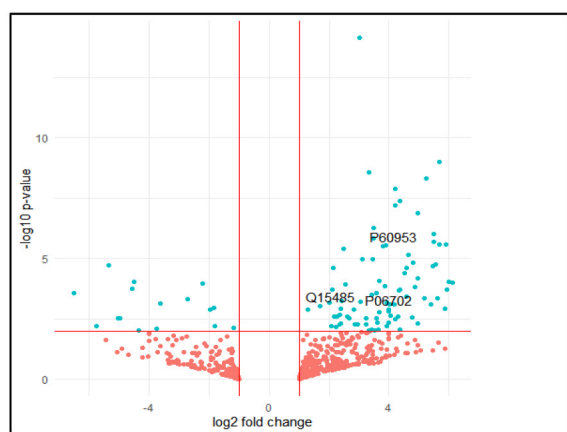
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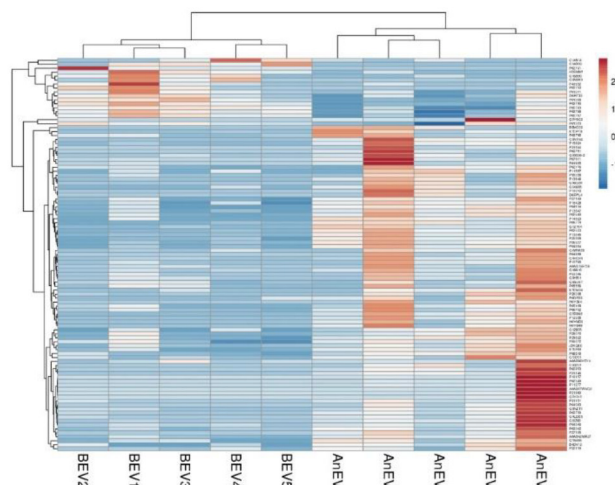
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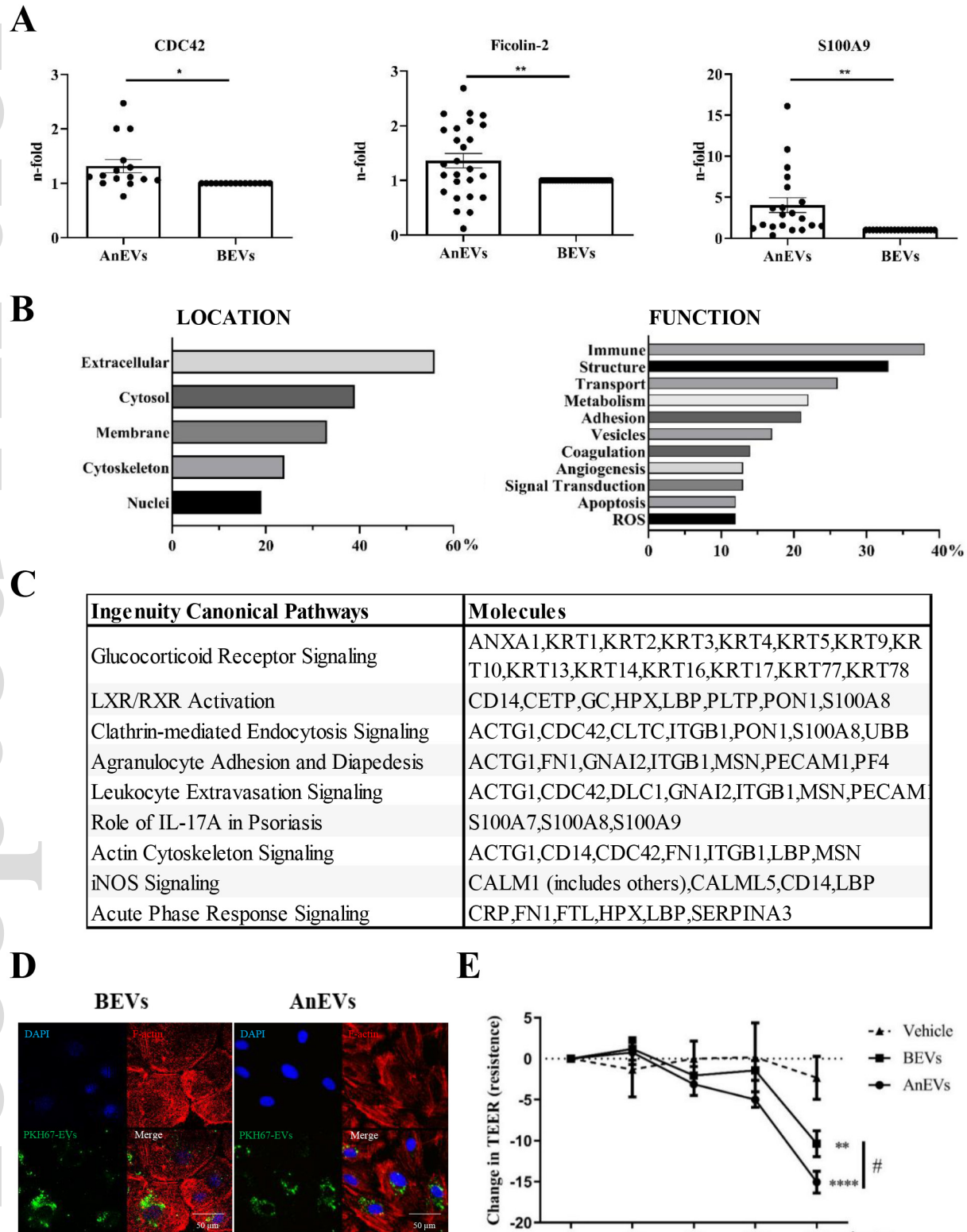


