

**Neutrophils produce pro-inflammatory or anti-inflammatory extracellular vesicles  
depending on the environmental conditions**

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**Summary sentence:** EVs generated under different physiologically or pathologically relevant conditions from neutrophils exert divergent and selective effects on cells and functions in their environment

**Running title:** Pro- or anti-inflammatory EVs from PMN

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### **Abbreviations**

EV: extracellular vesicle

aEV: antibacterial EV

apoEV: apoptotic EV

sEV: spontaneous EV

PMN: polymorphonuclear cell (here: neutrophilic granulocyte)

IL-1 $\beta$ : interleukin 1 beta

TNF $\alpha$ : tumor necrosis factor alpha

IL-6: interleukin 6

IL-8: interleukin 8

IL-10: interleukin 10

IL-12: interleukin 12

HBSS: Hank's balanced salt solution

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

FC: flow cytometry

FSC: forward scatter

SSC: side scatter

CD11b: cluster of differentiation molecule 11b

RPE: R-phycoerythrin

MFI: mean fluorescent intensity

PBS: phosphate-buffered saline

FBS: fetal bovine serum

fMLF: N-Formylmethionyl-leucyl-phenylalanine

ROS: reactive oxygen species

DMSO: dimethyl sulfoxide

PMA: phorbol 12-myristate 13-acetate

HUVEC: human umbilical vein endothelial cells

ELISA: enzyme-linked immunosorbent assay

VCAM-1: vascular cell adhesion molecule 1

BSA: bovine serum albumin

HRP: horseradish peroxidase

IgG: immunoglobulin G

TMB: 3,3',5,5'-tetramethylbenzidine

SEM: standard error of the mean

TP: thromboplastin

## **Abstract**

Extracellular vesicles are important elements of intercellular communication. A plethora of different, occasionally even opposite, physiological and pathological effects has been attributed to these vesicles in the last decade. A direct comparison of individual observations is however hampered by the significant differences in the way of elicitation, collection, handling and storage of the investigated vesicles. In the current work we carried out a careful comparative study on three, previously characterized types of extracellular vesicles produced by neutrophilic granulocytes. We investigated in parallel the modulation of multiple blood-related cells and functions by medium-sized vesicles. We show that extracellular vesicles released from resting neutrophils exert anti-inflammatory action by reducing production of reactive oxygen species and cytokine release from neutrophils. In contrast, vesicles generated upon encounter of neutrophils with opsonized particles, rather promote pro-inflammatory processes as they increase production of reactive oxygen species and cytokine secretion from neutrophils and activate endothelial cells. Extracellular vesicles released from apoptosing cells were mainly active in promoting coagulation. We thus propose that extracellular vesicles are “custom made”, acquiring selective capacities depending on environmental factors prevailing at the time of their biogenesis.

## Introduction

Generation of extracellular vesicles (EV) is a common property of cells. Intensive research of the last decade has revealed a multitude of different biological – both physiologic and pathologic – effects of EVs.<sup>1,2</sup> Following a significant number of preclinical studies<sup>3,4</sup> initial attempts of therapeutic applications using EVs or EV-related drug delivery have started.<sup>5,6</sup> However, comparative data on specificity and selectivity of the effect of defined EV populations are still scarce.<sup>7-10</sup>

Neutrophilic granulocytes (PMN) represent the most abundant population of leukocytes in circulating blood. As they are active in formation of EVs, PMN-derived EVs constitute a large fraction of EVs in normal blood. The number of PMN-derived EVs was reported to become significantly elevated in various pathologic conditions.<sup>2,11,12</sup> The effects of neutrophil-derived EVs have been extensively investigated on almost every blood-related cell type and function, including neutrophils themselves,<sup>13-16</sup> monocytes,<sup>17</sup> monocyte-derived macrophages<sup>17-25</sup> and dendritic cells,<sup>26</sup> lymphocytes,<sup>27</sup> endothelial cells<sup>28,29</sup> and coagulation.<sup>30</sup>

Most studies demonstrated dominant anti-inflammatory effect of PMN-EVs on the interacting cells, by decreasing the production of activating cytokines such as IL-1 $\beta$ , TNF $\alpha$ , IL-6, IL-8, IL-10 or IL-12<sup>17-21,26</sup> and increasing the secretion of TGF $\beta$  or resolving mediators.<sup>17,20,25,26</sup>

Opposing effects have also been reported, such as an increase in IL-6 and IL-8 production from, and expression of adhesion molecules on, endothelial cells;<sup>29</sup> enhanced superoxide, IL-6 and TNF $\alpha$  secretion from macrophages<sup>13,18</sup> and stimulation of LTB<sub>4</sub> synthesis in neutrophils.<sup>15</sup> Enhanced coagulation has also been reported.<sup>30</sup> These studies typically investigated the effects of PMN-EVs on one single cell type or function. A wide variety of EVs were applied, including true exosomes<sup>15</sup> and microvesicles/ectosomes produced spontaneously or upon various stimuli.<sup>13,17-21,25,26,29,30</sup> However, the differences between the

effects of differently produced EVs were only rarely analyzed.<sup>18,31</sup> Lastly, in many investigations EVs were stored frozen for undefined periods.

In previous work our group has characterized three different types of PMN-EVs in detail: those produced spontaneously in short incubation from resting cells (sEV), those produced by apoptotic cells in one to three days (apoEV), and those generated upon stimulation with opsonized particles.<sup>12,32</sup> Only the latter EV population was able to impair bacterial growth in a concentration dependent manner,<sup>12,33</sup> hence they were named “antibacterial EVs” (aEV). However, anti-bacterial capacity was lost under different storage conditions in a relatively short time.<sup>34</sup> In several tests sEV and apoEV were more similar to each other than either of them to aEV.<sup>32</sup> Hence the question arises whether sEV, apoEV and aEV only differ in their antibacterial capacity or also in their effects on other blood cells and functions.

The aim of the present study was to compare the effects of three, previously well characterized PMN-EV types, applied freshly after isolation, on cells and function they could affect in their natural environment by autocrine or paracrine mechanisms, such as neutrophils themselves, endothelial cells and coagulation of pooled human plasma. We demonstrate selective effects in all of the investigated functions.

## **Methods**

### ***Materials***

Hank’s balanced salt solution (HBSS) with calcium, magnesium and glucose was from GE Healthcare Life Sciences (South Logan, UT, USA), zymosan A was from Sigma Aldrich (St. Louis, MO, USA), Ficoll-Paque from GE Healthcare Bio-Sciences AB (Uppsala, Sweden), HEPES (pH 7.4) from Sigma. All other used reagents were of research grade.

Green fluorescent protein (GFP) expressing and chloramphenicol resistant *S. aureus* (USA300) was a kind gift from Professor William Nauseef (University of Iowa).

### ***Isolation of human PMN and monocytes***

Venous blood samples were drawn from healthy adult volunteers according to procedures approved by the National Ethical Committee (ETT-TUKEB No. BPR/021/01563-2/2015).

The age and gender distribution of our donors was the following: 32.5% of the donors were women, 67.5% men. Mean age was  $24.8 \pm 6.5$  years; the youngest donor was 19, the oldest 55 years old.

Neutrophils were obtained by dextran sedimentation followed by a 62.5% (v/v) Ficoll gradient centrifugation (Beckman Coulter Allegra X-15R, 1000 g, 20 min, 22°C) as previously described.<sup>35</sup> The mononuclear cell layer (consisting of lymphocytes and monocytes) was extracted by pipetting after the Ficoll gradient centrifugation step.

Contaminating red blood cells were removed by hypotonic lysis. Cells were finally resuspended in HBSS and kept on ice until use. The neutrophil preparations contained more than 95% PMN and less than 0.5% eosinophils.

### ***Opsonization***

Zymosan A (5 mg in 1 mL HBSS) was opsonized with 500  $\mu$ L pre-warmed pooled human serum for 25 min at 37°C. After opsonization, zymosan was centrifuged (5000 g, 5 min, 4°C, Hermle Z216MK 45° fixed angle rotor), and washed once in HBSS.

USA300 bacteria ( $OD_{600}=1.0$  in 900  $\mu$ L HBSS) were opsonized with 100  $\mu$ L pre-warmed pooled human serum for 25 min at 37°C. After opsonization, bacteria were centrifuged (5000 g, 5 min, 4°C), and washed once in HBSS.

### ***Preparation of EV fractions***

PMN ( $10^7$  cells in 1 mL HBSS) were left unstimulated or were activated by 0.5 mg/mL final concentration of opsonized zymosan A for 20 min at 37°C in a linear shaker (80 rpm).

Spontaneous cell death was initiated in HBSS by leaving PMN ( $2.5 \times 10^6$  cells per mL HBSS) unstimulated at 37°C for 24 h. After incubation, cells were sedimented (Hermle Z216MK 45° fixed angle rotor, 500 g, 5 min, 4°C). The supernatant was filtered through a 5 µm pore sterile filter (Sterile Millex Filter Unit, Millipore, Billerica, MA, USA). The filtered fraction was sedimented (15700 g, 10 min, 4°C) and the sediment was resuspended in HBSS at the original incubation volume unless indicated otherwise. By this procedure we got 3 different EV types as characterized previously:<sup>32</sup> activated EVs (aEVs) from opsonized zymosan A activated cells in 20 min, spontaneously generated EVs (sEVs) from unstimulated cells in 20 min and apoptotic EVs (apoEVs) from cells undergoing spontaneous cell death. Apoptotic EVs originated from the PMN preparation of the preceding day of the indicated experiment.

