**Supplementary material**

Cellular preparation and assays.

*Human VSMC.* Human vascular smooth muscle cells (hVSMC) were grown from explants of aortic fragments obtained after aneurysm repair operations, kindly donated from the University Hospital of Maastricht. Collection, storage, and use of tissue and human aortic samples were performed in agreement with the Dutch Code of Proper Secondary Use of Human Tissue. Cells were cultured in 5% CO2 at 37°C with complete DMEM (Gibco, Waltham, US) supplemented with 20% Foetal bovine serum (FBS), 1% penicillin and streptomycin and used between passages 3 and 12. Each experiment was carried repeaed in VSMCs from at least 3 different donors.

*Human Umbilical Vein Cord Endothelial Cells (HUVEC).* Cells were freshly isolated fromumbilical cords that were kindly donated by the midwifery staff of the Maternity Unit, Royal London Hospital (London, UK) with an approved protocol (East London & The City Local Research Ethics Committee reference 05/Q0603/34 ELCHA). Cells were cultured 0.5% gelatin coated T75 flasks, in 5% CO2 at 37 °C with complete Medium 199 (Gibco, Waltham, USA) containing 100 U penicillin, 100 mg/mL streptomycin, and 2.5 µg/mL fungizone (Gibco) supplemented with 20% Human serum (Sigma-Aldrich, UK), and used up to passage 4.

*Monocyte and polymorphonuclear cells (PMN) isolation.* All volunteers gave written, informed consent to blood collection and the procedure was approved by the Queen Mary Ethics of Research Committee (QMERC2014.61) for healthy controls. Blood (30 ml) drawn from healthy volunteers was anticoagulated with 0.32% w/v sodium citrate, centrifuged at 150x*g* for 20 min. After removal of platelet rich plasma, RosetteSep™ cocktail (StemCell Technology, Vancouver, Canada) was added (50 µl/ml of blood) for 20 min prior to layering over Histopaque™ 1077 (Sigma-Aldrich, Gillingham, UK). After 20 min, centrifugation at 1200x*g*, the monocyte layer was harvested, washed and cell concentration adjusted as needed.

For polymorphonuclear cells (PMN) isolation, whole blood was centrifuged at 130x*g* for 20 min, plasma removed, erythrocytes depleted on 6% w/v dextran (high molecular weight, Sigma-Aldrich, in PBS). Then, the leukocyte-rich fraction was layered over Histopaque and centrifuged for 30 min 450x*g* at room temperature. The PMN layer was harvested, washed and cell concentration adjusted as needed.

*Generation and isolation of monocyte and PMN EVs.* Monocytes (1x106 cells/mL) or PMN (20x106 cells/mL) were incubated with TNF-α (50 ng/mL; Sigma-Aldrich) for 60 min or 20 min, respectively, or vehicle at 37°C. Time and concentration were chosen on preliminary experiments and published data1, 2. Cell suspensions were centrifuged at 4,400x*g* at 4°C for 15 min to pellet cells and/or platelets, followed by a second centrifugation at 13,000x*g* at 4°C for 2 min to remove remaining contaminants (e.g. apoptotic bodies). EVs were enriched by centrifuging at 20,000x*g* at 4°C for 30 min, the supernatant was removed, and pellets were re-suspended in filtered sterile PBS. The physical characteristics of the EV populations produced were monitored by Nanosight™ and Imagestream™. Briefly, nearly 0.5 ml of EVs (between 106 to 108 EVs) in suspension were loaded onto the Nanosight NS300 with 488 nm scatter laser and high sensitivity camera (Malvern Instruments Ltd., Malvern, UK); five videos of 90 seconds each were recorded for each sample. Data analysis was performed with NTA2.1 software (Nanosight, Malvern, UK) using the following settings: detection threshold: 5–10; Blur: auto; minimum expected particle size: 20 nm. For imaging flow cytometry analysis, EVs were analysed and counted using fluorescence triggering on an ImageStreamx™ MKII imaging cytometer as described previously3. Briefly, vesicles were labelled with 50 μM BODIPY maleimide fluorescein or BODIPY Texas-Red (Life Technologies, Carlsbad, USA), and acquired as such or after labelling with anti-CD14-APC (2 μg/ml; clone 61D3; Biolegend) and anti-CD41-PE (2 μg/ml; clone HIP8; Biolegend). Fluorescence minus one controls, serial dilutions controls and antibody only were run as controls in order to exclude possibility of swarming effect and antibody aggregation.

