Proteomic Analysis of Vocal Fold Fibroblasts Exposed to Cigarette Smoke Extract: Exploring the Pathophysiology of Reinke’s Edema

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In Brief
To explore the pathophysiology of Reinke’s edema, we exposed human vocal fold fibroblasts to medium conditioned with cigarette smoke extract followed by quantitative mass spectrometry. Proteomic analyses revealed an upregulation of proteins related in oxidative stress response. Fibrillar collagens were downregulated, whereas hyaluronan levels were increased. The latter two lead to an altered ECM composition and tissue properties.

Highlights
- First global study on laryngeal cells cultured with cigarette smoke enriched medium.
- Vocal fold fibroblasts increased production of ECM component hyaluronan.
- Expression of several fibrillar collagens was reduced.

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Proteomic Analysis of Vocal Fold Fibroblasts Exposed to Cigarette Smoke Extract: Exploring the Pathophysiology of Reinke’s Edema*

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Reinke’s edema is a smoking-associated, benign, mostly bilateral lesion of the vocal folds leading to difficulties in breathing and voice problems. Pronounced histological changes such as damaged microvessels or immune cell infiltration have been described in the vocal fold connective tissue, the lamina propria. Thus, vocal fold fibroblasts, the main cell type of the lamina propria, have been postulated to play a critical role in disease mediation. Yet information about the pathophysiology is still scarce and treatment is only surgical, i.e. symptomatic. To explore the pathophysiology of Reinke’s edema, we exposed near-primary human vocal fold fibroblasts to medium conditioned with cigarette smoke extract for 24 h as well as 4 days followed by quantitative mass spectrometry.

Proteomic analyses after 24 h revealed that cigarette smoke increased proteins previously described to be involved in oxidative stress responses in other contexts. Correspondingly, gene sets linked to metabolism of xenobiotics and reactive oxygen species were significantly enriched among cigarette smoke-induced proteins. Among the proteins most downregulated by cigarette smoke, we identified fibrillar collagens COL1A1 and COL1A2; this reduction was validated by complementary methods. Further, we found a significant increase of UDP-glucose 6-dehydrogenase, generating a building block for biosynthesis of hyaluronan, another crucial component of the vocal fold lamina propria. In line with this result, hyaluronan levels were significantly increased because of cigarette smoke exposure. Long term treatment of 4 days did not lead to significant changes.

The current findings corroborate previous studies but also reveal new insights in possible disease mechanisms of Reinke’s edema. We postulate that changes in the composition of the vocal folds’ extracellular matrix – reduction of collagen fibrils, increase of hyaluronan – may lead to the clinical findings. This might ease the identification of better, disease-specific treatment options. Molecular & Cellular Proteomics 18: 1511–1525, 2019. DOI: 10.1074/mcp.RA119.001272.

Reinke’s edema (RE) is a common, mostly bilateral, benign lesion of the vocal folds (VF) characterized by an excessive edema of the superficial layer of the lamina propria, aka. Reinke space (supplemental Fig. S1). The disease occurs almost exclusively in smokers and is triggered by voice abuse (1, 2). The development of RE appears to be a unique tissue phenotype and differs significantly from inflammatory processes of tissues only several centimeters caudal, such as the tracheal mucosa.