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BEVs AnEVs
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Figure 2



SUPPORTING INFORMATION

Supplementary Material and Methods

Patient selection

The studied population included 86 paired peripheral blood samples (plasma) from 43 patients presenting anaphylaxis as a result of provocation tests and/or collected in the emergency departments and allergy units of the Fundación Jiménez Díaz and Hospital Central de la Cruz Roja; Madrid, Spain. Patients were followed up at the clinic, where an allergy work-up was performed as needed (skin prick tests, specific IgE measurements, and/or challenge tests) and the diagnosis was confirmed by an allergist. Serum tryptase levels were measured using UniCAP (Thermo Scientific).

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 the reaction. Patients defined as presenting anaphylaxis according to the 2006 National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network criteria were included [1]. For sampling, the inclusion and exclusion criteria detailed in the approved ethical protocols were considered. The study authors adhered to the tenets of the Declaration of Helsinki and patients were included after providing informed consent. The protocol was approved by the FJD Ethics Committee (PIC38/2016_FJD and PIC142/2016_FJD).

EV purification

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

Electron microscopy

Electron microscopy was carried out from 2 µg of EVs that were fixed in PBS-formaldehyde 2% (v/v) for 16 hours at 4°C and shaken. Particles were adsorbed on copper-carbon grids (copper-formvar/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 80 kV. 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 Solmeglaz Biotechnology Company (Spain). EV pellets were diluted in PBS to a final volume of 1 ml. Optimal concentrations were determined by pre-testing the particle per frame value (140–200 particles/frame). The manufacturer's default software settings for EVs (& liposomes) or nanospheres were selected accordingly. For each measurement, two cycles were performed by scanning 11 cell positions each and capturing 60 frames per position (video setting: high).

Western blot analysis

Total protein concentration of EVs was determined with the Bradford Protein Assay Kit (ThermoFisher) [2]. Samples were lysed in reducing conditions with β-mercaptoethanol and 30 µl of EVs were loaded per lane. EVs were analyzed by Western Blot with primary antibodies 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).

Digestion and Pre-Fractions of peptides

For protein 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. Briefly, proteins were reduced with 10 mM DTT for 1 hour at 37°C, then alkylated with 55 mM iodoacetamide for 1 hour at RT in darkness and finally digested with a 1/25 (w/w) ratio of recombinant trypsin (Trypsin sequencing grade; Roche) overnight at 37°C.

The tryptic peptides were pre-fractionated with High pH Reversed-Phase Peptide Fractionation Kit according to manufacturer (Thermo Scientific) instructions. The eluted peptides from 7 fractions were re-suspended in 12 µl of 2% acetonitrile/ 0.1% formic acid before nLC-MS/MS analysis. The supernatants were stored at -20°C until use.

Liquid Chromatography and mass spectrometer analysis

Peptide fractions were analyzed by reverse-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).

Peptides were first loaded onto an Acclaim PepMap 100 Trapping column (Thermo Scientific, 20 mm × 75 µm ID, 3 µm C18 resin with 100Å pore size) using buffer A (mobile phase A: 2% acetonitrile, 0.1% formic acid), and then separated on a C18 resin analytical column Easy Spray Column (Thermo Scientific, 500 mm × 75 µm ID, 2 µm C18 resin with 100Å pore size) with an integrated spray tip. Separation took place in a 120-minute gradient of 5% to 35% Buffer B (100% acetonitrile, 0.1% formic acid) in Buffer A at a constant flow rate of 250 nl/min.