To check possible TNF-α EV contamination, EVs were pelleted at 20000*xg* speed and re-suspended in 200 μL of filtered PBS. TNF-α concentration was measured by ELISA using human TNF-α ELISA kit (Thermo Fisher Scientific) according to manufacturer's instructions.

hVSMC activity assays.

*Calcification:* Human VSMC (2x105) were cultured in DMEM supplemented with 0.5% FBS, 1.8 mM CaCl2 prior to addition of EV subsets on a ratio of 10 EV/Cell. Concentrations and ratios of EVs were chosen based on preliminary experiments. As positive control, oxLDL was added at a final concentration of 50 ng/mL4. Cells were incubated at 5% CO2 at 37°C. Once calcified, cell media were removed and 120 µL of 0.1 M HCl added to solubilise calcium, measured with a Calcium assay kit (Randox reagents, The Netherlands). After the reaction, absorbance was read at 562 nm on Cytation 3 Cell Imaging Multi-Mode Reader (Biotek, Vermont, USA). Data are normalised to total protein content measured with BCA assay (ThermoFisher, UK), and presented as µg Ca2+ over µg protein ratio. The N number represents the number of different EV preparations isolated from distinct patient sample each time and used for each single biological replicate; all experiments were performed with 3 technical replicates.

*Proliferation:* Human VSMC (2x104) were seeded in DMEM with 0.5% FBS and 1% penicillin and streptomycin in a 96X E-Plate (Acea Bioscience, California, USA). The E-plate 96 is placed within the Real-Time Cell Analyzer (RTCA) single plate Station, which is kept in a humidified incubator and is connected to the RTCA Analyzer and RTCA Control Unit. Cell attachment, spreading and proliferation were monitored every 30 min using the xCELLigence system (Acea Bioscience, California, USA). After 24 hours, when the cells were in the log growth phase, the indicated EV concertation (at a ratio of 10 EV/Cell) or oxLDL (50 ng/mL) were added. Cells were monitored at 37°C at 5% CO2 for at least other 100 hours. Data were acquired and analysed with RTCA Software (version number 1.2.1.1002). The N number represents the number of different EV preparations isolated from distinct patient sample each time and used for each single biological replicate; all experiments were performed with 3 technical replicates.

*Fluorescent microscopy analysis:* hVSMCs (7.5x105) were incubated overnight in presence or absence of indicated treatments, at 5% CO2 and 37oC. After fixation in 4% paraformaldehyde (4°C, 30 min), cells were washed, blocked with 0.1% Triton and 0.2% BSA (T-PBS; for intracellular staining) for 30 min at room temperature, prior to addition of Phalloidin-AF488 (0.5 μg/mL; ThermoFisher scientific, UK). After 1 hr and counterstaining with DAPI (1 µ/mL; Sigma, UK), fluorescence was read on Cytation 3 Cell Imaging Multi-Mode Reader (ThermoFisher Scientific) at 465 nm for FITC staining, 590 nm AlexaFluor 488 and 365 nm for DAPI. Data were analysed with Gen5 Software (BioTek).

HUVEC flow chamber assay

HUVECs were cultured until confluence and seeded in μ-Slides VI 0.4 (80606, Ibidi) coated with 0.5% bovine gelatin. Confluent monolayers were stimulated with 10ng/mL of TNF-α (Sigma-Aldrich) or EVs overnight on a ratio of 10 EVs/Cell. Isolated PMNs as described above were pelleted and resuspended at 1x107 cell/mL in Dulbecco phosphate buffered saline (DPBS) with 0.1% bovine serum albumin (BSA) and kept on ice before use. Cells were then diluted to the final concentration of 1x106 cells/mL in DPBS (with Ca2+ and Mg2+) with 0.1% BSA immediately prior to the flow chamber assay and flowed across HUVEC layers using a shear stress of 1 dyne/cm2. The flow chamber slides were viewed using a Nikon Eclipse TE3000 with a x10 and x20 phase contrast objectives (Nikon). PMNs were perfused over the HUVEC monolayer for 8 minutes before starting recording and five, 30s frames were collected for each well from random fields using a Q-Imaging Retiga EXi Digital Video Camera (Q-Imaging) recorded and analysed using Image Pro-Plus software (Media cybernetics). Four different capture measurements were performed: first the total number of interacting cells were quantified as the number of PMNs firstly captured in the 10s frame, then they were further characterized as either rolling, transmigrated or fully adherent. Experiments were performed at least three times with different blood donors and different isolated HUVEC. While analysing them, the total number of interacting cells for each category (capture, rolling, transmigrated, adherent) was converted to number of cells/field. The N number represents the number of different EV preparations isolated from distinct patient sample each time and used for each single biological replicate; all experiments were performed with 3 technical replicates.