RE is commonly diagnosed in women (aged 50 years or older) leading to a significant decrease of the mean fundamental frequency and a hoarse and non-sustainable voice (3, 4). Because males have lower pitched voices than females, they are less likely to observe significant changes in their voice and are therefore less likely to seek treatment. Low pitched voices (females, < 130 Hz) result from the mass-loaded VF that oscillate at an inordinately low frequency (5). The swelling is typically bilateral but asymmetric in volume, enhancing irregular phonatory vibration and thus dysphonia. Even if a relation between smoking and voice abuse is suggested by several authors (6, 7), the initiating trauma leading to RE is not known. However, it is assumed that continuous exposure to chemical and mechanical stressors combined with a proliferation of microvessels leads to a damage of the capillary endothelium with subsequent extravasation of fluid into the potential space resulting in edema and hence swelling (1, 8). The edema is furthermore increased by the aerodynamic pressures that drive VF mucosal oscillation. The effects of laryngo-pharyngeal reflux (LPR) are discussed controversially in the development of RE, with some authors stating an additive effect of cigarette smoke and LPR (1), whereas others showed lower incidences (9).
Diagnosis is done by transnasal or transoral endoscopy of the larynx (laryngoscopy) in the outpatient clinic in the awake patient. Prominent vessels that run irregularly in the subepithelial space shimmering through the mucosa are characteristic for the disease. Immuno-histologically RE is characterized by a chaotic distribution of short and scattered connective fibers and an epithelial layer that is locally thickened and hyperplastic (8, 10, 11). The lamina propria of RE specimens contains interstitial and inflammatory cells (mainly macrophages and mastocytes) (8). A thickening of the basement membrane, as well as edematous lakes, extravascular erythrocytes and increased thickness of submucosal vessels in the lamina propria are considered as main histopathologic features (2, 12).

Several studies postulate that vocal fold fibroblasts (VFF) as the main cell type of the VF lamina propria play an important role in mediating this disease (6, 13, 14). It was shown that the VFF phenotype was altered in vitro by cultivation with cigarette smoke condensate containing medium and that an antioxidative protein was upregulated in these cells (6). Likewise, autophagy was induced by cigarette smoke extract (CSE) in human VFF reflecting a protective response to the significant increase of highly reactive oxygen species molecules (13).

In contrast to the aforementioned studies that investigated effects of CS on selected proteins (and thus biological processes) we employed proteome analysis to identify an unprecedented number of candidate proteins involved in the pathogenesis of RE.

EXPERIMENTAL PROCEDURES

Human Tissue Samples and Cell Culture—Near-primary human vocal fold fibroblasts (np-hVFF, n = 5) were derived from VF mucosal specimens either obtained from surgery (ENT University Hospital, Medical University of Graz), or post mortem (Diagnostic & Research Institute of Pathology, Medical University Graz). None of the subjects had a smoking history, subject data is summarized in supplemental Table S1. Procedures were approved by the local ethics committee. Processing of samples was performed as described previously (15). Briefly, tissue pieces were allowed to adhere on the bottom of 12-well plates before addition of growth medium (GM), consisting of 75% Dulbecco's Modified Eagle Medium (DMEM) (4.5 g/L Glucose; Sigma Aldrich, Vienna, Austria) and 25% F12 Nutrient Mix (Life Technologies, Waltham, Massachusetts) supplemented with 5% Fetal Bovine Serum (FBS; Biowest, Nuaille, France), 1x Insulin-Transferrin-Selenium (Life Technologies), 200 nM 3,3',5-Triliodo-l-thyronine (Sigma Aldrich), 100 ng/ml human Insulin-like Growth Factor-I, 12.5 ng/ml human Fibroblast growth factor-2 (both Thermo Scientific, Waltham, Massachusetts) and 100 μg/ml Normocin (Invivogen, San Diego, California). Samples were incubated under standard conditions (37 °C, 5% CO2 and GM was partially replaced every 3–4 days until robust outgrowth of fibroblastoid cells. Subsequently, cells were expanded over 3–4 passages (using 0.25% Trypsin/EDTA (Sigma Aldrich) for cell detachment) to generate cryostocks in 90% FBS/10% DMSO which were stored in liquid nitrogen. RT-qPCR performed at this stage confirmed the absence of epithelial, endothelial, and skeletal muscle markers (unpublished data). Cells between passages 4 and 9 were used for proteomic analyses (see below).

Immortalized human VFF (16) were kindly provided by Prof. Thibeault, University of Wisconsin-Madison and were cultivated in standard medium (SM), consisting of DMEM (4.5 g/L Glucose), 10% FBS, and 100 μg/ml Normocin as previously described (17). For experiments, cells were seeded in SM at a density of 15,000 cells/cm², unless indicated otherwise. GM was replaced the following day with CSE or air-bubbled control (ABC) medium. For wells destined for analysis of collagen, CSE and ABC media were mixed with ABC medium containing Ficoll® PM400 (final concentration 16.7 mg/ml), Ficoll® PM70 (final concentration 25 mg/ml), and L-ascorbic acid (final concentration 100 μM; all Sigma Aldrich). As previously described by others (18) and us (17, 19), this “macromolecular crowding” enhances post-translational processing of fibrillar collagen in vitro.