Data acquisition was performed with a Q-Exactive HF. All data were acquired using data-dependent acquisition (DDA) and in positive mode with Xcalibur 4.0 software. The MS1 scans were acquired at an m/z range of 350–1,700 Da with mass resolution of 60,000 and automatic gain control (AGC) target of 3E6 at a maximum Ion Time (ITmax) of 20 milliseconds. The threshold to trigger MS2 scans was 1.2E3. For MS2 scan, we selected the 15 most abundant precursors with charges of 2 to 4+ in MS1 scan with a dynamic exclusion of 20 seconds for higher energy collisional dissociation (HCD) fragmentation. The normalized collision energy (NCE) was 20%. The resolved fragments were scanned at mass resolution of 30,000 and in an ITmax of 100 milliseconds.

Protein Quantification and Data Analysis

Peptide identification from raw data was carried out using Mascot v. 2.6.1 search engine through the Protein Discoverer 2.3 Software (Thermo Scientific) to generate Mascot result files. A database search was performed against Human Proteome UP000005640 (93804 sequences) and contaminant database (247 sequences). The following parameters were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites allowed, and tolerances of 10 ppm for precursor ions and 0.02 Da for MS/MS fragment ions. The searches allowed for optional methionine oxidation and methionine loss plus acetyl protein N-terminal and fixed carbamidomethylation of cysteine. A search against a decoy database (integrated decoy approach) was used to FDR calculate. The Mascot Scores were adjusted by a percolator algorithm. The acceptance criteria for protein identification were FDR < 1% and at least one unique peptide identified with high confidence (CI>95%).

Protein quantification was performed by spectral counting with Proline 2.0 software (PL) [3]. Spectral counting (SC) was considered for the statistical analysis and imported to R. This method consists in counting the number of spectra which match peptides to measure the abundance of the protein to which they belong in samples. Only the total number of PSMs that identify the specific peptides belonging to a protein or a protein group were considered (named Specific SC in Proline) and shared peptides by different protein groups are excluded from identification. Differential statistical analysis applied with our data is proposed by Pham *et al.* 2010 [4], using paired the beta nominal test between two conditions. Proteins were considered statistically differential between two groups visualized in volcano plot with: at least 6 spectral counts summed in each group, p value lower than 0.01, and fold change (FC) higher than 1.5.

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. The gene list contained in the main canonical pathways observed by IPA was 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 on endothelial barrier integrity was evaluated using Transwell 24-well cell culture inserts (TWs) as previously described [2]. HMVEC-L *in vitro* TW systems were incubated with 50 and 100 µg/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.

Acting as a control for 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 (Supplementary Figure 1).

PKH67 EVs staining , immunofluorescence, and quantification

AnEVs were stained with 100 µl of the green fluorescent membrane dye PKH67 4 µM (Sigma). After 5 minutes at RT, the reaction was stopped and the mix was incubated for 1 minute at RT with 200 µl of 5% BSA in PBS (Sigma). Then, an Exosome Spin Column (Invitrogen) was used to eliminate the staining excess. This column was previously hydrated with 600 µl of a 5-mM EDTA/PBS solution for 15 minutes at RT after shaking it to remove air bubbles. Subsequently, to eliminate the excess of interstitial liquid, the spin column was centrifuged at 3,000 rpm for 2 minutes at RT. The column was placed in a 1.5-ml Eppendorf and 100 µl of the stained EV solution was added, followed by a similar centrifugation. Stained vesicles were obtained in the eluted fraction.

To evaluate the interaction between BEVs/AnEVs and endothelial cells (EC), *in vitro* confocal microscopy was performed. HMVEC-L were seeded in P8 chambers (IBIDI) and incubated with PKH67-EVs for 5 hours. Next, cells were fixed with 4% paraformaldehyde for 15 minutes at RT, washed in PBS-Tween 0.1% solution, permeabilized with Triton 0.1% for 5 minutes at 4°C, and blocked with 2% BSA for 30 minutes at RT. After the cells were rinsed in PBS-Tween, they were incubated with Texas Red-X phalloidin (1:40) for 30

minutes followed by a 10-minute incubation with DAPI (1:1000). Images were acquired in an inverted confocal microscope (LSM700; Carl Zeiss, Oberkochen, Germany). Quantification of PKH67 EV uptake was carried out by using Image J software.