As zymosan residues arising from the cell activation are an inherent, inseparable part of aEV fractions after the EV isolation process, we prepared a control sample for aEV measurements which contained the same amount of zymosan as aEV isolates. To achieve this, half of the aEV batch was sedimented (15700 g, 10 min, 4°C), resuspended in distilled water at the original volume, vortexed for 10 min, then sedimented again (15700 g, 10 min, 4°C) and resuspended in HBSS at the same volume as the aEV sample. By this means relevant EV fractions were destroyed due to hypotonic lysis and mechanical disruption, zymosan particles however are resistant to both. We refer to this sample as “lysed aEV”.

### ***Characterization of the size distribution of PMN-derived EVs***

Dynamic light scattering (DLS) measurements were performed at room temperature with an equipment consisting of a goniometer system (ALV GmbH, Langen, Germany), a diode-



pumped solid-state laser light source (Melles Griot 58-BLS-301, 457nm, 150mW) and a light detector (Hamamatsu H7155 PMT module). The evaluation software yielded the autocorrelation function of scattered light intensity which was further analyzed by the maximum entropy method (MEM), from where the different contributions of this function were determined. The radius of the particles was calculated using sphere approximation.<sup>36</sup> For nanoparticle tracking analysis (NTA) samples were resuspended in 1 mL of PBS to reach appropriate particle concentration range for the measurement. Particle size distribution and concentration were analyzed on ZetaView PMX120 instrument (Particle Metrix, Germany). For each measurement, 11 cell positions were scanned at 25°C (in 2 cycles) with the following camera settings: shutter speed -100, sensitivity - 75, frame rate - 7.5, video quality - medium (30 frames). The videos were analyzed by the ZetaView Analyze software 8.05.10 with a minimum area of 5, maximum area of 1000, and a minimum brightness of 20.

#### ***Transmission electron microscopic (TEM) investigation of the PMN-derived EVs***

EV-containing pellets were processed as described in our previous papers.<sup>12,36</sup> Briefly, pelleted EVs were fixed with 4% paraformaldehyde at room temperature for 1 hour, rinsed by PBS and post-fixed in 1% osmium tetroxide (OsO<sub>4</sub>) for 20 min. After rinsing with distilled water, pellets were dehydrated by a series of increasing ethanol concentrations, including block staining with 1% uranyl-acetate in 50% ethanol for 30 min, finally embedded in Taab 812 (Taab; Aldermaston, UK). Following polymerization at 60°C for 12 hours, 50-60 nm ultrathin sections were cut using a Leica UCT ultramicrotome (Leica Microsystems, UK) and examined using a Hitachi 7100 transmission electron microscope (Hitachi Ltd., Japan). Electron micrographs were made by Veleta 2k x 2k MegaPixel side-mounted TEM CCD camera (Olympus). Contrast/brightness of electron micrographs was edited by Adobe Photoshop CS4 (Adobe Systems Incorporated, CA, USA).

### ***Antibacterial activity of different types of PMN-derived EVs***

Opsonized bacteria ( $5 \times 10^7/50 \mu\text{L}$  HBSS ) were added to 500  $\mu\text{L}$  EV (derived from  $5 \times 10^6$  PMN) suspended in HBSS. During a 40 min co-incubation step at  $37^\circ\text{C}$ , the bacterial count decreases or increases depending on the samples' antibacterial effect and the growth of bacteria. At the end of the incubation, 2 mL ice-cold stopping solution (1mg/mL saponin in HBSS) was added to stop the incubation and lyse EVs. After a freezing step at  $-80^\circ\text{C}$  for 20 min, samples were thawed to room temperature and inoculated into LB broth. Bacterial growth was followed as changes in OD using a shaking microplate reader (Labsystems iEMS Reader MF, Thermo Scientific) for 8 hours, at  $37^\circ\text{C}$ , at 650 nm. After the end of growth phase the initial bacterial counts were calculated indirectly using an equation similar to PCR calculation, as described previously.<sup>35</sup>

### ***Investigation of the EV uptake by leukocytes***

All aEVs, sEVs and apoEVs were stained with PKH67 (Sigma) in 4  $\mu\text{M}$  final concentration for 5 min. To wash out unbound PKH67, after sedimentation of the EVs (15700 g, 10 min,  $4^\circ\text{C}$ ), the pellet was resuspended in HBSS at double of the original volume. After 10 min incubation at room temperature, EVs were sedimented again (15700 g, 10 min,  $4^\circ\text{C}$ ) and resuspended at the original volume. One part of the EVs was pelleted for a 3<sup>rd</sup> time (15700 g, 10 min,  $4^\circ\text{C}$ ) and the supernatant was used as control for unspecific PKH binding. PMN (50  $\mu\text{L}$  of  $5 \times 10^6/\text{mL}$ ) or mononuclear cell suspension (50  $\mu\text{L}$  of  $10^7/\text{mL}$ ) was added to 500  $\mu\text{L}$  aEV, sEV, apoEV sample or to the control supernatant. EVs and cells were coincubated for 45 min in a linear shaker (80 rpm) at  $37^\circ\text{C}$ . aEV and sEV samples were prepared from  $10^7$  cells while apoEV samples were derived from  $1.25 \times 10^6$  cells.

For flow cytometric (FC) detection of EV uptake a Becton Dickinson FACSCalibur flow cytometer was used with the following settings: flow rate was held under 1000 events/s; FSC= E-1 (log); SSC=320 V (log); 530/30 nm detector (FL1)=500 V (log).

FC data were analyzed with Flowing Software 2.5.1 (Turku Centre for Biotechnology, Finland).

PMN and monocytes were gated out based on their FSC-SSC characteristics, cell gates were defined in previous measurements with anti-CD11b-RPE antibodies (Dako, Glostrup, Denmark). Absolute change in geometric mean of FL1 (green) fluorescent intensity ( $\Delta$ MFI) of the indicated cell types was compared to the change measured in supernatant control samples after 45 min incubation.

The uptake was also confirmed by confocal microscopic images (Zeiss LSM710 confocal laser scanning microscope equipped with EC Plan-Neoflural, Zeiss 40x/1.30 Oil DIC objective). Excitation and emission wavelengths were 488 and 494-651 nm, resp. Similar to FC experiments 50  $\mu$ L of  $5 \times 10^6$ /mL PMN or  $10^7$ /mL mononuclear cell suspension was added to 500  $\mu$ L aEV, sEV, apoEV sample or the control supernatant and incubated on a cover slip at 37°C. Samples were analyzed at 0 and 45 min with ZEN software (Zeiss).

### ***Measurement of phagocytic activity of PMN***

PMN (120  $\mu$ L of  $5 \times 10^6$ /mL) were added to 480  $\mu$ L aEV, lysed aEV, sEV, apoEV sample or HBSS at 37°C in a linear shaker (80 rpm) for 45 min. aEV, lysed aEV and sEV samples were prepared from  $1.92 \times 10^7$  cells while apoEV samples were derived from  $0.96 \times 10^7$  cells.

In order to determine the phagocytic capacity, five different concentrations of opsonized *USA300* bacteria were used ( $1 \times 10^8$ ,  $3 \times 10^8$ ,  $1 \times 10^9$ ,  $3 \times 10^9$  and  $1 \times 10^{10}$ /mL). From each concentration 10  $\mu$ L was added to 100  $\mu$ L of the pretreated PMN populations at 37°C in a digital heating/shaking drybath for 20 min. Phagocytosis was stopped by adding 1 mL of ice-

cold PBS to each sample. Uptake of *USA300* bacteria was detected with FC with the following settings: flow rate was held under 1000 events/s; FSC= E-1 (log); SSC=320 V (log); 530/30nm detector (FL1)=480 V (log). PMN were gated out based on their FSC-SSC appearance. Autofluorescence intensity was measured with a PMN sample without bacteria. Geometric mean of FL1 (green) fluorescent intensity of PMN and percentage of PMN above the autofluorescence threshold were measured.

Similarly, kinetics of the phagocytic process was investigated by co-incubating 600  $\mu\text{L}$  of the abovementioned pretreated PMN populations with 60  $\mu\text{L}$  of  $3 \times 10^8/\text{mL}$  opsonized *USA300* bacteria at  $37^\circ\text{C}$  in a digital heating/shaking drybath (750 rpm) for 20 min. At 0, 5, 10, 15 and 20 min 100  $\mu\text{L}$  of each suspension was added to 1 mL of ice-cold PBS. FL1 fluorescence was measured instantaneously with FC.

#### ***Determination of the migratory potential of PMN***

PMN (120  $\mu\text{L}$  of  $5 \times 10^6/\text{mL}$ ) were added to 480  $\mu\text{L}$  aEV, lysed aEV, sEV, apoEV sample or HBSS at  $37^\circ\text{C}$  in a linear shaker (80 rpm) for 45 min. aEV, lysed aEV and sEV samples were prepared from  $1.92 \times 10^7$  cells while apoEV samples were derived from  $0.96 \times 10^7$  cells. The pretreated PMN samples were placed in the wells of a 3  $\mu\text{m}$  pore Corning transwell cell culture plate coated with 10% FBS. Every well contained  $2 \times 10^5$  cells. As a chemoattractant, 100 nM fMLF was used. After 1 h incubation at  $37^\circ\text{C}$ , the transwell plate was centrifuged (Eppendorf 5810 R swing-bucket plate rotor, 3220 g, 3 min,  $4^\circ\text{C}$ ). Transmigrated cells were counted using an acid phosphatase assay<sup>37</sup> in a plate reader (Labsystems iEMS Reader MF, Thermo Scientific).

