EXTRACELLULAR VESICLES DERIVED FROM ENDOTHELIAL PROGENITOR CELLS PROTECT GLOMERULAR ENDOTHELIAL CELLS AND PODOCYTES FROM COMPLEMENT- AND CYTOKINE-MEDIATED INJURY

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Background:

- Glomerulonephritis (GN) is an inflammatory kidney disease that potentially leads to chronic kidney disease (CKD) because of accelerated glomerular cell senescence.
- Glomerular endothelial cells (GEC) and podocytes (Podo) are the primary targets during GN.
- Inflammatory reaction of GN is mediated by activation of the complement cascade (Compl) and pro-inflammatory cytokines (CK), such as Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6).
- Endothelial Progenitor Cells (EPC) are bone-marrow hematopoietic stem cells that repair injured endothelium by paracrine mechanisms.
- Extracellular Vesicles (EV) are microparticles involved in paracrine intercellular communication by the transfer proteins and RNAs, particularly microRNAs, to target cells.

Aim of the study:

- Evaluation of the protective effect of EPC-derived EVss on GECs and Podo cultured *in vitro* in detrimental conditions that mimic GN microenvironment.

Methods:

- Experiments were conducted on GECs and Podo isolated from human renal glomeruli and cultured with TNF-α, IL-6 and the Compl protein C5a.
- EPCs were isolated from peripheral blood of healthy volunteers and characterized for endothelial and stem cell markers.
- EVs were isolated from EPC supernatants by ultracentrifugation and characterized for size, concentration (Nanosight), protein (FACS) and RNA (RT-PCR) expression.

Results:

- EVs labelled with red dye PKH26 were internalized in GECs and Podo through a L-selectin-dependent mechanism (Figure 1).
- In GECs, EVs enhanced the formation of capillary-like structures and cell migration (Figure 2) by modulating gene expression (Figure 3) and inducing the release of growth factors (VEGF-A and HGF, Figure 4).
- In the presence of CK and C5a, EVs protected GECs from apoptosis by decreasing oxidative stress (Figure 5) and prevented leukocyte adhesion by downregulating adhesion molecules (ICAM-1, VCAM-1, E-selectin, Figure 6).
- On Podo, EVs inhibited apoptosis and prevented nephrin shedding induced by CK and C5a (Figure 7).
- In a co-culture model of GECs and Podo that mimic glomerular filtration barrier, EVs preserved cell function and perme-selectivity from inflammatory-mediated damage (Figure 8).
- Of note, RNase pre-treatment of EV abrogated their protective effects, suggesting the crucial role of RNA transfer from EV to damaged GECs.

Conclusion:

- EPC-derived EVs preserved GECs and Podo function from Compl- and CK-induced damage, suggesting their potential role as therapeutic agents for drug-resistant GN.
- EPC-derived EVs limit glomerular senescence and fibrosis induced by Compl activation and chronic inflammation.

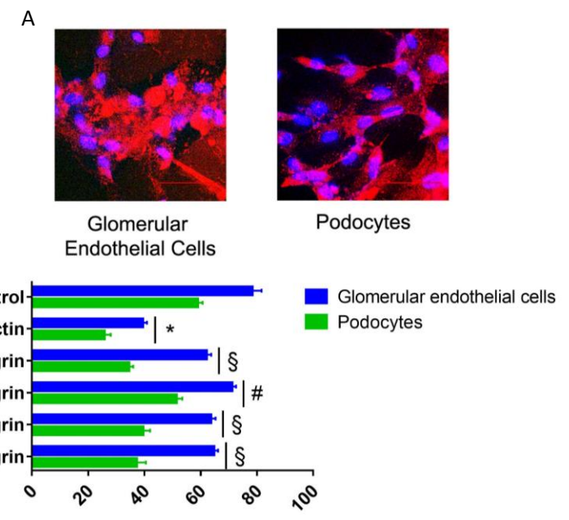

Figure 1. (**A**) Representative confocal microscopy micrographs of Internalization of PKH26 red dye-labelled EPC-derived EVs in human GECs and podocytes *in vitro*. (**B**) Graph showing FACS analysis of PKH26-labelled EV internalization in GECs and podocytes. EVs were pre-incubated with 1 μg/mL blocking mAb directed to L-selectin, α4, α6, β1, αVβ3 integrin. (* $p < 0.05$ L-selectin vs. Positive Control; § $p < 0.05$ α4, β1 or αVβ3 vs. L-selectin; * $p < 0.05$ α4, β1 or αvβ3 vs. Positive Control).