Statistical Analysis

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

SUPPLEMENTAL TABLES AND FIGURES

Supplementary Table 1. Clinical features of anaphylactic reactions included in this study. The studied population included 43 anaphylactic patients ranging in age from 20-76 years (45.2 ± 2.3); 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 the classification by Brown [5]. The mild group was grade 1 (12% of the population), moderate was grade 2 (56%), and severe was grade 3 (32%). Severe anaphylaxis includes those with cardiovascular involvement and hypotension. The percentage of drugs administered was as follows: epinephrine (53%), β 2-adrenergic agonist (14%), histamine receptor antagonist type 1 (65%) and 2 (21%), and steroids (67%). In addition, 39% of patients presented elevated tryptase ($>11.4 \mu\text{g/l}$) [6] in the acute phase, with a mean value of $11.8 \pm 1.8 \mu\text{g/l}$ in our group. The columns titled “used to” indicate experimental utility of each EV patient paired of samples by means of an “x”. Numbers (¹⁻⁵) indicate the pool of EVs analysed by MS/MS and letters (^{a-g}) indicate the patient used by NTA. 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 (heart rate), SatO₂ (oxygen saturation). Treatments: Ep (epinephrine), β 2A (β 2-adrenergic agonist), H1RA (histamine 1-receptor antagonist), H2RA (histamine 2-receptor antagonist), St (Steroids). Used to: MS/MS, NTA, Validations (S100A, Ficolin-2, and/or CDC42), *in vitro* (vascular permeability).

Extracellular vesicles in anaphylaxis

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Patients			Signs and symptoms										Tryptase		Treatments					Used to				
Gender	Age	Etiology	Sk	Mu	Di	Re	Cv/Ne	SBP	DBP	Hr	SatO2	Severity	Acute	Basal	Ep	β2A	H1 AR	H2 AR	Cs	MS/MS	NTA	Validations	in vitro	
F	44	Drug	x		x	x		-	-	-	-	2	4.2	6.07			x		x				x	
M	56	Drug	x	x	x	x		160	90	93	93%	3	8.47	4.07									x	
M	42	Food	x		x	x	x	-	-	-	-	3	14.2	4.85						x ¹	x	x	x	
F	31	Drug	x					109	-	94	98%	1	2.74	3.85			x	x	x			x		
F	81	Drug	x	x				150	69	68	98%	1	6.65	7.94			x		x			x		
F	65	Drug	x		x	x	x	123	72	68	95%	2	6.94	5.37									x	
