Supplementary Material

for the following *Cell Mol Life Sci* article

**Platelet-released extracellular vesicles: The effects of thrombin activation**

*Running head:* Platelet microvesicles and the effect of thrombin

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## SUPPLEMENTARY TABLES

**Supplementary Table 1. Cell surface molecules for platelet extracellular vesicle (pEV) identification and characterization**

| Marker | Alternative name     | Expression             | Conjugation | Clone | Company          |
|--------|----------------------|------------------------|-------------|-------|------------------|
| Annexin V | PS-binding protein   | Widely expressed       | PE          | -     | Molecular Probes |
| CD41a  | αIIbβ3-integrin       | Platelets              | FITC        | HIP8  | BD Pharmingen    |
| CD61   | β3-integrin          | Platelets, leukocytes  | FITC        | VI-PL2| BD Pharmingen    |
| CD62P  | P-selectin           | Activated platelets    | FITC        | AK-4  | BD Pharmingen    |
| PAC1   | αIIbβ3-integrin       | Activated platelets    | FITC        | PAC1  | BD Biosciences   |
| IgG1κ  | -                    | -                      | FITC        | MPOC21| BD Pharmingen    |

BD indicates Becton Dickinson; FITC indicates fluorescein isothiocyanate; PE, phycoerythrin; PS, phosphatidylserine
Supplementary Table 2. KEGG pathways of all identified pEV proteins

| KEGG ID | KEGG pathway                                      | Organism        | Nº of proteins |
|---------|--------------------------------------------------|-----------------|----------------|
| hsa01100| Metabolic pathways                               | Homo sapiens    | 21             |
| hsa04810| Regulation of actin cytoskeleton                 | Homo sapiens    | 16             |
| hsa04510| Focal adhesion                                   | Homo sapiens    | 14             |
| hsa04670| Leukocyte transendothelial migration             | Homo sapiens    | 11             |
| hsa04610| Complement and coagulation cascades              | Homo sapiens    | 10             |

Table S2 shows the KEGG-pathways to which 10 or more proteins in pEVs were annotated.

Supplementary Table 3. Panther pathways of all identified pEV proteins

| Panther ID | Panther pathway                                | Organism        | Nº of proteins | P-value     |
|------------|-----------------------------------------------|-----------------|----------------|-------------|
| P00011     | Blood coagulation                             | Homo sapiens    | 8              | 1.11E-06    |
| P00034     | Integrin signaling pathway                     | Homo sapiens    | 11             | 9.92E-05    |
| P00049     | Parkinson disease                              | Homo sapiens    | 7              | 2.47E-03    |
| P00024     | Glycolysis                                     | Homo sapiens    | 4              | 7.81E-03    |
| P00016     | Cytoskeletal regulation by Rho GTPase          | Homo sapiens    | 6              | 2.51E-02    |

Results, which in this case are ordered by expected P-value (binomial statistical tool)

Supplementary Table 4. FunRich biological pathways of all identified pEV proteins

| Biological pathway                              | Percentage of genes | P-value*  |
|------------------------------------------------|---------------------|-----------|
| Platelet degranulation                          | 11.3                | 6,254E-11 |
| Response to elevated platelet cytosolic Ca^{2+} | 12.4                | 1,134E-10 |
| Platelet activation, signaling and aggregation | 17.5                | 1,758E-08 |
| Hemostasis                                      | 24.7                | 7,729E-07 |
| Smooth Muscle Contraction                       | 6.2                 | 6,623E-04 |
| Common Pathway of Coagulation                   | 5.2                 | 2,024E-03 |
| Muscle contraction                              | 7.2                 | 6,138E-03 |
| Formation of Fibrin Clot (Clotting Cascade)     | 6.2                 | 6,376E-03 |
| EphrinB-EPHB pathway                            | 7.2                 | 2,387E-02 |
| Integrons in angiogenesis                       | 7.2                 | 3,643E-02 |

* P-value corrected by Bonferroni method
Supplementary Table 5. IPA analysis differential pEV proteins from baseline non-activated platelets and thrombin-stimulated platelets

| T-pEVs vs B-pEVs                                      | P-value / Score |
|------------------------------------------------------|-----------------|
| Intrinsic prothrombin activation pathway             | 3.2·10⁻⁷        |
| Coagulation system                                   | 8.1·10⁻⁷        |
| Extrinsic prothrombin activation pathway             | 4.8·10⁻⁶        |
| Regulation of actin-based motility by Rho            | 8.5·10⁻⁶        |
| RhoA signaling                                       | 2.8·10⁻⁵        |

| Top networks                                         |                  |
|------------------------------------------------------|-----------------|
| Cellular Assembly and Organization, Cellular Function and Maintenance, Developmental Disorder | 55              |
| Gene Expression, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function | 18              |
| Cellular Function and Maintenance, Cardiovascular Disease, Cardiovascular System Development and Function | 11              |