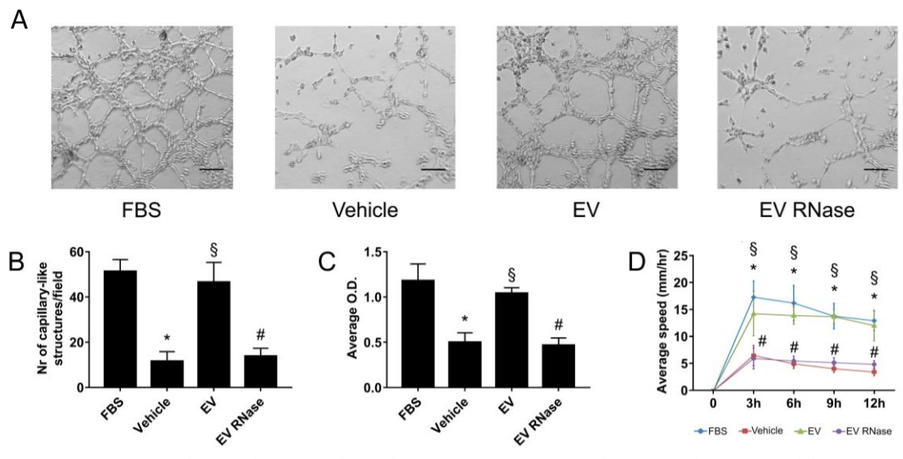

Figure 2. EPC-derived EVs induced GEC angiogenesis by stimulating proliferation and migration. (**A**) Representative light microscopy images and (**B**) count of GECs cultured on Matrigel *in vitro*. (**C**) Analysis of GEC proliferation by BrdU assay and (**D**) migration test on six-well plates after 3, 6, 9, and 12 h. * $p < 0.05$ vehicle vs. FBS; § $p < 0.05$ EV vs. vehicle; # $p < 0.05$ EV RNase vs. EV.

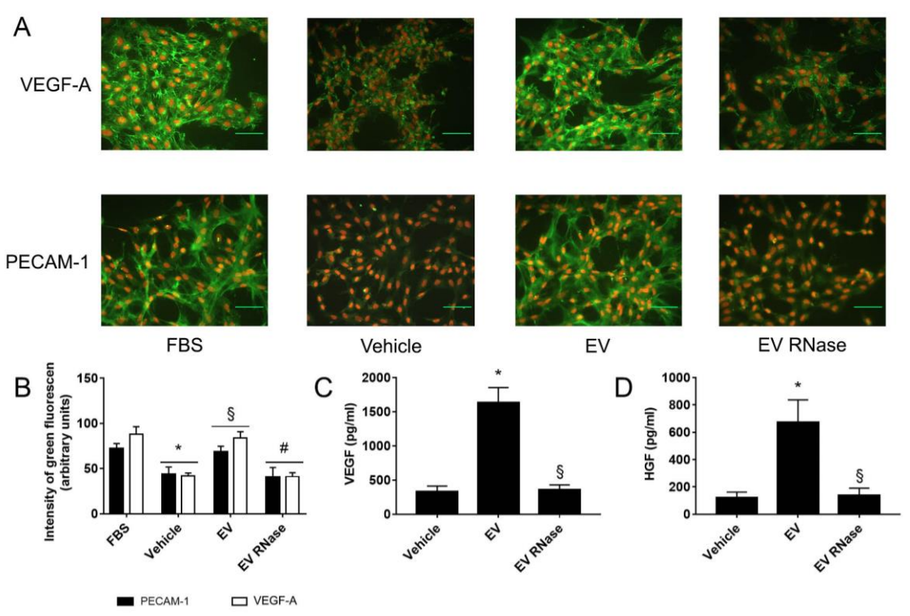

Figure 4. (**A**) Representative immunofluorescence micrographs and (**B**) quantification on fluorescence intensity of platelet/endothelial cell adhesion molecule (PECAM-1) and vascular endothelial growth factor (VEGF-A) expression (green staining) in GECs. Nuclei were counterstained with 1 μg/mL propidium iodide; scale bar: 50 μm. Quantification of (**C**) VEGF and (**D**) HGF on GEC supernatants by ELISA. * $p < 0.05$ vehicle vs. FBS; § $p < 0.05$ EV vs. vehicle; # $p < 0.05$ EV RNase vs. EV.