F	51	Drug	x				x	120	80	90	95%	2	24.9	9.33			x		x		x ⁵			
F	38	Drug	x					120	70	80	98%	1	12.5	13.3							x ⁵			
M	44	Drug	x				x	161	127	102	98%	2	12.8	4.87							x ⁴			
F	31	Drug	x		x	x		93	71	72	88%	2	3.92	5.43	x		x		x			x	x	
F	40	Drug	x	x				120	60	70	99%	1	5.56	5.51								x		
F	49	Drug	x	x	x	x		130	80	70	94%	2	5.32	5.82	x				x			x		
F	46	Drug	x			x	x	-	-	-	-	3	27.2	3.45	x		x	x	x		x ³	x	x	x
F	20	Food			x	x	x	110	45	105	66	3	17.6	6.18					x		x ²	x	x	x
M	57	Others	x	x		x		136	100	100	95%	2	15.6	8.18							x ¹			
F	57	Drug	x		x		x	-	-	-	-	2	5.67	3.26	x		x		x			x		
F	29	Drug	x			x	x	96	69	70	99%	3	3.06	3.36									x	
M	47	Drug	x	x		x	x	75	55	112	98%	3	6.19	5.99	x		x		x			x		
M	50	Drug	x	x	x	x	x	90	60	90	92%	3	12	8.63	x		x		x		x ⁴	x	x	
F	25	Drug	x			x		110	60	85	-	2	3.6	4.74	x	x	x					x	x	
M	47	Drug	x				x	170	80	78	89%	3	9.8	6.77	x			x	x			x		
M	42	Others	x	x		x		129	68	70	97%	2	8.12	5.94	x	x	x		x			x		
F	51	Food	x	x	x	x		132	80	70	97%	2	7.98	4.07	x		x		x			x	x	
F	56	Drug	x			x	x	190	100	130	70%	3	7.6	6.16	x		x	x	x			x		
F	49	Drug	x			x		145	95	103	98%	2	3.12	3.69		x	x		x			x	x	
M	51	Drug	x		x		x	110	80	-	98%	2	4.25	3.91	x		x		x			x		
M	73	Others	x			x	x	ICU	-	-	-	3	13	4.38			x	x	x		x ²	x		
M	22	Others	x	x		x		127	73	71	98%	2	28.6	4.94	x		x		x		x ³	x	x	x
F	25	Others	x	x		x	x	136	75	110	-	3	18	3.74	x		x	x	x			x	x	
F	39	Food	x		x			-	-	-	-	2	8.64	7.41								x	x	
F	22	Drug	x		x	x	x	120	75	130	91%	2	5.56	4.49	x		x					x		
F	33	Food	x	x		x		123	78	93	98%	2	12.6	7.2	x		x	x	x			x		
M	62	Drug		x	x	x		165	82	98	96%	2	7.36	6.01	x		x	x	x			x	x	
F	47	Drug	x		x	x	x					3	15.2	8.48									x	
F	76	Drug	x		x	x	x	91	49	76	93%	3	48	7.83		x	x		x			x		
M	45	Food	x	x	x	x		131	94	84	98%	2	18.4	6.87	x		x	x	x			x		
F	36	Food	x	x	x	x	x	108	75	100	98%	2	4.34	3.75	x							x		
F	25	Food	x			x	x	-	-	132	96%	2	6.56	3.98			x		x			x		
M	33	Drug			x	x		125	106	128	95%	2	5.34	4.38	x	x	x		x			x		
F	37	Drug	x		x	x	x	150	90	100	94%	2	10.8	7.35	x							x		
F	49	Others	x	x	x	x	x	97	60	-	98%	2	14.8	3.28	x		x		x			x		
M	69	Drug	x	x		x	x	50	40	120	80%	3	45.5	4.68	x	x	x		x			x		
F	70	Drug	x					158	78	93	97%	1	3.75	4.33			x		x			x		