Generation of CSE—Based on previous studies (14, 20, 21) we set up a device to generate CSE consisting of a 100 ml Erlenmeyer flask with a 5 mm polypropylene tube that was guided through the flask’s rubber cap and connected to a 200 μm borosilicate glass frit at its lower end. The lateral opening of the Erlenmeyer flask was connected to a 60 ml syringe via a silicone tube. For extraction, full-strength Marlboro® cigarettes were inserted into the upper end of the polypropylene tube, lighted and smoked by applying negative pressure via the syringe. By this, the cigarette smoke was bubbled into 60 ml of medium, consisting of DMEM, 10 mM HEPES, 1% FBS, and 100 μg/ml Normocin, Sigma Aldrich. During extraction, the medium was continuously mixed via a magnetic stir bar (7 mm length, 600 rpm). For each cigarette, 6 to 7 cycles of smoking (50 ml over 20 s) and 20 s pause were applied, after which the remainder of the tobacco-filled cylinder had a length of ~5 mm. The total extraction process consisted of 3 cigarettes. Subsequently, the medium was subjected to sterile filtration (0.22 μm) and absorbance was measured from 200 nm to 620 nm in 10 nm steps using UV star microwell plates (Greiner Bio One, Frickenhausen, Germany). To quantify the entirety of extracted substances, the absorbance of ABC medium was subtracted at each wavelength, which revealed an absorption maximum at 240 nm (supplemental Fig. S2A). To minimize variations in the extraction procedure between replicate experiments, we used ABC medium to dilute the original CSE to an OD240 of 1 (again blanked against ABC medium), which was defined as 100% CSE. Likewise, further CSE dilutions were made using ABC medium. CSE and ABC media were always freshly prepared the day on which they were applied to the cells.

Flow Cytometry—Cells (2 T25 flasks per condition) were trypsinized and pooled in SM, followed by centrifugation at 170 × g for 5 min. Cell pellets were resuspended in 500 μl PBS, and 3 μl Propidium Iodide (PI, Thermo Scientific, #B5000Pi) were added shortly before measurement. Samples were analyzed on an LSR II flow cytometer (BD Biosciences, San Jose, California) using Side scatter-area/Forward scatter-area (SSC-A/FSC-A) to gate out debris and cell doublets, respectively. PI was measured using a blue (488 nm) laser combined with a 600 nm longpass filter and a 610/20 bandpass filter. Cells without PI, as well
as cells with PI exposed to heating (80 °C/10 min) were included as controls.

Lactate Dehydrogenase (LDH) Assay—Quantification of cytotoxicity was performed with cell culture supernatants using the Pierce™ LDH Cytotoxicity Assay Kit (Thermo Scientific). For each experiment, a maximum LDH activity control was run in parallel to the conditions of interest by seeding cells (at the same density) in 2 wells of a 24-well plate. Forty-five minutes before sampling of supernatants, 10× lysis buffer was added to these wells, followed by further incubation at 37 °C and 5% CO₂. Subsequently, all samples were processed according to the manufacturer’s instructions.

Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) Experiments—For each condition (ABC or 5% CSE), cells were seeded into 2 T75 flasks and 1 T25 flask and proliferated in GM until 50% optical confluence. Subsequently, the medium was removed, and cells were washed twice with PBS before addition of either 5% CSE or ABC medium. 24 h or 4 days later, supernatants were sampled for LDH activity and cells in T75 flasks were tryspinized followed by addition of SM and centrifugation at 170 × g for 5 min. Cell pellets were resuspended in 10 ml PBS, the total number of cells was determined using a hemocytometer, and cells were centrifuged at 170 × g for 5 min. Subsequently, the cell pellets were resuspended in 1 ml PBS for a second wash step (170 × g for 5 min) before PBS was carefully aspirated and pellets were snap-frozen in liquid nitrogen. Cells in T25 flasks were washed twice with PBS before addition of QIAZOL reagent (700 µl per flask). Samples were stored at −80 °C.