#### ***Measurement of ROS production of PMN***

PMN (200  $\mu\text{L}$  of  $5 \times 10^6/\text{mL}$ ) were added to 2000  $\mu\text{L}$  aEV, lysed aEV, sEV, apoEV sample or HBSS at  $37^\circ\text{C}$  in a linear shaker (80 rpm) for 45 min. aEV, lysed aEV and sEV samples were prepared from  $4 \times 10^7$  cells while apoEV samples were derived from  $2 \times 10^7$  cells.

Lucigenin (5 mg/mL N,N'-Dimethyl-9,9'-biacridinium dinitrate dissolved in dimethyl sulfoxide [DMSO], both from Sigma) was added in 1:100 volume ratio to the pretreated cells.

White flat-bottom 96-well plates were coated with 10% FBS at room temperature for 1 h.

Three parallel 180  $\mu\text{L}$  samples of pretreated PMN were activated in the coated wells with 20  $\mu\text{L}$  1  $\mu\text{M}$  PMA. Changes in the luminescence were recorded for 90 min at  $37^\circ\text{C}$  with gentle shaking in a Varioskan multimode microplate reader (Thermo Fisher Scientific) every minute.

#### ***Quantification of IL-8 secretion of PMN***

PMN (120  $\mu\text{L}$  of  $2,5 \times 10^7/\text{mL}$ ) were added to 480  $\mu\text{L}$  aEV, lysed aEV, sEV, apoEV sample or HBSS at  $37^\circ\text{C}$  in a linear shaker (80 rpm) for 3 h. aEV, lysed aEV and sEV samples were prepared from  $1.92 \times 10^7$  cells while apoEV samples were derived from  $0.96 \times 10^7$  cells.

Cells were centrifuged (500 g, 10 min,  $4^\circ\text{C}$ ) and supernatants were analyzed for IL-8 with a human CXCL8/IL-8 DuoSet sandwich ELISA kit according to the manufacturer's instructions (R&D Systems)<sup>38</sup> in a plate reader (Labsystems iEMS Reader MF, Thermo Scientific).

#### ***Effect of EVs on coagulation***

Turbidimetry was performed to study the EVs prothrombotic properties by registering the absorbance of samples at 340 nm at  $37^\circ\text{C}$  with a CLARIOStar microplate reader (BMG LABTECH, Ortenberg, Germany) as described previously.<sup>39-41</sup>

For clotting assays aEV, lysed aEV and sEV samples were prepared from  $6.5 \times 10^7$  cells while apoEV samples were derived from  $2 \times 10^7$  cells.

To compare the positive effect of the aforementioned different types of EVs on the initiation of clotting in plasma, the change of absorbance was followed in microtiter plates. EVs were added to recalcified citrated human pooled plasma to reach a total volume of 104  $\mu$ L with 12.5 mM calcium.

Furthermore, to assess the effects of EVs on the dynamics of clotting, the above described mixture was supplemented with 5  $\mu$ L 100x diluted recombinant thromboplastin (TP) Dia-PT R (Diagon Kft, Budapest, Hungary) and the clotting curves were analyzed. A self-designed script running under the Matlab software (The Mathworks, Natick, MA, USA) was used to determine the maximum absorbance, and times to reach 10/50/90% of maximum absorbance.

#### ***Preparation and culture of human umbilical vein endothelial cells (HUVECs)***

Cells were harvested from fresh umbilical cords obtained during normal delivery of healthy neonates (according to Helsinki Protocol, Semmelweis University Institutional Review Board specifically approved this study, (permission number: SETUKEB 141/2015), and all participants provided their written informed consent to participate in this study), by collagenase digestion as described earlier.<sup>42,43</sup> HUVECs were kept in gelatin-precoated flasks in MCDB131 medium (Life Technologies) completed with 5% heat-inactivated fetal calf serum, 2 ng/mL human recombinant epidermal growth factor (R&D Systems), 1 ng/mL human recombinant basic fibroblast growth factor (Sigma), 0.3% Insulin Transferrin Selenium (Life Technologies), 1% Chemically Defined Lipid Concentrate (Life Technologies), 1% Glutamax (Life Technologies), 1% Penicillin-Streptomycin antibiotics (Sigma), 5  $\mu$ g/mL ascorbic acid (Sigma), 250 nM hydrocortisone (Sigma), 10 mM HEPES (Sigma), and 7.5 U/mL heparin hereinafter referred to as Comp-MCDB131. Each experiment was performed on at least three independent primary HUVEC cultures from different individuals.

### ***Measurement of cytokine production of HUVECs by sandwich ELISA***

Confluent layers ( $10^4$  cell/well) of HUVECs were cultured in 96-well plates for 24 h in 100  $\mu$ L Comp-MCDB131 medium supplemented with 20  $\mu$ L EV sample. aEV, lysed aEV and sEV samples were prepared from  $5 \times 10^6$  cells while apoEV samples were derived from  $3.33 \times 10^6$  cells. IL-8 was measured in a plate reader (Infinite M1000 PRO, Tecan Group Ltd) by CXCL8/IL-8 DuoSet sandwich ELISA kit according to the manufacturer's protocol (R&D Systems) as described previously.<sup>42,43</sup>

### ***Detection of adhesion molecules by cellular ELISA***

HUVECs were cultured in 96-well plates at confluent concentration in 100  $\mu$ L Comp-MCDB131 medium for 1 day, then treated with different EV populations in 100  $\mu$ L Comp-MCDB131 supplemented with 20  $\mu$ L EV sample. aEV, lysed aEV and sEV samples were prepared from  $5 \times 10^6$  cells while apoEV samples were derived from  $3.33 \times 10^6$  cells. Previous studies have shown<sup>42</sup> that maximum expression of E-selectin and VCAM-1 were at 6 and 24 h, respectively, thus we detected the expression of adhesion molecules at these time points. Supernatants were removed for cytokine ELISA, cells were fixed in 1:1 methanol and acetone mixture and incubated with mouse-anti-human E-selectin or mouse-antihuman VCAM-1 (both from Bender MedSystems, GmbH, Vienna, Austria) for 1 h. Cells were washed with PBS-Tween containing 1% BSA then incubated with HRP labelled goat-anti-mouse IgG (SouthernBiotech, Birmingham, AL) for 1 h. Color reaction was developed by 3,3',5,5'-tetramethylbenzidine (TMB, Thermo Fisher Scientific) and detected in a plate reader (Infinite M1000 PRO, Tecan Group Ltd).

### ***Proteomic analysis of PMN-derived EVs***

Proteomic analysis was performed as previously described.<sup>32</sup> Briefly samples (45 µg) were lysed using 2% sodium dodecyl sulfate (SDS) at 65°C for 30 min, reduced, alkylated, and digested using trypsin (Promega, Madison, WI, USA).<sup>44</sup> Peptides were isolated through a YM-10 filter, desalted and concentrated using NEST Group C18 PROTOTM UltraMicroSpin columns. Desalted samples were separated offline into seven strong cation exchange (SCX) fractions using SCX MicroTrapTM (Michrom-Bruker, Auburn, CA, USA) prior to analysis by 1D-RP (C18) nanoflow UHPLC and nanoelectrospray-MS<sup>45</sup> on the Thermo LTQ-Orbitrap ELITE MS platform.

Data were acquired using Orbitrap ELITE in ETD decision tree method. All MS1 was acquired with the FTMS and MS2 acquired with the ITMS. All MS data were searched using PD1.4 with Sequest and Mascot (v2.4) in a decoy database search strategy against UniprotKB.

Search data results file were imported into Scaffold (v4.3.4 Proteome Software Inc., Portland, OR) to control for <1.0% FDR with Peptide and Protein Prophet. Peptide and protein identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet<sup>46</sup> or the Protein Prophet<sup>47</sup> algorithm, respectively. Comparison of protein abundance among the EV groups was determined in Scaffold as the exponentially modified Protein Abundance Index (emPAI), as described by Ishihama et al.<sup>48</sup>

Proteomic data were analyzed further by the FunRich (version 3.1.3.) program to compare EV samples to each other or to the current FunRich (heatmap and integrin interactome) and UniProt human database (functional analysis).<sup>49,50</sup>

### ***Statistics***



Plasma clotting results without thromboplastin were analyzed by Fisher's exact test, all other comparisons between two groups were analyzed by two-tailed Student's t tests or ANOVA. Exact statistical tests are indicated in the figure legends. All bar graphs show mean and  $\pm$  SEM. Difference was taken significant if P value was  $<0.05$ . \* represents  $P < 0.05$ ; \*\* represents  $P < 0.01$ ; \*\*\* represents  $P < 0.001$ . Statistical analysis was performed using GraphPad Prism 8 for Windows (La Jolla, California, USA).

In every experiment, "n" indicates the number of independent experiments from different donors, unless stated otherwise in the figure legend (Fig. 5.).

Biological variance between individual donors was considerable and also showed seasonal variations. Therefore in addition to the summarized data we present the normalized and paired values of individual experiments as well.

## **Results**

### ***Characterization of PMN-derived EVs***

First, we characterized the basic physical properties of the three types of PMN-derived EVs: those produced upon stimulation with opsonized zymosan (aEV) or spontaneously from fresh (sEV) or apoptotic cells (apoEV). The size distribution of the EV preparations was investigated by dynamic light scattering (Fig. 1A). A broad peak was detected around 200 nm that disappeared upon treatment with Triton X-100 supporting the vesicular nature of the preparation. No significant differences were found among the 3 types of EVs. Nano-track analysis (NTA) was performed to quantitate both the number and the size of the EV populations. As shown in Fig. 2B, there were about twice as many particles in the aEV than in the sEV preparation. This difference corresponds to our earlier data obtained with flow cytometry.<sup>12</sup> There was no difference in the median diameter of sEV and aEV, whereas apoEV proved to be slightly larger (Fig. 1C). Electron microscopic images support that all

three EV preparations contained membrane surrounded vesicles corresponding to “medium-size” EVs (Fig. 1D-F).