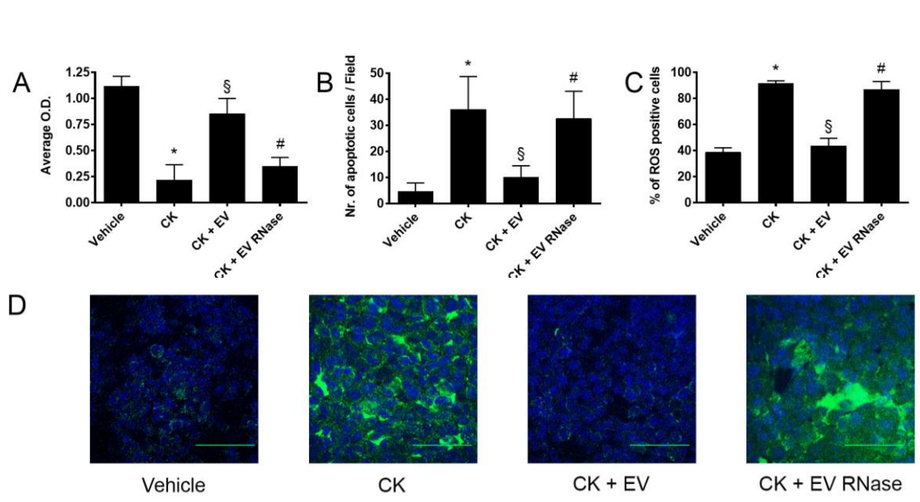

Figure 5. (**A**) Graphs showing GEC cytotoxicity by XTT assay and (**B**) apoptosis by TUNEL assay. (**C**) FACS analysis and (**D**) representative micrographs of ROS expression of GEC (green staining) by confocal microscopy studies (scale bar 50 μm). Nuclei were counterstained in blue by 2.5 μg/mL Hoechst. * $p < 0.05$ CK vs. vehicle; § $p < 0.05$ CK + EV vs. CK; # $p < 0.05$ CK + EV RNase vs. CK + EV.

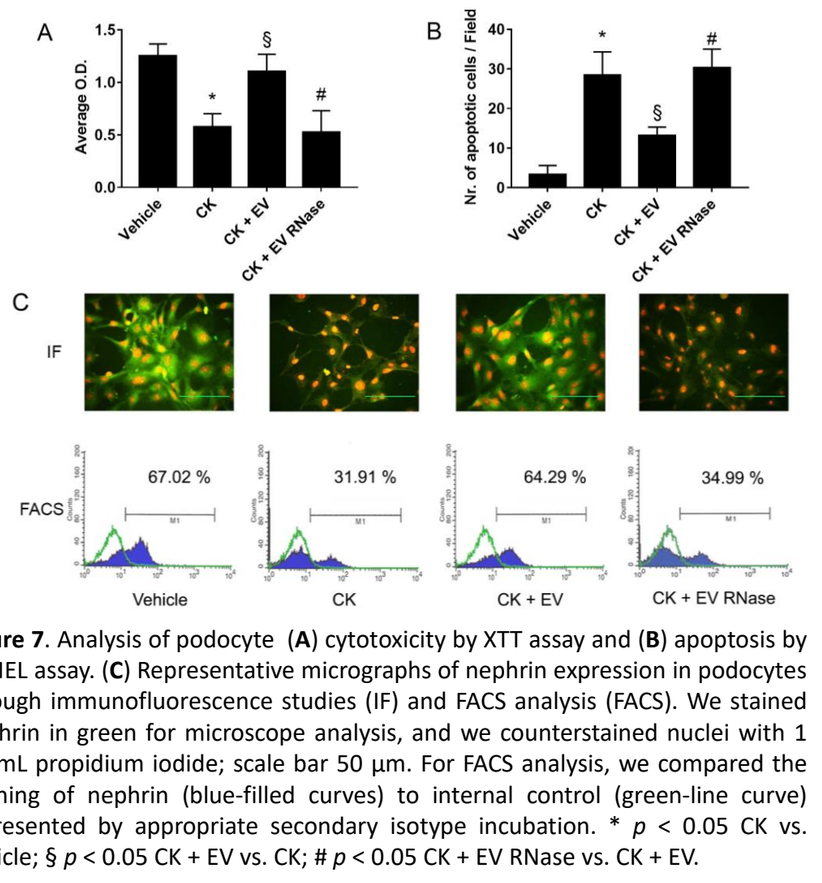

Figure 7. Analysis of podocyte (**A**) cytotoxicity by XTT assay and (**B**) apoptosis by TUNEL assay. (**C**) Representative micrographs of nephrin expression in podocytes through immunofluorescence studies (IF) and FACS analysis (FACS). We stained nephrin in green for microscope analysis, and we counterstained nuclei with 1 μg/mL propidium iodide; scale bar 50 μm. For FACS analysis, we compared the staining of nephrin (blue-filled curves) to internal control (green-line curve) represented by appropriate secondary isotype incubation. * $p < 0.05$ CK vs. vehicle; § $p < 0.05$ CK + EV vs. CK; # $p < 0.05$ CK + EV RNase vs. CK + EV.