Samples were lysed in 100 µl lysis buffer (100 mM Tris pH 8.0, 10 mM TCEP, 40 mM 2-chloroacetamide and 1% SDS) by sonication for 2 min on ice. The lysates were heat denatured, reduced and alkylated for 10 min at 90 °C. The supernatant was collected after removal of cell debris by centrifugation at 1000 × g (10 min, room temperature). Protein was determined by BCA-RAC assay (Thermo Scientific). Sev- enty-five micrograms protein was subjected to acetone precipitation. The pellet was re-dissolved in TFE-digestion buffer (25% 2,2,2-trifluoroethanol (TFE) in 100 mM Tris-HCl, pH = 8.5 and predigested with rLysC (Promega, Mannheim, Germany, enzyme/protein m/m 1:100) overnight. Peptides were acidified with formic acid (final concentration of 0.1%). Five hundred nanograms per sample were ana- lyzed by nano-HPLC (Dionex Ultimate 3000) equipped with a C18, 5 µm, 100 Å, 500 l lysis buffer (100 mM Tris pH 8.0, 155–165 min: 95% B; 165–165.1 min: 4% B; 165.1–180 min: 4% B). The sample was ionized in the nanospray source equipped with stainless steel emitters (Thermo Fisher Scientific, Vi- enna) and analyzed in a Thermo Orbitrap velos pro mass spectrom- eter in positive ion mode by alternating full scan MS (m/z 300 to 2000, 60,000 resolution) in the ICR cell and MS/MS by CID of the 10 most intense peaks in the ion trap with dynamic exclusion enabled.

Samples collected from immortalized hVFF after 4 days of expo- sure to CSE were desalted using stage tips(22) and analyzed using Dionex 3000 nanoRSLC coupled to a Bruker MaxiSill ETD. The em- ployed column was an Aurora UHPLC pulled emitter column (length 250 mm, inner diameter 0.75 µm, particle size 1.6 µm, pore size 120 Å, Ionopticks, Melbourne, Australia). Samples were directly loaded onto the analytical column and separated using a constant flow rate of 300 nL/min and the following gradient. A: H₂O 0.1% formic acid, B: ACN 0.1% formic acid; 0 min 2% B; 18 min 2% B; 100 min 25% B; 107 min 35% B; 108 min 95% B; 118 min 95% B; 118 min 2% B; 133 min 2% B. Mass spectrometer was operated in positive DDA mode (top 20).

Processing of LC-MS/MS Data—The LC-MS/MS data were ana- lyzed by MaxQuant by searching the public Swissprot human data- base (13202466 residues, 20304 sequences, downloaded on Nov 8th, 2017) and common contaminants. Carbamidomethylation on Cys was entered as fixed modification, oxidation on methionine as vari- able modification. Detailed search criteria were used as follows: tryp- sin, max. missed cleavage sites: 2; search mode: MS/MS ion search with decoy database search included; precursor mass tolerance ± 0.006 Da; product mass tolerance ± 0.02 ppm; acceptance parameters for identification: 1% PSM FDR; 1% protein FDR. In addition, a label free quantitation (LFQ) was performed using MaxQuant, version 1.5.8.3 (23), requiring a minimum of 2 ratio counts of quantified razor and unique peptides. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (24) partner repository and can be retrieved via the data set identifier PXD010640. Further processing of proteomics data was performed with R (www.r-project.org). Proteins with peptide counts (razer+ unique) ≤ 1 were removed, and, for each biological replicate, paired log2-transformed fold changes (pFC; 5%CE/ABC) of LFQ intensity values were calculated. Proteins with pFC > 0.01 were filtered, sorted according to average pFC, and used for heat map visualization using the ‘pheateam’ package. Hierarchical clustering of samples was conducted using the “complete linkage” method of the hclust function from the “stats” pack- age. The top and bottom 2% of proteins (i.e. those most dynamically regulated by CSE) were further used to conduct a paired t test, followed by applying the Benjamini-Hochberg correction for multiple testing. To infer biological categories that are significantly affected by CSE, we employed Gene Set Enrichment Analysis (GSEA) (25, 26) using the javaGSEA desktop application with preset values of gene set sizes. Gene sets with a FDR-corrected p value < 0.1 were considered as significantly enriched.