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Supplementary Table 2. Panel of the 99 statistically significant differential proteins detected in anaphylaxis-derived EVs. FC: Fold change (AnEVs/BEVs)

Symbol	Gene	Name	Specific peptides	Log2 Ratio (Fc)	p-value
P19013	KRT4	Keratin, type II cytoskeletal 4	10	99999,96	1,98E-06
P60953	CDC42	Cell division control protein 42 homolog	4	99804,39	3E-06
Q5D862	FLG2	Filaggrin-2	11	99652,1	9,705E-07
Q8N1N4	KRT78	Keratin, type II cytoskeletal 78	7	98281,25	1,466E-05
P31146	CORO1A	Coronin-1A	6	97102,85	9,976E-05
P12035	KRT3	Keratin, type II cytoskeletal 3	2	97042,07	9,405E-05
P02549	SPTA1	Spectrin alpha chain, erythrocytic 1	55	215,8767	6,284E-08
P11277	SPTB	Spectrin beta chain, erythrocytic	48	167,1398	5,289E-07
P27105	STOM	Erythrocyte band 7 integral membrane protein	8	84,39976	1,404E-06
K7EPT8	GFAP	Glial fibrillary acidic protein (Fragment)	1	61,5786	7,421E-15
H0YMD0	ANXA2	Annexin (Fragment)	9	35,6317	0,0001989
Q7Z794	KRT77	Keratin, type II cytoskeletal 1b	9	35,31161	2,562E-06
P15924	DSP	Desmoplakin	43	34,06902	9,545E-10
H0Y5H9	SERPINB4	Serpin B4 (Fragment)	7	31,01294	0,0004461
P31944	CASP14	Caspase-14	11	28,01735	0,0004532
P02751	FN1	Fibronectin	3	23,78926	0,0027645
P13646	KRT13	Keratin, type I cytoskeletal 13	8	22,87735	2,369E-05
H0Y394	HDLBP	Vigilin (Fragment)	4	22,85756	0,0003789
P04899	GNAI2	Guanine nucleotide-binding protein G(i) subunit alpha-2	9	20,91872	0,0007712
D6RFL4	CD14	Monocyte differentiation antigen CD14 (Fragment)	5	20,02135	0,0007868
B5MC02	STEAP2	Metalloreductase STEAP2	2	17,55204	0,0012215
P26038	MSN	Moesin	21	16,68444	1,676E-05
P07911	UMOD	Uromodulin	13	16,16272	0,0007895
P02533	KRT14	Keratin, type I cytoskeletal 14	18	15,72125	4,665E-09
Q8IZ83	ALDH16A1	Aldehyde dehydrogenase family 16 member A1	20	14,87132	1,312E-07
Q9HD89	RETN	Resistin	5	14,82815	6,681E-05
P08779	KRT16	Keratin, type I cytoskeletal 16	22	13,10514	4,052E-08
A0A087WVQ6	CLTC	Clathrin heavy chain	21	12,9159	0,0025893
P02730	SLC4A1	Band 3 anion transport protein	21	12,65532	1,27E-08
P81605	DCD	Dermcidin	2	12,18108	0,0022146
Q96QB1	DLC1	Rho GTPase-activating protein 7	2	11,7656	0,0060257
Q4LDE5	SVEP1	Sushi, von Willebrand factor type A, EGF and pentraxin domain	27	11,59297	0,0001349
P04083	ANXA1	Annexin A1	6	10,95036	0,0089157
O00231	PSMD11	26S proteasome non-ATPase regulatory subunit 11	5	10,53407	0,0096541
Q04695	KRT17	Keratin, type I cytoskeletal 17	12	10,37407	1,069E-05
Q00536-2	CDK16	Isoform 2 of Cyclin-dependent kinase 16	2	10,30638	0,0003249
P13645	KRT10	Keratin, type I cytoskeletal 10	50	9,940046	2,712E-09
P14923	JUP	Junction plakoglobin	22	9,321253	1,029E-05
K7EM38	ACTG1	Actin, cytoplasmic 2 (Fragment)	1	9,146439	0,0006388
P10720	PF4V1	Platelet factor 4 variant	1	8,51651	0,0013325
P21980	TGM2	Protein-glutamine gamma-glutamyltransferase 2	12	8,013709	0,0029686
Q16610	ECM1	Extracellular matrix protein 1	11	7,824185	0,0024044
Q9NZT1	CALML5	Calmodulin-like protein 5	10	7,623431	0,0001201
A0A0A0MRJ7	F5	Coagulation factor V	52	7,452809	4,007E-06
Q9Y6C2	EMILIN1	EMILIN-1	16	7,303118	0,0005677
Q9Y315	DERA	Deoxyribose-phosphate aldolase	13	7,213505	0,0050208
P53396	ACLY	ATP-citrate synthase	17	7,151889	0,0012224
P16157	ANK1	Ankyrin-1	14	7,070543	0,005273
P0DP23	CALM1	Calmodulin-1	4	6,814475	0,0025553
P55058	PLTP	Phospholipid transfer protein	5	6,693328	0,0065592