The P-scores (−log10 [P-values]) reflect the probabilities of such associations occurring by chance, with the threshold value for significance set as 1.25; as evident the scores are highly significant. Score of top networks are a measure of the number of focus proteins in a network.
**Supplementary Table 6. FunRich biological pathways of differential pEV proteins from baseline non-activated platelets and thrombin-stimulated platelets**

| Biological pathway                                      | Percentage of genes | P-value*  |
|----------------------------------------------------------|---------------------|-----------|
| Common Pathway of Coagulation                            | 13.6                | 0.005     |
| Formation of Fibrin Clot (Clotting Cascade)              | 13.6                | 0.046     |
| Beta3 integrin cell surface interactions                 | 13.6                | 0.102     |
| Hemostasis                                               | 27.3                | 0.316     |
| Cell-extracellular matrix interactions                   | 9.1                 | 0.317     |
| Integrin cell surface interactions                       | 13.6                | 0.460     |

*P*-value corrected by Bonferroni method

**Supplementary Table 7. Reactome biological pathways of differential pEV proteins from baseline non-activated platelets and thrombin-stimulated platelets**

| Biological pathway                                      | Organism           | P-value*  |
|----------------------------------------------------------|--------------------|-----------|
| Platelet degranulation                                   | Homo sapiens       | 1.42E-8   |
| Response to elevated platelet cytosolic Ca\(^{2+}\)     | Homo sapiens       | 2.08E-8   |
| Platelet activation, signaling and aggregation           | Homo sapiens       | 3.56E-7   |
| Hemostasis                                               | Homo sapiens       | 8.42E-6   |
| Common Pathway of Fibrin                                 | Homo sapiens       | 1.04E-6   |
| Clot Formation                                           | Homo sapiens       | 1.48E-6   |

*P*-value corrected by Bonferroni method
Supplementary Fig. 1 Flow cytometric characterization of platelet-derived extracellular vesicles. Determination of forward scatter and side scatter characteristics of platelets (PLTs) and pEVs in suspension. Platelets were activated with thrombin (0.5 uNIH/mL) during 3 minutes at 37°C. The vesicle gate was established based on light scattering properties and size, using microspheres for calibration and defining pEVs as events both smaller than 1 μm and smaller than unstimulated platelets.
Supplementary Fig. 2 Comparison of platelet-derived subfractions. (a) Flamingo staining pattern of protein extracts of extracellular vesicles and EV-free releasate from baseline non-activated (B) and thrombin-stimulated (T) human platelets. Note that the pattern of bands present in both samples is different. (b) Representative image of western blot against flotillin-1 on pEV samples. Scatter plots with bars showing the quantitative variations in spot intensity in the different studied groups (B, baseline non-activated and T, thrombin-stimulated, n=3/group). Total protein normalization analysis was performed with Ponceau S. Data are expressed in arbitrary units (AU) as mean ± SEM. Differences were analyzed by 2-sided unpaired Student T-test.
Supplementary Fig. 3 Venn diagram of total proteins in pEV fraction compared to Vesiclepedia and Exocarta databases. The list of pEV proteins identified was compared with the list of human extracellular vesicle proteome contained in Vesiclepedia, ExoCarta and ExoCarta top-100 databases as shown by the Venn diagrams.
Supplementary Fig. 4 Protocadherin α4 identification. Representative spectrum of PCDHA4 identified by mass spectrometry showing the matched peptides of the trypsin digested protein sequence with the theoretical database peptides, and identification parameters of MASCOT search.
Supplementary Fig. 5 Analysis of differentially regulated proteins by Ingenuity Pathways Analysis Core Analysis. Top interactions for differentially regulated pEV proteins from thrombin-stimulated platelets correspond to the *Cellular Assembly and Organization, Cellular Function and Maintenance, Developmental Disorder* interactome. Proteins identified by differential analysis are shown as shaded nodes with their gene names. Up-regulated proteins appear in red and down-regulated proteins in green. The intensity of color is representative of the change in protein intensity. Solid lines represent direct interactions, dotted represent indirect interactions. Arrows from one node to another indicate that this node acts upon the other. Lines without arrows represent binding. Node shapes are: double circle = complex or group; notched triangle = kinase; wavy shape = enzyme; circle = other. Proteins known to be involved in platelet activation by thrombin are indicated by a black solid arrow.
Supplementary Fig. 6 Protein-protein interaction network of thrombin-induced differential expressed proteins in pEVs. Potential interactions between differential expressed proteins and functional clusters in pEVs from thrombin-activated platelets were determined using the search tool for retrieval of interacting genes (STRING) database.