Next we show the major functional difference between the three types of PMN-derived EVs: only aEVs are able to impair bacterial growth (hence the name of antibacterial EVs), whereas sEV and apoEV lack this property (Fig. 1G).

In order to test the effect of the different EV types on neutrophil functions under stable conditions, we followed the fate of fluorescently labeled EVs upon encounter with PMN by flow cytometry. Monocytes served as a positive control. Fig. S1A presents the original data in form of dot plots on the fluorescence distribution at the beginning and at the end of the 45 min incubation time in a representative experiment. Summarized data of the increase of mean fluorescent intensity ( $\Delta$ MFI) are provided in Fig. S1B. At 45 min a measurable increase of MFI occurred with all three EV populations in both cell types indicating that all three types of EVs get associated with PMN. With confocal microscopic imaging we could verify that EVs are engulfed in PMN, as opposed to staying only attached on the surface of the cells (Fig. S1C).

In all the following experiments cells were pretreated with EVs for 45 min, thus allowing sufficient time for uptake of vesicles.

### ***Effect of PMN-derived EVs on resting and activated PMN***

First, we measured the effect of PMN-derived EVs on ROS production. EVs isolated as described in Methods section, do not produce any detectable amount of ROS on their own.<sup>12,32</sup> None of the three different types of PMN-derived EVs have any significant effect on the basal superoxide production of resting neutrophils (Fig. 2A, E). Next we tested whether EVs had any influence on stimulated ROS production. We applied as stimulator the pharmacological

agent phorbol 12-myristate 13-acetate (PMA). PMA-induced superoxide production starts after a typical lag phase of variable length. We chose as characteristic parameters ROS production in the early phase at 10 min and at the maximum which occurred between 30 to 40 min. Opsonized zymosan is an inherent component of the aEV preparation and it may have various effects on PMN on its own. To assess the true effect of EVs, in these experiments control PMN were treated with the lysed fraction of the aEV preparation (details see in Methods). Fig. 2 shows both the summarized data of the absolute values (Fig. 2A, E) and the paired data related to the relevant control from each experiment (Fig. 2B-D and F-H). After 10 min in the presence of sEV or apoEV, ROS production was significantly diminished (Fig. 2A, C-D). In contrast, in the presence of aEVs there was a significant and consistent increase of ROS production as compared to the control (Fig. 2A, B). Maximal ROS production was also significantly and consistently higher than the control in the presence of aEV and lower in the presence of sEV (Fig. 2E-G). The difference in the presence of apoEV was not significant (Fig. 2E, H). We thus observed opposing effect of aEV and sEV upon stimulated ROS production. A third type of effect was observed with apoEVs: by reducing the early but not affecting the maximal ROS production they induced a right-shift of the time curve.

Alteration of cytokine secretion upon EV treatment was investigated previously in monocytes,<sup>17-21,25</sup> but not in PMN. Therefore, we tested cytokine secretion from neutrophils after encountering the different EV populations. Resting PMN produce low amount of IL-8 which was dramatically increased by opsonized zymosan, which served as positive control (Fig. 3A). Even the zymosan remnants present in the aEV preparation were able to increase IL-8 secretion approximately fourfold (lysed aEV column in Fig. 3A). In order to test exclusively the effect of the different EV preparations, all the samples contained the same amount of lysed aEV. As summarized in Figure 3A, IL-8 release was significantly increased by aEV, but decreased by sEV. These changes were consistently observed in all experiments

(Fig. 3B-C). In contrast, IL-8 release in the presence of apoEV showed no significant change (Fig. 3D).

In the following experiments we compared phagocytosis, another basic neutrophil function, in the absence or following pretreatment by different types of PMN derived extracellular vesicles. In Fig. S2 we show both the kinetics of uptake of fluorescent *S. aureus* (panels A, C, E) and the maximal uptake in case of different ratios of bacteria to PMN (panels B, D, F). None of the EVs had any significant effect on the engulfment of fully opsonized bacteria. Finally, we tested the effect of EVs on neutrophil migration in a chemotactic gradient (Fig. S3). Again, none of the EVs had significant or consistent effect.

Taken together, our results show that aEV and sEV have opposite effects on ROS production and cytokine secretion, whereas apoEV only delayed ROS production. Phagocytosis and chemotactic migration were not influenced by any of the EVs.

### ***Effect of PMN-derived EVs on endothelial cells***

The first reports on biological effects of PMN-derived EVs showed an increase of pro-inflammatory cytokine secretion from endothelial cells.<sup>28,29</sup> In view of the observed opposing effect of sEV and aEV on IL-8 secretion from neutrophils, we tested their effect also on human umbilical vein endothelial cells (HUVEC). In this setting only aEV stimulated a significant and reproducible increase of IL-8 secretion (Fig. 4A, D) whereas sEV and apoEV had no consistent effect (Fig. S3A, D).

To gain further insight in dissimilar effectivity of neutrophil-derived EVs, we tested two activation markers on the endothelial cells: E-selectin and vascular cell adhesion molecule 1 (VCAM-1). The expression of both surface markers was significantly and reproducibly

increased by aEVs (Fig. 4B, C, E, F). In contrast, neither sEV nor apoEV had any consistent effect (Fig. S3).

Our data obtained on endothelial cells further support the diverging effect of EVs generated by resting (sEV) or activated (aEV) neutrophils.

### ***Effect of PMN-derived EVs on coagulation***

Increased blood clotting was reported as a common property of EVs released from different cell types.<sup>2</sup> Based on our above results we asked whether all EV types have similar capacity in enhancing coagulation.

We tested the system in two different settings. First we explored the pro-coagulant activity of the EVs themselves (in the absence of added TP). In the experiments presented in Fig. 5A-F we detected the frequency of coagulation in the presence of the different types of EVs. Panels A-C provide the exact numbers of cases where coagulation did or did not occur which allowed the statistical analysis of data, whereas panels D-F present the ratio of events where coagulation did happen. For aEVs the frequency of coagulation was almost the same in case of intact or lysed aEVs, suggesting that coagulation was initiated by some other component (e.g. opsonized zymosan remnant) but not the EVs themselves. In cases of both sEV and apoEV the frequency of coagulation was significantly higher in the presence of EVs than in their absence. ApoEV proved to be the most effective, initiating coagulation in over one third of the measurements.

In the second test coagulation time was measured in a system initiated by TP. As shown in Fig. 5G-I, the presence of apoEV reduced coagulation time significantly and consistently. The presence of sEV resulted in a decreasing tendency but the effect was not statistically significant. Similar to the previous test, the effect of aEV was weak.

Our results on coagulation support the functional diversity of the different types of PMN-derived EVs, with apoEV having the largest and aEV the smallest effect.

### ***Proteomic analysis of PMN-derived EVs***

We carried out proteomic analysis of the three distinct EV preparations in order to relate protein composition to the observed functional divergences. A total of 774 proteins could be identified in the 3 EV populations. The variety of proteins in aEVs is less than the half of that in the other two EV types (284 vs. 636 and 705 respectively) and the number of unique proteins is also remarkably lower (Fig. 6A). The differences in the abundance of individual proteins compared to the average of the three EV types is shown in the heat map of Fig. 6B. A large cluster of proteins is significantly underrepresented and another cluster significantly overrepresented in aEVs compared to either sEV or apoEV, however the latter two samples also showed characteristic differences. Next, the abundance of specific groups of proteins was analyzed (Fig. 6C). The origin of proteins shows that aEVs contain more proteins of plasma membrane and less proteins of nucleoplasmic origin than either sEV or apoEV, and they are also enriched in components of focal adhesions and exosomes. Categorizing proteins according to biological function shows that aEVs contain more proteins involved in cell adhesion and immune response than the other two EV types, whereas proteins associated to the MAPK cascade are less abundant. As for molecular functions, several types of binding proteins, including integrin binding proteins are enriched in aEVs.

Recently we have identified Mac1 integrin as the critical surface receptor that initiates formation of aEVs.<sup>36</sup> Previously we showed a potential role of Mac1 in the aggregation of bacteria and aEVs related to impaired bacterial killing.<sup>12</sup> Therefore we analyzed in detail the interactome of integrins identified in the distinct EV preparations. As indicated by the

proportion of red dots in Fig. 6D, aEVs contain a higher proportion of proteins interacting with integrins.

## **Discussion**

Using PMN as model cell type, we show in this study that EVs generated under different physiologically or pathologically relevant conditions from the same cell exert divergent and selective effects on cells and functions in their environment (Table 1). In previous studies we demonstrated the difference between aEV and sEV in their action on bacterial growth and in their protein composition.<sup>12,32</sup> This line of observations is now extended by demonstrating their opposing effect on ROS production and IL-8 secretion from PMN, and their distinct effects on endothelial cells. Importantly, we also show differences in their influence on coagulation.

Interestingly, we observed some differences between the effects of sEV and apoEV as well. The latter type did not decrease maximal ROS production and IL-8 release from neutrophils, but had a strong and clear pro-coagulant effect. Production of sEV seems to be a constitutive property of neutrophils. In our hands no inhibitor or genetic deficiency of receptors or signaling molecules had any influence on sEV generation.<sup>36,51</sup> Neutrophils being short-lived cells which go in spontaneous apoptosis, it could be envisaged that sEV are produced by a few cells going into apoptosis during the short (20 min) incubation time before we collect the vesicles. However, the observed differences in the actions and the protein composition between sEV and apoEV indicate separate EV populations.