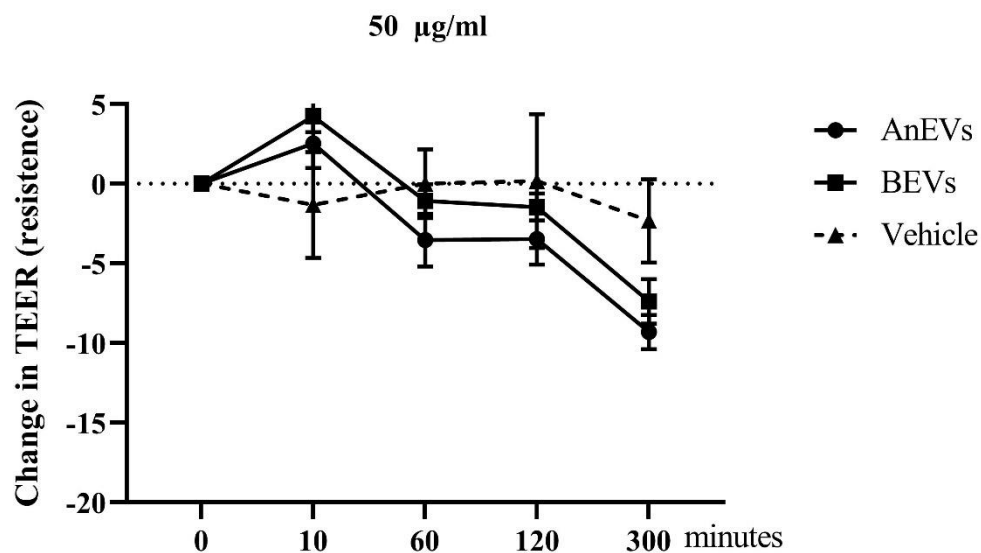
Symbol	Gene	Name	Specific peptides	Log2 Ratio (Fc)	p-value
A0A075B738	PECAM1	Platelet endothelial cell adhesion molecule	16	6,501658	0,0025363
P31151	S100A7	Protein S100-A7	9	6,280955	0,0001895
P05109	S100A8	Protein S100-A8	11	6,024987	0,0006555
P08519	LPA	Apolipoprotein(a)	42	5,453327	2,099E-05
B4DV12	UBB	Polyubiquitin-B	3	4,964349	0,0047538
P13647	KRT5	Keratin, type II cytoskeletal 5	18	4,872108	0,0001555
P35527	KRT9	Keratin, type I cytoskeletal 9	78	4,646119	6,729E-06
P35908	KRT2	Keratin, type II cytoskeletal 2 epidermal	57	4,520535	3,975E-05
P27169	PON1	Serum paraoxonase/arylesterase 1	14	4,357064	0,00019
P28070	PSMB4	Proteasome subunit beta type-4	6	4,35054	0,0087652
P06702	S100A9	Protein S100-A9	13	4,330399	0,0002036
Q9HBI1	PARVB	Beta-parvin	8	4,20526	0,0032089
Q8WWZ8	OIT3	Oncoprotein-induced transcript 3 protein	14	4	0,0015549
P11597	CETP	Cholesteryl ester transfer protein	11	4	0,0012775
Q12805	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	8	4	0,0046342
P18428	LBP	Lipopolysaccharide-binding protein	11	3,936541	0,0006501
P04264	KRT1	Keratin, type II cytoskeletal 1	71	3,900468	2,772E-06
P04040	CAT	Catalase	36	3,677063	0,0016152
Q13201	MMRN1	Multimerin-1	33	3,668466	8,09E-05
P29622	SERPINA4	Kallistatin	11	3,62269	0,0045007
J3KQ66	RELN	Reelin	30	3,570716	0,0028897
P04114	APOB	Apolipoprotein B-100	264	3,568745	0,0002572
P32119	PRDX2	Peroxisredoxin-2	9	3,426252	0,0089584
P02792	FTL	Ferritin light chain	12	3,335186	0,0093918
K7ER19	APOC1	Apolipoprotein C-I (Fragment)	6	3,235332	0,0029086
P02776	PF4	Platelet factor 4	4	3,218949	0,005254
P55072	VCP	Transitional endoplasmic reticulum ATPase	44	2,958376	0,0050836
P02042	HBD	Hemoglobin subunit delta	6	2,845074	0,0052127
Q86UX7	FERMT3	Fermitin family homolog 3	30	2,351766	0,0020431
P02649	APOE	Apolipoprotein E	29	2,152154	2,44E-05
P05556	ITGB1	Integrin beta-1	16	2,073517	0,0060058
Q15485	FCN2	Ficolin-2	20	1,696653	0,0009602
A0A286Y4Y4	IGHG2	Immunoglobulin heavy constant gamma 2 (Fragment)	12	1,282761	0,0013366
P01023	A2M	Alpha-2-macroglobulin	144	-1,177754	0,0072036
P01011	SERPINA3	Alpha-1-antichymotrypsin	15	-1,803576	0,0061578
P02768	ALB	Serum albumin	84	-1,854315	0,001107
D6RF35	GC	Vitamin D-binding protein	30	-1,971534	0,0012864
P02787	TF	Serotransferrin	58	-2,220968	0,0001087
P01008	SERPINC1	Antithrombin-III	26	-3,633864	0,0007471
O14514	ADGRB1	Adhesion G protein-coupled receptor B1	4	-4,338067	0,0091217
P02790	HPX	Hemopexin	23	-4,554568	0,0001838
P43652	AFM	Afamin	14	-4,960374	0,0030167
P02763	ORM1	Alpha-1-acid glycoprotein 1	6	-5,35757	1,89E-05
Q16880	UGT8	2-hydroxyacylsphingosine 1-beta-galactosyltransferase	1	-8,145471	0,0004877
P02750	LRG1	Leucine-rich alpha-2-glycoprotein	6	-11,24569	0,0081574
H3BMM1	MYO9A	Unconventional myosin-Ixa	2	-13,5409	8,98E-05
Q8N8K9	KIAA1958	Uncharacterized protein KIAA1958	2	-15,08366	0,0028649
O94993	SOX30	Transcription factor SOX-30	1	-17,21976	0,0063023
P02741	CRP	C-reactive protein	4	-96908,15	0,0002779