EVs isolation and characterisation

Isolation and characterization of plasma EVs from ASCOT samples by Nanoparticle tracking analysis and Imaging flow cytometry. Twenty microliters of platelet reach plasma (PRP) were depleted of platelets and apoptotic bodies by centrifugation at 13,000x*g* for 2 min. EVs were enriched by centrifuging at 20,000x*g* at 4°C for 30 min, the supernatant was removed, and pellets were re-suspended in filtered sterile PBS. Approximately 0.5 ml of EV suspension (containing between 106 to 108 particles) were loaded onto the Nanosight NS300 with 488 nm scatter laser and high sensitivity camera (Malvern Instruments Ltd., Malvern, UK). Data analysis was performed with NTA2.1 software (Nanosight, Malvern, UK) as indicated above. Moreover, EVs were analysed and counted using fluorescence triggering on an ImageStreamx™ MKII imaging cytometer as described in the previous section. Briefly, vesicles were labelled with 50 μM BODIPY maleimide fluorescein (Life Technologies, Carlsbad, USA), and acquired directly or after labelling with either 2 μg/ml anti-CD14-APC, 2 μg/ml anti-CD41-PE or 1 μg/ml anti-CD146-AF647 (clone PIH12; Biolegend). Fluorescence minus one (FMO) controls were used for gating all protein antigen-positive events. Approximately 20,000 events were acquired per sample.

For transmission electron microscopy (TEM) analysis copper grids (400 mesh, Agar Scientific, Essex, UK) were pre-coated with 1% formvar (Agar Scientific, Essex, UK) solution prepared in chloroform. 5-10 μL of EV suspension (1x108/ml) was pipetted directly onto TEM grids and left 5-10 minutes to allow the EVs to adhere. TEM Grids were washed with distilled water and stained in 2% uranyl acetate for 2 min. A JEOL 1400+ TEM (Tokyo, JPN) equipped with an AMT XR16 CCD camera (AMT, Massachusetts, USA) was used to acquire images taken between x8,000 and ×20,000 magnification.

Presence of a select group of proteins identified by proteomic analysis was confirmed through standard SDS-PAGE, loading extracts from ∼30 × 106 EVs per lane (Millipore, Watford, UK). Western blot was conducted with specific antibodies against Annexin A1 (AnxA1; 5 μg/ml), CD9 (CD9; 5 μg/ml; SystemBIO), and anti-β-actin (ACTB; 5 μg/ml; clone AC-74, Sigma-Aldrich), Calnexin (CNX, 1 µg/ml; Abcam; Cat# ab22595), overnight at 4 °C followed by a 1 h incubation with either an HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Dako). Proteins were detected using Luminata™ Forte Western HRP Substrate (Millipore) visualized on Hyperfilm™ (GE Healthcare).

Statistical analysis

Statistical analysis and graphing of the cell culturing experiments were performed in GraphPad Prism 6 Software. Data distribution analysis was performed using Shapiro-Wilk test. Differences of variances by one way analysis of variance (ANOVA) followed by post-hoc Bonferroni for normally distributed data. A p value <0.05 was considered statistically significant. Flow cytometry images and plots were analysed with IDEAS 6.2 software. All statistical analysis and graphing regarding ASCOT sample characterization were performed in STATA 15.1. Descriptive characteristic statistics are presented with mean and standard deviation (SD) or frequencies and percentages, and descriptive EV statistics are presented with median and inter-quartile range (IQR).

Paired t-tests were conducted to compare crude mean differences in EVs between cases and controls. In addition, the robust Wilcoxon matched-pairs signed-rank test was also used to test for distributional differences in EVs between cases and controls, while relaxing the assumption of normality of EV distribution. These tests were also repeated, comparing EVs between those randomised to atorvastatin or placebo during the ASCOT trial.

Conditional logistic regression was used to estimate odds ratios (OR), with accompanying 95% confidence intervals (CI) and p-values, comparing the odds of being a case to the odds of being a control. Crude ORs were estimated, as well as adjusted ORs, adjusted for sex and baseline SBP.

References

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