In the current study we compare three types of EVs which are present under different conditions in circulating blood.<sup>12</sup> sEV and apoEV are produced from resting, not specifically stimulated cells. They have no effect or mitigate neutrophil and endothelial cell activation.

These findings are consistent with numerous previous reports on anti-inflammatory effects of PMN-derived EVs on monocytes and macrophages.<sup>14,20,23,52</sup> In contrast, aEVs are produced upon stimulation by opsonized particles, typical under infectious conditions.<sup>12</sup> Our present data indicate that aEVs activate select pro-inflammatory functions in both neutrophils and endothelial cells. These observations are consistent with data on pro-inflammatory properties of PMN-derived EVs.<sup>15,17,18,24,25,28,29</sup>

Finally, it is important to note that neither phagocytosis nor chemotactic migration were affected by any of the EVs, supporting the selective nature of EV actions.

Many previous studies have concluded that EVs are able both to stimulate and to dampen immune functions.<sup>3,53,54</sup> However, those studies summarized the effects of EVs issued from very different sources and actions on most different players of the complicated immune reaction. The novelty of our study resides in demonstrating that the same cells are able to transmit either anti-inflammatory or pro-inflammatory signals via EVs, depending on the environmental cues.

The divergent effects communicated by the different types of EVs are unlikely to be caused by one common mechanism. The time scale of the demonstrated effects alone suggests different mechanisms. Coagulation occurs in a few minutes, hence differences in the surface components can be envisaged as the decisive factors. Alteration of ROS production was evident in 10 to 30 min, suggesting posttranslational modification rather than alteration of gene expression as potential mechanism. Cytokine secretion from PMN and HUVEC as well as appearance of HUVEC surface markers occurred after several hours, suggesting an alteration in gene expression. The observed differences in protein composition (Fig. 6A), abundance (Fig. 6B) and pattern (Fig. 6C,D) among the 3 types of EVs can account for both short-term and long-term functional alterations. The specific signaling pathways involved in



the diverging or opposing effects revealed in this study, have to be deciphered in future investigations.

Production of EVs with diverse and selective effect is probably not the unique property of neutrophilic granulocytes. Numerous studies demonstrated differences in the composition of EVs secreted from the same cell under different conditions. In contrast, functional differences were tested only by a few publications.<sup>18,31</sup> In the present study we revealed that EV effects can be divergent and even antagonistic depending on the environmental conditions prevailing at time of EV biogenesis. At the dawn of therapeutic usage of EVs and EV-related preparations, we call the attention to the need of detailed comparative examination of functional properties of extracellular vesicles.

### **Authorship**

FK carried out most of the experiments, prepared the figures and participated in writing of the manuscript, EG and VJF provided expertise and were involved in experiments on HUVEC and coagulation, resp., DSV carried out DLS measurements, DK carried out NTA measurements, MLM and KRM did the proteomic analysis, ÁML analyzed proteomic data, ÁML and EL designed and supervised the study and wrote most part of the manuscript, EL obtained financing.

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### **Conflict of Interest**

The authors have no conflict of interest to declare.

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## Figure legends

**Figure 1. Characterization of EV samples.** **A** Size distribution spectra of EVs measured by DLS. Broken line represents 0.1% Triton X-100 treated aEV. Representative results out of three similar experiments. **B** Particle size distribution of EVs measured by NTA. ApoEV were measured in a 10-fold dilution in order to stay within optimal detection ranges. Representative results out of three similar experiments. **C** Particle median diameter of EVs measured by NTA. Data were compared by using RM one-way ANOVA coupled with Sidak's post hoc test; n=3. Error bars represent mean +SEM. **D-F** Representative electron microscopic images of sEV (**D**), aEV (**E**), apoEV (**F**). Original magnification is 30 000x. Representative pictures out of three similar experiments. **G** Bacterial survival in the presence of different types of EVs released from  $5 \times 10^6$  PMN. Data were compared by using RM one-way ANOVA coupled with Sidak's post hoc test; n=4. Error bars represent mean +SEM. 100% represents the initial bacterial count.

**Figure 2. Effect of EVs on the ROS production of PMN.** PMN were pretreated for 45 min with the indicated EV or control, then left unstimulated or activated with 100 nM PMA. ROS production was determined at 10 min after activation (**A-D**) and at the peak intensity of the curve, typically at 30 to 40 min (**E-H**). **A** and **E** show the summarized ROS production of the EV-pretreated PMN, **B-D** and **F-H** show the normalized data pairs from each experiment. Data were normalized to their adequate controls ("aEV" to "Lysed aEV", "sEV" and "apoEV" to "No EV"). Raw data were compared using paired Student's t-test; n=13 for aEV & sEV; n=7 for apoEV. Error bars represent mean +SEM.

**Figure 3. Effect of EVs on the IL-8 production of PMN.** PMN were treated for 3 h with one of the three EV populations or their controls. IL-8 amount of the supernatant was quantified with ELISA. **A** shows the summarized changes in IL-8 production of the EV-treated cells. **B-D** show the normalized data pairs from each experiment. Data were normalized to their adequate controls (“aEV” to “Lysed aEV”, “sEV” and “apoEV” to “No EV”). Raw data were compared using paired Student’s t-test; n=15 for aEV; n=7 for sEV; n=8 for apoEV. Error bars represent mean +SEM.

**Figure 4. Effect of EVs on endothelial cells.** HUVEC were pretreated for 6 h (E-Selectin) or 24 h (VCAM-1 & IL-8) with one of the three EV populations or their controls. IL-8 amount of the supernatant was quantified with ELISA (**A, D**). E-Selectin and VCAM-1 expression was determined by cellular ELISA (**B, C, E, F**). **A-C** show the summarized changes in IL-8 secretion, E-Selectin and VCAM-1 expression of the EV-treated cells. **D-F** show the normalized data pairs for aEV or control treated cells from each experiment. Data were normalized to their adequate controls (“aEV” to “Lysed aEV”, “sEV” and “apoEV” to “No EV”). Raw data were compared using paired Student’s t-test; n=5. Error bars represent mean +SEM.

**Figure 5. Effect of EVs on coagulation.** One of the three EV populations or their controls were mixed with pooled citrated human plasma in the absence (**A-F**) or presence (**G-I**) of thromboplastin followed by recalcification with Ca-HEPES. **A-C** show the absolute numbers of coagulated and not coagulated wells in each sample. **D-F** represent the percentage of coagulated wells based on the same data. **G-I** show the time needed for 50% of the coagulation process in the thromboplastin treated samples (raw data pairs). The dotted lines