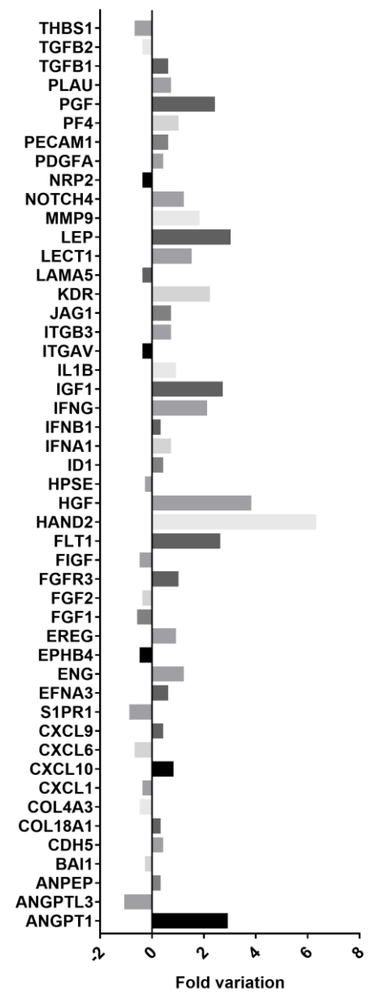

Figure 3. Graph showing the fold variation of mRNA expression by RT-PCR analysis of angiogenesis-related genes in GECs stimulated with EVs compared to untreated GECs

Figure 6. (**A**) Representative images and (**B**) graphs showing the count of adherent PBMCs (black columns) and PMNs (white columns) to GECs. scale bar 50 μm. (**C**) FACS analysis of ICAM-1, VCAM-1, and E-selectin in GECs. * $p < 0.05$ CK vs. vehicle; § $p < 0.05$ CK + EV vs. CK; # $p < 0.05$ CK + EV RNase vs. CK + EV.

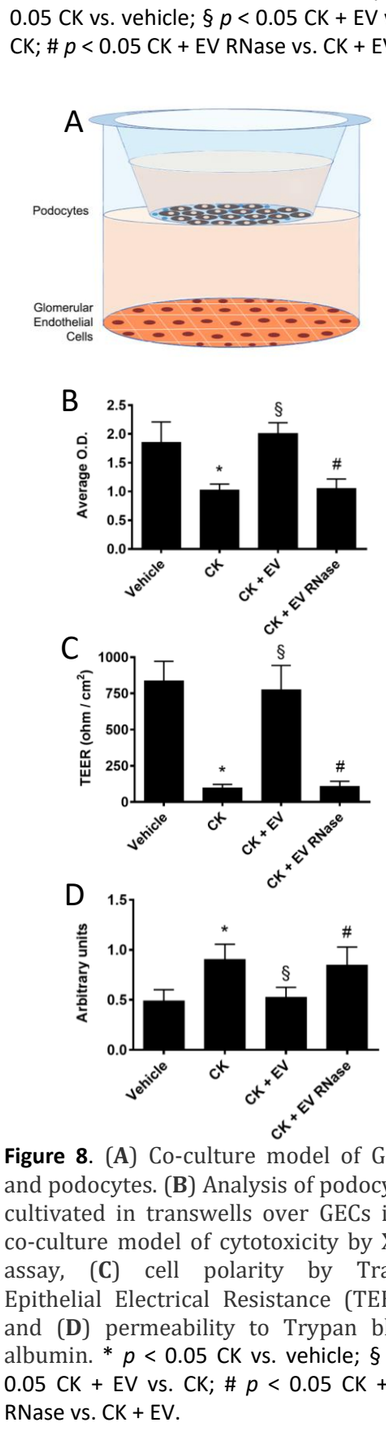

Figure 8. (**A**) Co-culture model of GECs and podocytes. (**B**) Analysis of podocytes cultivated in transwells over GECs in a co-culture model of cytotoxicity by XTT assay, (**C**) cell polarity by Trans-Epithelial Electrical Resistance (TEER), and (**D**) permeability to Trypan blue-albumin. * $p < 0.05$ CK vs. vehicle; § $p < 0.05$ CK + EV vs. CK; # $p < 0.05$ CK + EV RNase vs. CK + EV.