Isolation and Analysis of RNA—Cells were harvested with QIAZOL reagent (QIAGEN, Hilden, Germany) and total RNA was isolated using the miRNeasy Mini kit (QIAGEN) according to the manufacturer’s instructions. Purified RNA was eluted in 30 µl RNase-free water and concentration was determined using a NanoDrop 2000c spectropho- tometer (Thermo Scientific). Reverse transcription (RT), as well as RT quantitative PCR (RT-qPCR) were performed as previously described (15). Primer sequences are provided in supplemental Table S2. Crossing threshold (CT) values of technical triplicates were averaged and relative quantification of all mRNAs of interest was performed based on the 2−ΔΔC₅₀ method (27) with two modifications: (1) the geometric mean of the C₅₀ values of B2M and UXT reference RNAs was used as internal normalization factor; (2) for all samples and mRNAs of interest, the same CT value (hVFF-E7 cells/ABC medium/ B2M) was used as external reference, i.e. to calculate ΔΔC₅₀ values; this enables to infer not only differences in expression for a particular mRNA between distinct samples, but also to assess the abundance of distinct mRNAs relative to each other.

SDS-PAGE and Silver Stain—Analysis of fibrillar collagens was performed as in a previous study (17). Briefly, cells in 24-well plates were washed with PBS and incubated with digestion buffer (0.25 mg/ml Pepsin, 0.005% Triton X-100, 0.01% Phenol red in 250 mM HCl; 100 µl per well; all Sigma Aldrich) for 2 h at room temperature with shaking (200 rpm). Subsequently, pH was neutralized by addition of 30 µl NaOH (1 N, Sigma Aldrich) per well. Twenty-one microliters of sample were mixed with 7 µl Laemmli sample buffer (4x) and applied to 3–8% Criterion XT Tris-Acetate gels (bio-rad, Hercules, California). VitroColl® human type I collagen solution (Advanced BioMatrix, San Diego, California) was used as standard and applied on a separate
lane (0.16 μg in 18 μl ddH₂O; pre-mixed with 6 μl Laemmli sample buffer (4×)). Electrophoresis was run at 200 V for 80 min. For fixation, the gel was placed in a solution of 40% EtOH and 10% acetic acid in ddH₂O (Roth GmbH, Graz, Austria) and incubated with agitation (50 rpm) at room temperature for 1 h. Subsequently, the gel was stained using the SilverQuestTM Silver Staining kit (Thermo Scientific) according to the manufacturer’s protocol. Gel images were acquired with the ChemiDoc Touch system and densitometric analysis was conducted using ImageLabTM software (bio-rad).

Fluorescence Microscopy—Cells were seeded into 4-well Lab-Tek™ II chamber slides (Thermo Scientific). For visualization of HA, cells were fixed by incubation with 100% MeOH (Roth GmbH, 10 min at −20 °C) followed by air-drying in the fume hood for 30 min. Blocking was accomplished by incubation with 3% bovine serum albumin (BSA, Sigma Aldrich) in PBS (45 min at room temperature), and cells were incubated overnight at 4 °C with 2 μg/ml of biotinylated HA binding protein (HABP; amsbio, Abingdon, UK, #AMS.HKD-BC41; diluted in Antibody Diluent) for 1 h at room temperature. Subsequently, nuclei were counterstained by incubation with Hoechst 33342 (Dako, Glostrup, Denmark). The next day, cells were incubated with Streptavidin-conjugated Alexa Fluor™ 594 (Thermo Scientific, #S11227; 1:250 in Antibody Diluent) for 1h at room temperature. Subsequently, nuclei were counterstained with Streptomyces hyalurolyticus hyaluronidase (Sigma, Aldrich, #H1136; dissolved in 0.01% BSA/PBS) for 15 min at 37 °C immediately before fixation. For staining of type I collagen (col-I), cells were fixed by incubation with 4% paraformaldehyde in PBS (20 min at room temperature), followed by incubation with 100% MeOH (10 min at −20 °C). Blocking was performed with 20% FBS and 10% normal goat serum (Cell Signaling, Danvers, Massachusetts) in PBS (45 min at room temperature), and cells were incubated overnight at 4 °C with mouse monoclonal anti-col-I antibody (Sigma, Aldrich, #C2456E; diluted 1:500 with Antibody Diluent). On the next day, cells were incubated with Alexa Fluor® 594 conjugated goat anti-mouse IgG (H+L) (Invitrogen, Carlsbad, California, #A11005; diluted 1:1000 in Antibody Diluent). Counterstaining of nuclei was performed by incubation with Hoechst 33342 (1 μg/ml in Antibody Diluent) for 15 min at room temperature, and slides were covered with Fluorescence Mounting medium. Between each of the aforementioned steps, cells were washed thrice with 0.1% BSA in PBS (5 min at room temperature). To confirm specificity of HA staining, 1 well of a chamber slide was incubated with 20 U/ml of Streptomyces hyalurolyticus hyaluronidase (Sigma, Aldrich, #H1136; dissolved in 0.01% BSA/PBS) for 15 min at 37 °C immediately after fixation.