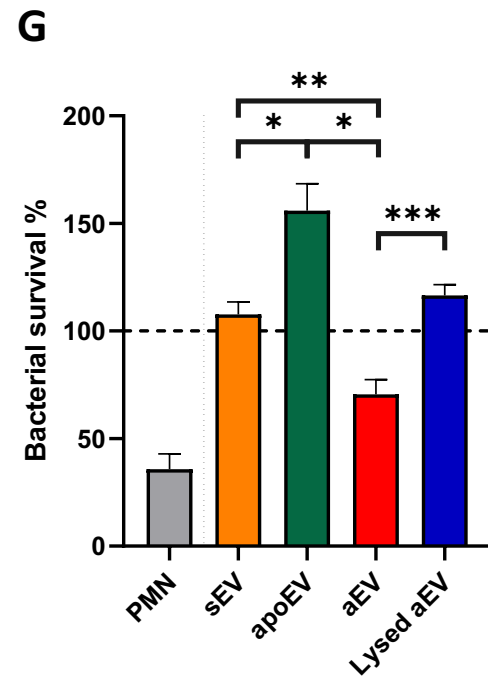
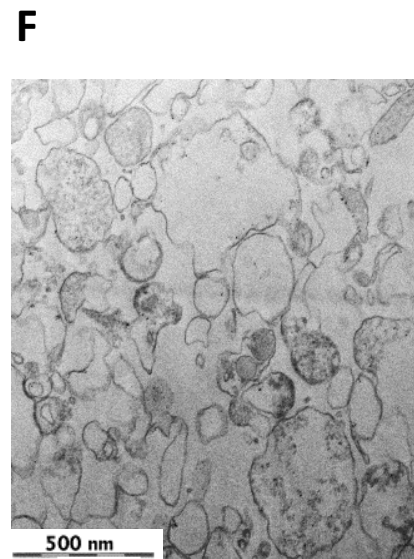
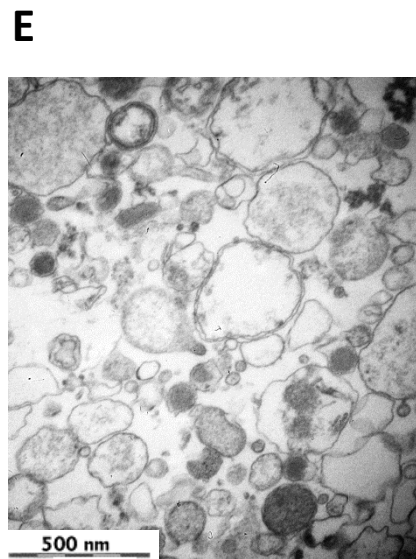
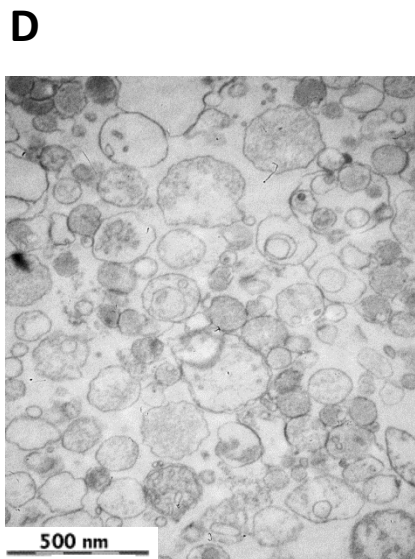
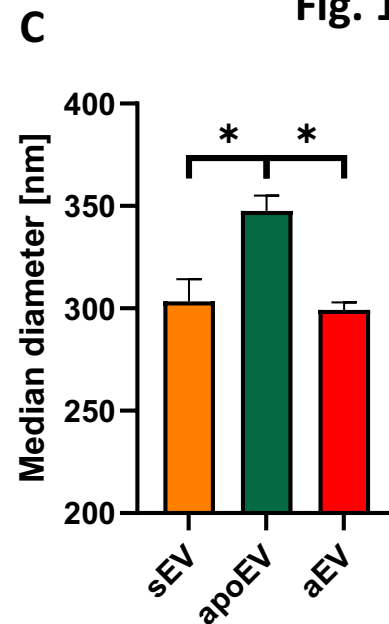
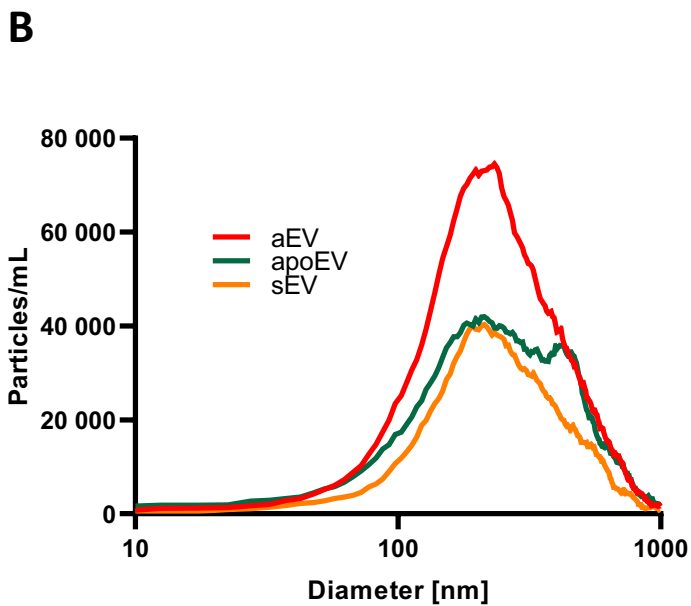
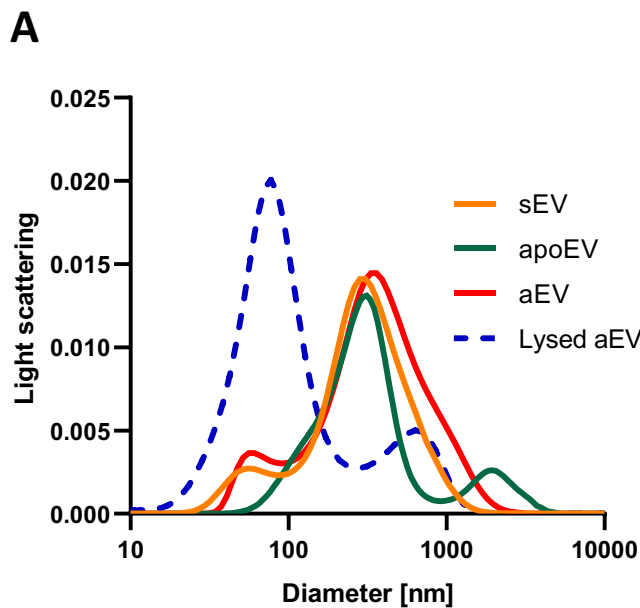
on **G-I** show the average coagulation time of the “No EV” samples. Data were compared using Fisher’s exact test (**A-F**) and paired Student’s t-test (**G-I**). n=29 wells from 7 donors for aEV & sEV; n=30 wells from 6 donors for apoEV (**A-F**). n=5 from 5 donors (**G-I**).

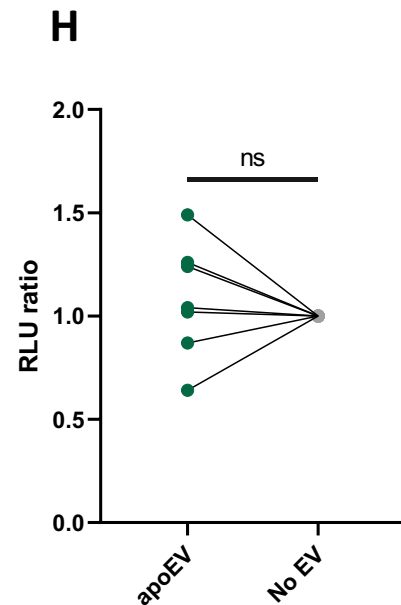
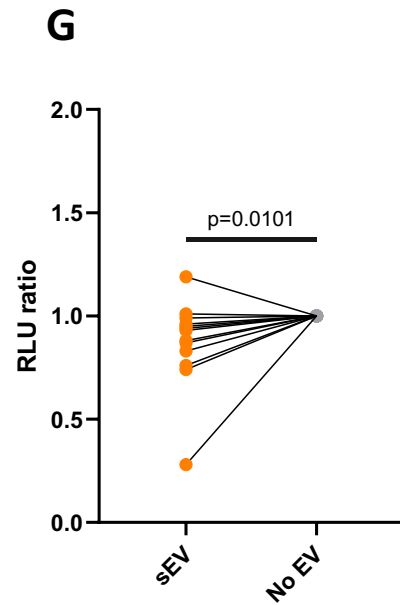
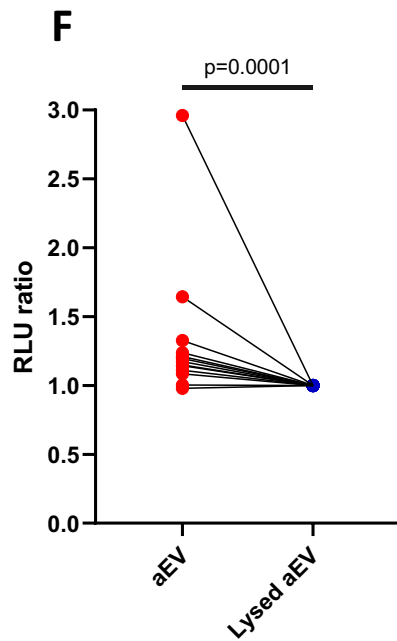
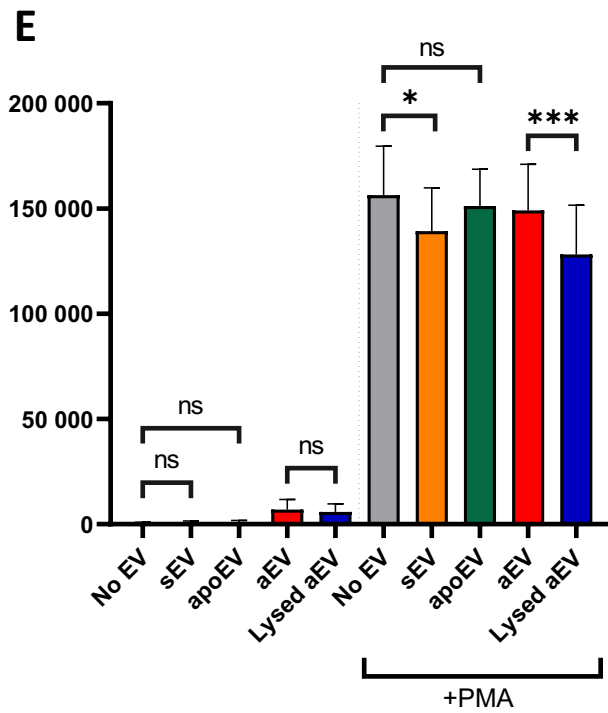
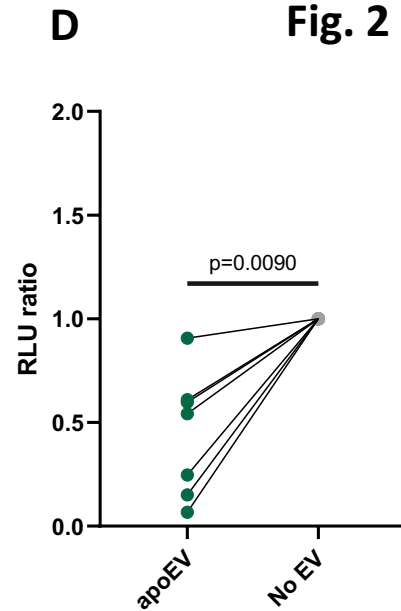
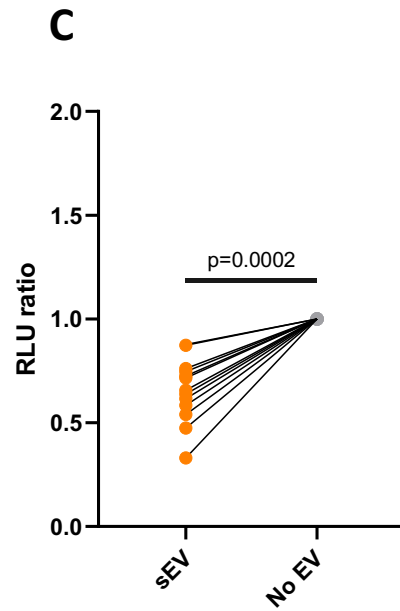
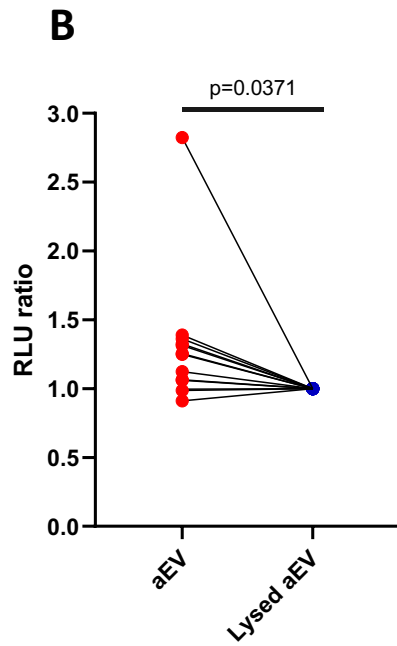
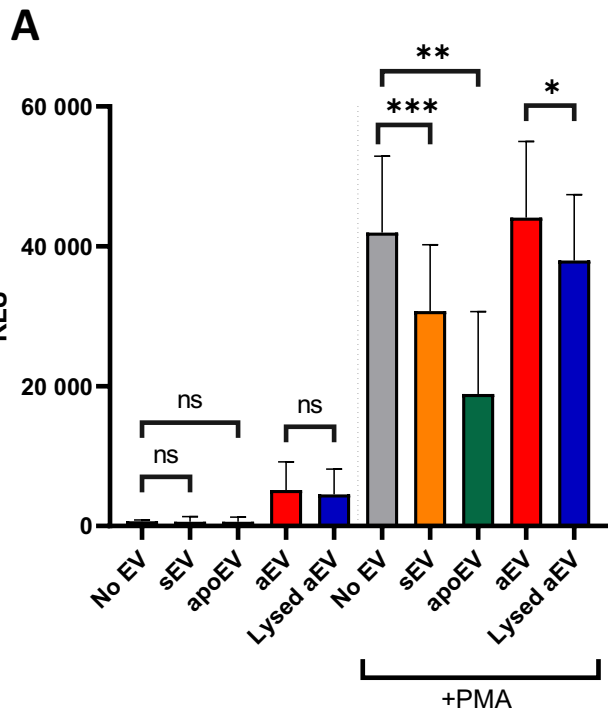
**Figure 6. Proteomic analysis of EV populations.** **A** Comparison of protein presence in different EV populations using Venn diagram. Equal protein amount was analyzed (45 µg). The size of the set is proportional to the number of identified proteins. **B** Protein enrichment heat map of the three different EV populations normalized to each row. Proteins are clustered according to the calculated dendrogram by FunRich. **C** Analysis of protein content according to cellular origin, biological process and molecular function. **D** Integrin interactome of sEV, apoEV and aEV. Red nodes represent proteins that are part of the integrin interactome. Blue nodes represent identified proteins that are not the part of the integrin interactome. Percentage of integrin interactome proteins to all proteins is indicated.

