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Supplementary information

Material and Methods

Preparation of media to treat HCAECs

Conditioned medium for Indirect (I) treatment. An MM6 cell suspension was adjusted to 2×10^6 cells/mL in starvation medium and 0.5 mL/well was seeded in a 24-well plate. After 2 hours of incubation at 37°C, the cells were exposed to several concentrations of 3R4F/THS2.2 aqueous extract or PBS (vehicle control) for 2 hours. Culture supernatants (conditioned media) and cell lysates were collected and stored at -80°C for the adhesion assay and mRNA extraction, respectively.

Unconditioned medium for Direct (D) treatment. In parallel, 3R4F/THS2.2 aqueous extract dilutions or PBS (vehicle control) were incubated for 2 hours at 37°C without MM6 cells, and then stored at −80°C for the adhesion assay.

In vitro adhesion assay

At the end of the 4-hour treatments, HCAECs were washed once with PBS, stained with 1 µg/mL Hoechst 33342 (Sigma, #14533, Buchs, Switzerland) for 15 minutes at 37°C, and washed twice with PBS. In parallel MM6 cells were re-suspended at $10X10^6$ cells/mL in 5µM Draq5 (Brunschwig, Basel, Switzerland) and incubated for 15 minutes at 37°C. Then, 2.5×10^5 freshly Draq5-stained MM6 cells per well were added on top of the stained HCAECs. After 45 minutes of incubation at 37°C, the cells were washed three times with PBS and fixed for 15 minutes with 4% formaldehyde (Sigma, #F1635, Buchs, Switzerland). The adherent MM6 cells and HCAECs were counted using an ArrayScan (Cellomics, Langenselbold, Germany). The adhesion rate was calculated by dividing the number of adherent MM6 cells by the total number of HCAECs in the well multiplied by 100 (number of adherent MM6 cells per 100 HCAECs). The experiment was conducted at least in triplicate for each condition.

Measurement of inflammatory markers in MM6 cell supernatants

Tumor necrosis factor α (TNFα) levels in MM6 cell-conditioned media were measured using an enzyme linked immunosorbent assay (ELISA) kit for cytokine detection (R&D Systems, #DY210, Abingdon, United Kingdom) based on the quantitative sandwich enzyme immunoassay technique (http:// www.rndsystems.com/Products/dy210/AssayProcedure).

A panel of 45 inflammatory markers (Human InflammationMAP® v1.0) including TNFα was quantified by Myriad-Rules Based Medicine (Austin, Texas, USA) in MM6 cell-conditioned media using multiplexed immunoassays (Multi-analyte profiling) (https://rbm.myriad.com/products-services/ humanmap-services/human-inflammationmap/).

Cell viability assay

The viability of MM6 cells and HCAECs was measured using resazurin following treatments with a/sbPBS for 2 hours and conditioned media, unconditioned media or fresh THS2.2 or 3R4F aqueous extract for 4 hours. The cells were incubated in starvation medium containing resazurin (60 µg/mL; Sigma, #R07017-5G, Buchs, Switzerland) for 2 hours. Fluorescence was measured at 560/590 nm with the FLUOstar OPTIMA Microplate Reader (BMG Labtech, Ortenberg, Germany).

RNA preparation and transcriptome analysis

RNA from MM6 and HCAECs (triplicate samples) was extracted using respectively the RNeasy Mini kit (Qiagen, #74104, Hilden, Germany) and RNeasy Micro Kit (Qiagen, #74004, CatHilden, Germany) then stored at -80°C. Purified RNA was quantified using a Nanodrop ND-1000 (Thermo, Waltham, MA, USA). RNA integrity was determined using an Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit (Agilent Technologies, #5067-1511, Santa Clara, CA, USA). All RNA samples had an RNA integrity number of >8.8. Transcriptome analysis for the HCAECs was performed using the Nugen Ovation amplification system V2 (Nugen, #3100-A01, San Carlos, CA, USA) for the HCAECs according to the manufacturer's instructions. After synthesis of double-stranded cDNA from 50 ng total RNA, SPIA (single primer isothermal amplification) was followed by the fragmentation and biotinylation of the cDNA. Transcriptome analysis for the MM6 was performed using the Affymetrix HT (High Throughput) 3'IVT Express protocol (Affymetrix, #901253, Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, after synthesis of double-stranded cDNA from 100 ng total RNA, the fragmented cRNA was biotinylated during the IVT (in vitro transcription) step. The degree of amplified and fragmented nucleic acids were checked by capillary electrophoresis using the Agilent 2100 Bioanalyzer. The biotinylated and fragmented cRNA were hybridized onto a GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, #900467, Santa Clara, CA, USA) overnight at 60 rpm in a 45°C GeneChip® Hybridization Oven 645 (Affymetrix, Santa Clara, CA, USA). The arrays were washed and stained on a GeneChip® Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA) followed by antibody signal amplification, washing, and staining with streptavidin r-phycoerythrin (Affymetrix, #900720, Santa Clara, CA, USA). The arrays were scanned using an Affymetrix GeneChip® Scanner 3000 7G generating the CEL files that were visually inspected for artifacts. The Affymetrix® Expression Console™ Software was used to check the quality of chips by applying default parameters.