213 **Supplementary Table 3.** Disease or function annotation obtained from IPA.

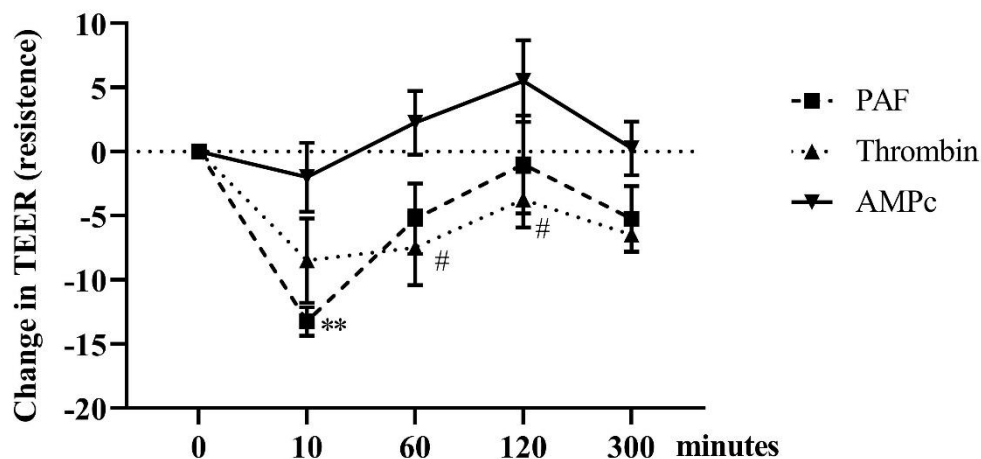
Diseases or Functions Annotation	p-value	Molecules	Total Molecules
Degranulation of cells	1,79E-22	ACLY,ANXA1,ANXA2,CALM3,CALML5,CAT,CD14,CORO1A,DERA,DSP,ECM1,F5,FERMT3,FLG2,FN1,FTL,JUP,KRT1,LRG1,MMRN1,PECAM1,PF4,PSMD11,RETN,S100A7,S100A8,S100A9,SERPINA3,SERPINA4,STOM,VCP	31
Degranulation of neutrophils	1,51E-18	ACLY,ANXA1,ANXA2,CALML5,CAT,CD14,DERA,DSP,FLG2,FTL,JUP,KRT1,LRG1,PECAM1,PF4,PSMD11,RETN,S100A7,S100A8,S100A9,SERPINA3,STOM,VCP	23
Keratinization of epidermis	1,91E-18	CASP14,DSP,JUP,KRT1,KRT10,KRT13,KRT14,KRT16,KRT17,KRT2,KRT3,KRT4,KRT5,KRT77,KRT78,KRT9	16
Formation of skin	1,69E-17	ANXA1,CALML5,CASP14,DSP,FLG2,FN1,ITGB1,JUP,KRT1,KRT10,KRT13,KRT14,KRT16,KRT17,KRT2,KRT3,KRT4,KRT5,KRT77,KRT78,KRT9,S100A7,SVEP1,TGM2	24
Degranulation of phagocytes	2,97E-17	ACLY,ANXA1,ANXA2,CALML5,CAT,CD14,CORO1A,DERA,DSP,FLG2,FTL,JUP,KRT1,LRG1,PECAM1,PF4,PSMD11,RETN,S100A7,S100A8,S100A9,SERPINA3,STOM,VCP	24
Adhesion of blood cells	9,1E-16	ADGRB1,ANXA1,CD14,CDC42,CORO1A,CRP,FERMT3,FN1,GNAI2,ITGB1,LBP,MSN,PECAM1,PF4,PLTP,PRDX2,S100A8,S100A9,SLC4A1,TGM2,UMOD	21
Binding of blood cells	1,02E-15	ADGRB1,ANXA1,CD14,CDC42,CORO1A,CRP,F5,FERMT3,FN1,GNAI2,ITGB1,LBP,MSN,PECAM1,PF4,PLTP,PRDX2,S100A8,S100A9,SLC4A1,TGM2,UMOD	22
Adhesion of immune cells	3,19E-15	ADGRB1,ANXA1,CD14,CDC42,CORO1A,CRP,FERMT3,FN1,GNAI2,ITGB1,LBP,MSN,PECAM1,PF4,PLTP,PRDX2,S100A8,S100A9,TGM2,UMOD	20
Inflammation of organ	3,27E-15	ANXA1,CAT,CD14,CORO1A,CRP,DCD,DSP,ECM1,F5,FCN2,FN1,GC,GNAI2,HPX,ITGB1,JUP,KRT1,KRT10,KRT14,KRT16,KRT17,KRT5,LBP,LRG1,MSN,PECAM1,PF4,PON1,PRDX2,S100A7,S100A8,S100A9,SERPINA3,SERPINB4,TGM2,UMOD,VCP	37
Immediate hypersensitivity	4,85E-15	ANXA1,CAT,CORO1A,DCD,ECM1,JUP,KRT1,KRT10,KRT16,KRT17,KRT5,LBP,MSN,PECAM1,S100A7,S100A8,S100A9,SERPINB4,VCP	19
Binding of myeloid cells	1,36E-14	ADGRB1,ANXA1,CD14,CORO1A,CRP,FERMT3,FN1,ITGB1,LBP,MSN,PECAM1,PF4,PLTP,PRDX2,S100A8,S100A9	16
Allergy	8,36E-14	ANXA1,CAT,CORO1A,DCD,ECM1,JUP,KRT1,KRT10,KRT16,KRT17,KRT5,LBP,MSN,PECAM1,S100A7,S100A8,S100A9,SERPINB4,TGM2,VCP	20

Supplementary Figure 1. A) The graphic shows the change in TEER measurements after addition of 50 $\mu\text{g/ml}$ of BEVs and AnEVs (n=13 patients). We performed a 2-way ANOVA followed by the Bonferroni test. B) TEER measurements in response to vasoactive agents (PAF and thrombin) and the endothelial barrier stabilizers (cAMP) in the HMVEC-L monolayer (** p= 0.0039, # p $\leq$ 0.05 vs cAMP) n=4.

A



B



Supplemental References

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