## Tables

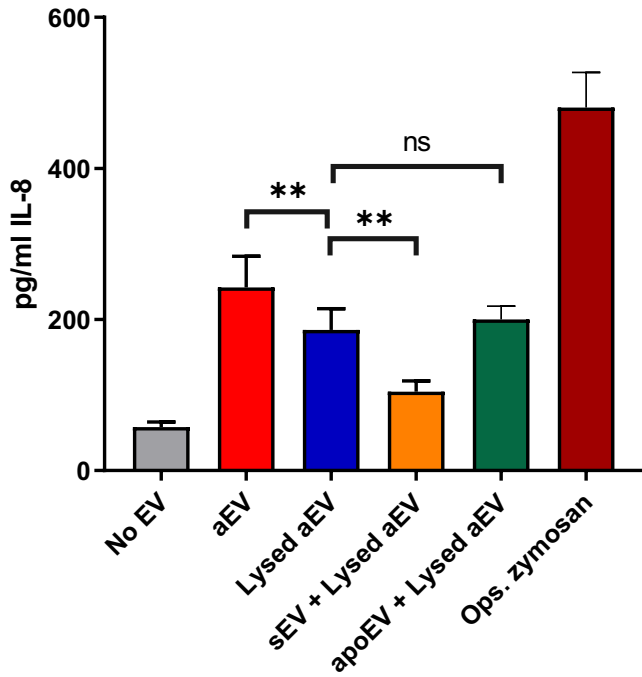
**Table 1. Summarized effects of PMN-derived EVs.** Arrows represent the observed statistically significant changes upon pretreatment with different EV populations compared to their adequate controls.

	<b>aEV</b>	<b>sEV</b>	<b>apoEV</b>
<b>Maximal ROS production</b>	↑	↓	-
<b>Early ROS production</b>	↑	↓	↓
<b>IL-8 production of PMN</b>	↑	↓	-
<b>IL-8 secretion of HUVEC</b>	↑	-	-
<b>E-selectin expression of HUVEC</b>	↑	-	-
<b>VCAM-1 expression of HUVEC</b>	↑	-	-
<b>Coagulation (no TP)</b>	-	↑	↑
<b>Coagulation time (TP)</b>	-	-	↑
<b>Phagocytosis</b>	-	-	-
<b>Migration</b>	-	-	-

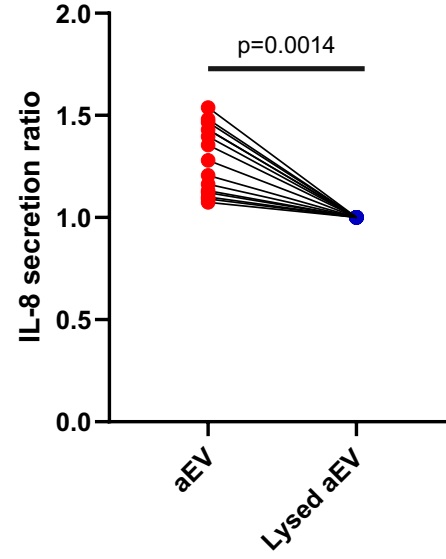




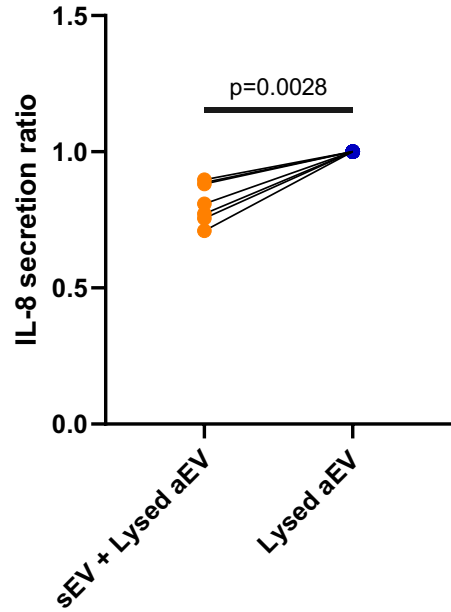
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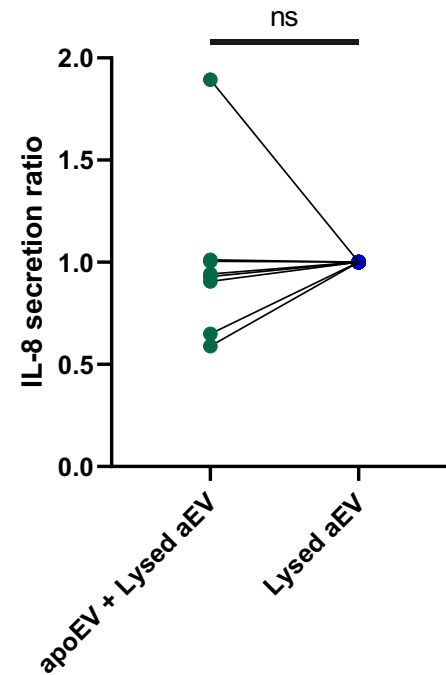
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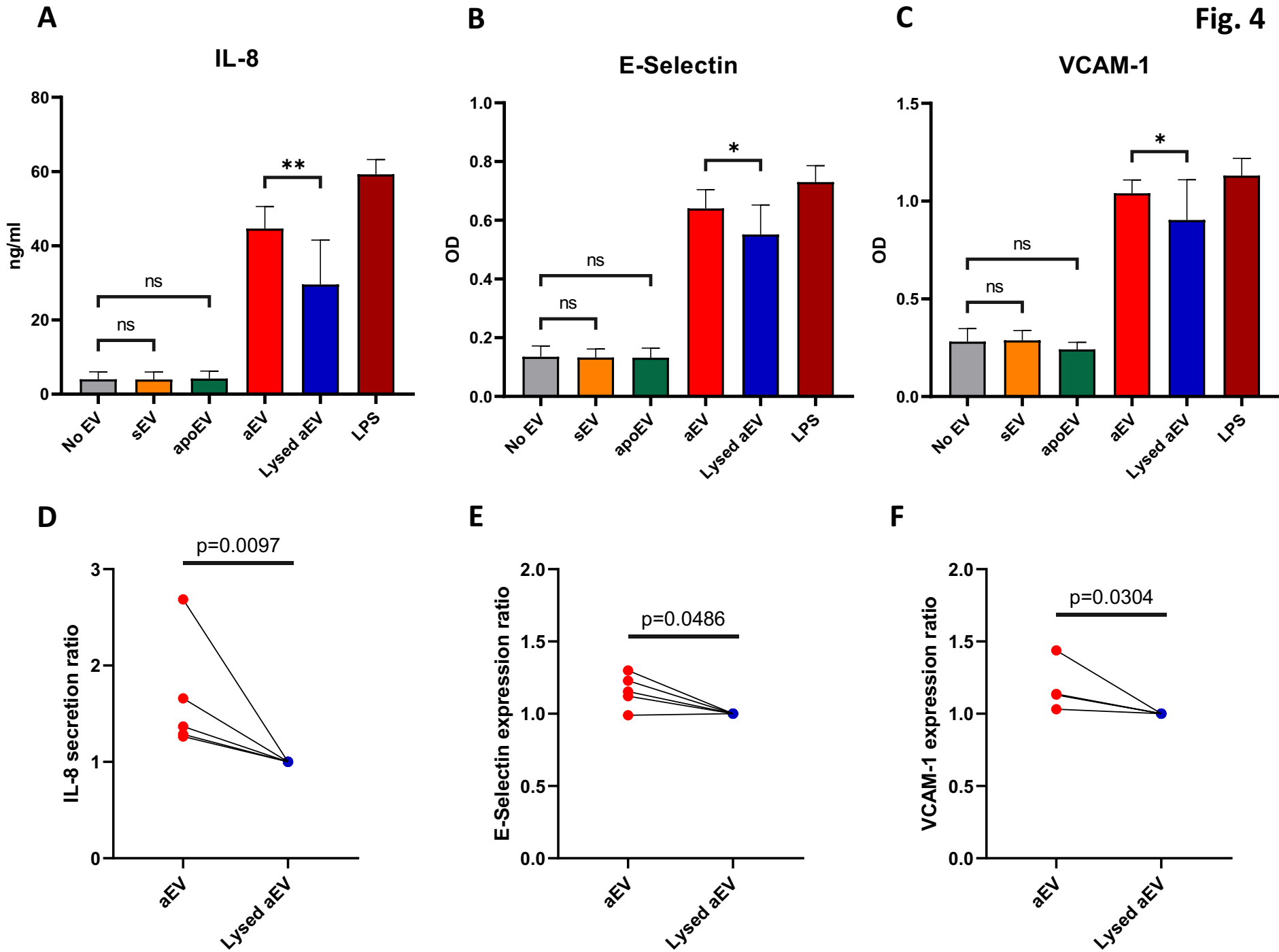
**C**