For staining of type I collagen (col-I), cells were fixed by incubation with 4% paraformaldehyde in PBS (20 min at room temperature), followed by incubation with 100% MeOH (10 min at −20 °C). Blocking was performed with 20% FBS and 10% normal goat serum (Cell Signaling, Danvers, Massachusetts) in PBS (45 min at room temperature), and cells were incubated overnight at 4 °C with mouse monoclonal anti-col-I antibody (Sigma, Aldrich, #C2456E; diluted 1:500 with Antibody Diluent). On the next day, cells were incubated with Alexa Fluor® 594 conjugated goat anti-mouse IgG (H+L) (Invitrogen, Carlsbad, California, #A11005; diluted 1:1000 in Antibody Diluent). Counterstaining of nuclei was performed by incubation with Hoechst 33342 (1 μg/ml in Antibody Diluent) for 15 min at room temperature, and slides were covered with Fluorescence Mounting medium. Between each of the previous steps, cells were washed thrice with 0.1% BSA in PBS (5 min at room temperature). For each biological replicate, 1 well of a chamber slide was processed without incubation with primary antibody (i.e. incubation solely with Antibody Diluent) which served as negative control.

Fluorescence microscopy was performed with a Nikon A1R confocal laser scanning microscope using a CFI Plan Apochromat Lambda objective (20x magnification). Images (1024 × 1024 pixels) were acquired with NIS Elements Imaging Software (Nikon, Tokyo, Japan) using the Galvano scanner mode. Hoechst 33342 (nuclei staining) was excited using a violet diode laser with an emission wavelength at 402.6 nm. Alexa Fluor 594 (HA or col-I staining) was excited using a diode pumped solid state (DPSS) laser with an emission wavelength at 561.3 nm.

Enzyme-Linked Immunosorbent Assay (ELISA)—Cell culture supernatants were centrifuged (300 × g/4 °C/10 min), decanted to eliminate any cell debris, and stored at −20 °C. Hyaluronan (HA) was measured using the Quantikine® ELISA kit (R&D Systems, Minneapolis, Minnesota; #DHYAL0) according to the manufacturer’s protocol. Samples were pre-diluted 1:30 with Calibrator Diluent RD5–18. HA standards and samples were assayed on the same plate in technical duplicates, and optical density (OD) values at 540 nm were subtracted from OD values at 450 nm. A 4-parameter logistic curve-fit was applied to the HA standards to infer the concentration of samples.

Magnetic Luminex Assay—Cell culture supernatants were centrifuged (300 × g/4 °C/10 min), decanted to eliminate any cell debris, and stored at −80 °C. Human Magnetic Luminex Assay® (R&D Systems; LXSAMD-13) was conducted using a multiplex plate measuring mono- cell cytokine/chemoattractant protein 1 (MCP-1), interleukin 6 (II-6), interleukin 8 (II-8), vascular endothelial growth factor (VEGF) -A, -C, and -D, hepatocyte growth factor (HGF) and basic fibroblast growth factor (FGF-basic) according to the manufacturer’s protocol on the Bio-Plex 200 assay reader (bio-rad). Final concentrations were calculated using Bio-Plex Manager Software Version 6.2 (bio-rad).