Supplementary figure legends

Supplementary Figure 1. Experimental set up of the adhesion assay using 3R4F aqueous extract. Schematic of the experimental set up for the treatment of HCAECs *via* three different treatment modalities prior to functional and molecular analyses. Conditioned media were generated for the "indirect" (I) treatment by treating MM6 cells with freshly generated sbPBS, and collecting and freezing supernatants at -80°C after 2 h. Unconditioned media generated for "direct" (D) treatment were subjected to the same medium dilution, temperature, and freeze/thaw conditions, but in the absence of MM6 cells. HCAECs were then treated with conditioned (I) or unconditioned (D) media. For "fresh direct" (FD) treatment, freshly generated sbPBS diluted in the same medium was directly used to treat HCAECs. After 4 h, the adhesion assay was conducted or cells were collected for transcriptome analysis. The experimental set up enabled deconvolution of the effects of various components present in the media, such as soluble MM6 mediators as well as stable and unstable CS compounds in relation to cell adhesion and underlying molecular changes assessed by transcriptomics data analysis. Abbreviations: MM6, Mono Mac-6; HCAECs, human coronary artery endothelial cells; CTL; control; SM; starvation medium; CM; conditioned medium; CS, cigarette smoke

Supplementary Figure 2: Concentration-response experiments with 3R4F aqueous extract and THS2.2 sbPBS. Experiments using (A) indirect/direct or (B) fresh direct exposures were conducted with a range of concentrations of 3R4F/THS2.2 aqueous extracts measuring MM6-HCAEC adhesion rate, TNFα release by MM6 cells, cell viability and count. The results correspond to independent experiments (ExpA-E) including 3-4 replicates (wells) for each (C) Schema supporting the rationale for the final selection of 3R4F and THS2.2 aqueous extract concentrations for the main experimental phase. Concentrations that promoted maximum response with MM6 and HCAEC cell death/loss below ~25% were chosen.

Supplementary Figure 3. Experimental design, adhesion rate and HCAEC cell count. (A) Design of the main experimental phase conducted with MM6 cells and HCAECs including 3 main factors: aqueous extract (two levels: 3R4F and THS2.2), concentrations (2-3 levels depending on the item), HCAEC exposure modality (3 levels: indirect, direct and fresh direct). (B) Results (adhesion rate and HCAEC count) of the adhesion assay originating from the same I/D and FD experiments for which HCAEC and MM6 cell transcriptomics data were generated (see details in Supplementary Figure 1).

Supplementary Figure 4. Pairwise gene expression fold-change correlation plots for HCAECs. The plots display gene expression fold-changes (log_2 scale) of SRP X and Y. r and p correspond to the Pearson correlation coefficient and associated *p*-value, respectively.

Supplementary Figure 5. Heatmap of NPA scores computed with HCAEC systems response profiles for 57 subnetworks. Blue star indicates that the NPA score is significant with regards to experimental variation and, O and K statistics with $p < 0.05$. Subnetworks are grouped by networks representative of higher level biological processes. Red and purple correspond to 3R4F and THS2.2 SRPs, respectively.

Supplementary Figure 6. Heatmap of enrichment scores for HCAECs including names of gene sets (see Figure 4 legend).

Supplementary Figure 7. Pairwise gene expression fold change correlation plots for MM6 cells. The plots display gene expression fold changes (log_2 scale) of SRP X and Y. r and p correspond to the Pearson correlation coefficient and associated *p*-value, respectively.

Supplementary Figure 8. Heatmap of NPA scores computed with MM6 cell SRPs for 54 subnetworks. A blue star indicates that NPA score is significant with regards to experimental variation and, O and K statistics with $p < 0.05$. The subnetworks are grouped by network representative of higher level biological processes. Red and purple colors correspond to 3R4F and THS2.2 SRPs, respectively.

Supplementary Figure 9. Heatmap of enrichment scores for MM6 cells including names of gene sets (see Figure 7 legend).

Supplemetary table legends

Supplementary table 1: Relative Harmful and Potentially Harmful Constituents (HPHC) Yields on a per mg Nicotine Basis Measured in 3R4F and THS2.2 Mainstream Smoke and Aerosol, respectively

Supplementary table 2: Genes Differentially Expressed by THS2.2 aqueous extract (FDR<0.05) and not by 3R4F aqueous extract in HCAECs.

Supplemetary table 3: List of Subnetworks used for NPA and BIF Computational Analysis of HCAECs and MM6 Transcriptomics Data.

Supplementary table 4: Backbone Scores of Subnetwork Nodes Present in Figure 3B Computed for each SRP. Footnotes: Values are expressed as percent of total contribution. The sign provides the regulation directionality of a node. Leading nodes contribute 80% of the NPA score.

Supplementary table 5: Genes Differentially Expressed by THS2.2 aqueous extract (FDR<0.05) and not by 3R4F aqueous extract in HCAECs

Supplemetary table 6: Panel of 45 Inflammatory Markers Measured in Conditioned Media using xMAP Technology. Footnotes: Analytes with limit of detection (LOD) below were excluded. Abbreviations: 0_15perc or 0_75perc: starvation medium with 15 or 75% (v/v) PBS (PBS control); significance level: **p*-value ≤ 0.05, ***p*-value ≤ 0.01, ****p*-value ≤ 0.001; LogPVal: -$\log_{10}$ raw-p-value; Lower/UpperCLMean: lower/upper bound of the 95% confidence interval for the mean difference between comparison groups.