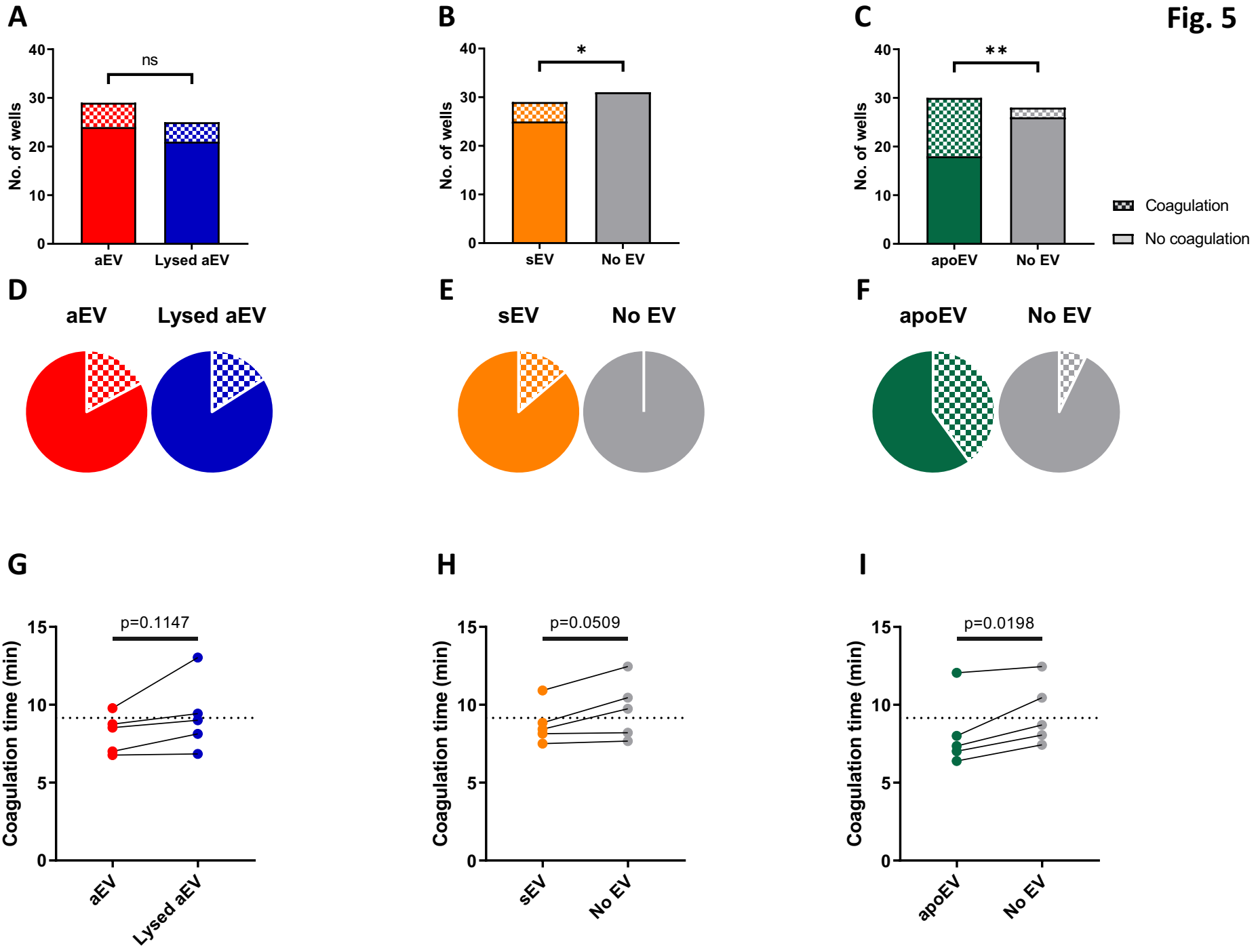


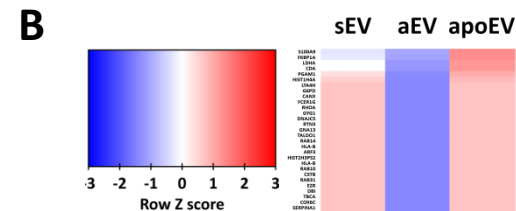
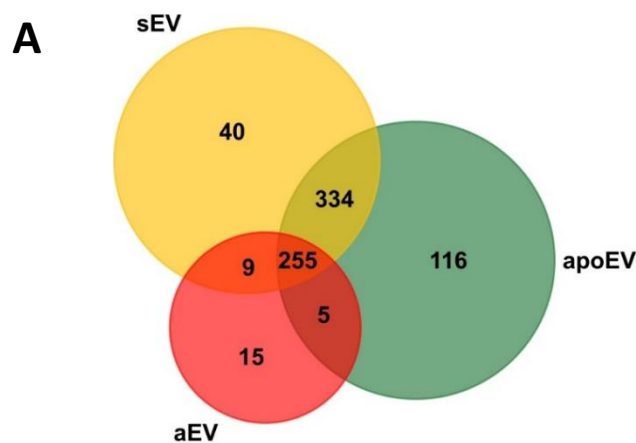
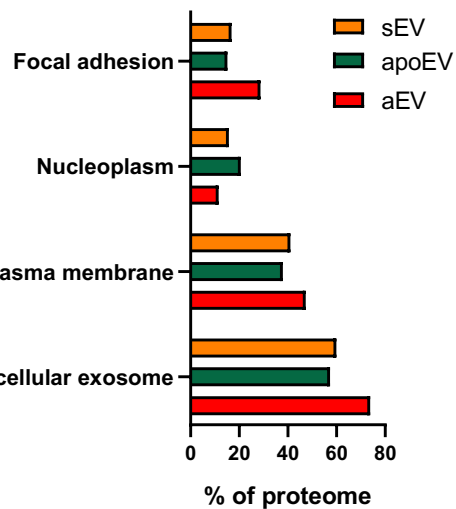
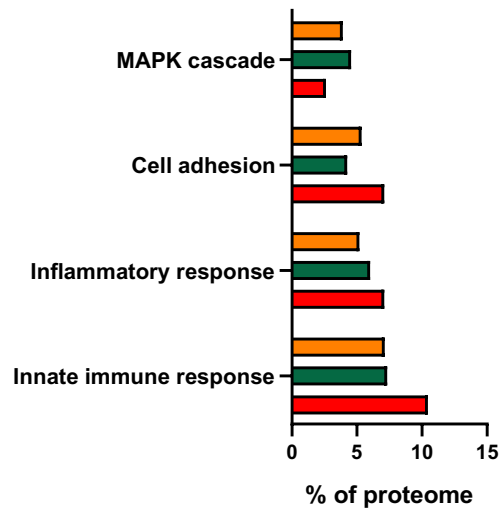
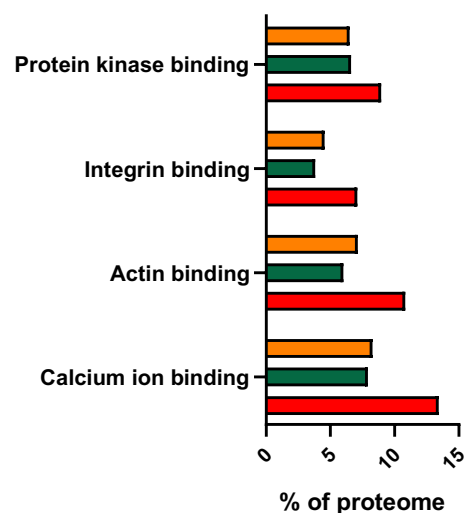
**D**









**Fig. 6****C****Cellular origin****Biological process****Molecular function****D**