Experimental Design and Statistical Rationale—The primary aim of this study was to investigate effects of an almost obligatory noxious agent for development of RE (i.e. CSE) on a cell type that likely participates in pathogenesis (i.e. VFF). To minimize the risk of identifying donor-specific effects, we performed the proteomic screening with 6 true biological replicates, i.e. 6 cell populations originating from different individuals. As in most instances RE is diagnosed in females, we chose cells originating exclusively from female donors. Further, the donor age ranged from 49 to 77 years (median 63 years), reflecting the age group in which RE is typically diagnosed. To reduce the risk of identifying in vitro artifacts, the major part of our proteomic screening was performed with cells that had been cultivated for only few passages. However, as cells from healthy human VF cannot be obtained on a routine basis, the project was designed to include an immortalized human VFF line for the proteomic screening. The underlying rationale was to use these cells for subsequent validation experiments, provided that the proteomic signature of this cell line would be like the signature of near-primary cells.

The overall goal of the proteomic screen was to generate a descriptive dataset, founded on a robust and stringent experimental design, from which hypotheses with potential relevance for the pathogenesis of RE could be deduced. For protein identification a stringent false discovery rate of 1% was used as threshold and for quantification at least two quantified peptides were required. In addition to analyzing the proteomic data for proteins that are significantly affected by CSE in their abundance (employing paired t-tests; including Benjamini-Hochberg correction for multiple testing), we also paid attention to the most extremely deregulated proteins regardless of actual q-values. Further functional analyses/validation experiments were performed in at least 3 replicates and analyzed by paired t-tests (p values < 0.05 were considered statistically significant). The identification of significantly affected biological categories based on the entire set of robustly detected proteins was performed with Gene Set Enrichment Analysis (GSEA); this analysis was conducted according to the GSEA user guide.

Statistical Testing and generation of graphs was performed with R (proteomic data) and GraphPad Prism 7 (other data).

RESULTS

Exposure of Vocal Fold Fibroblasts to CSE for Proteomic Profiling—A plethora of substances present in cigarettes is known to possess toxic properties (28). After having established a protocol for reproducible generation of CSE (see experimental procedures and supplemental Fig. S2A), we characterized the effects of varying CSE dilutions on VFF viability. Following 24 h of incubation, the confluence of wells exposed to 100%, 50%, or 25% CSE was substantially reduced compared with control cells, whereas a considerable
amount of detached cells and debris could be observed (supplementary Fig. S2B). This toxic effect was less pronounced for cells exposed to 10% CSE, and not observed for cells incubated with 5% CSE. These microscopic observations were corroborated by a quantitative viability analysis performed by flow cytometry (supplemental Fig. S2C). We thus performed label-free quantitative proteomics at a concentration of 5% CSE to obtain a global view of the molecular changes elicited in VFF by the complex mixture of cigarette smoke substances. The experiments employed five np-hVFF populations, as well as an immortalized hVFF population (hVFF-E7). Again, the chosen CSE concentration did not elicit cytotoxic effects in any of the biological replicates (supplemental Fig. S3). The number of identified proteins with robust abundance was similar across all analyzed samples (1923 proteins on S3). The number of identified proteins with robust abundance in any of the biological replicates (supplemental Fig. S2). This toxic effect was less pronounced for cells exposed to 10% CSE, and not observed for cells incubated with 5% CSE. These microscopic observations were corroborated by a quantitative viability analysis performed by flow cytometry (supplemental Fig. S2C). We thus performed label-free quantitative proteomics at a concentration of 5% CSE to obtain a global view of the molecular changes elicited in VFF by the complex mixture of cigarette smoke substances. The experiments employed five np-hVFF populations, as well as an immortalized hVFF population (hVFF-E7). Again, the chosen CSE concentration did not elicit cytotoxic effects in any of the biological replicates (supplemental Fig. S3). The number of identified proteins with robust abundance was similar across all analyzed samples (1923 proteins on average, S.D. = 58.7; Fig. 1A). CSE treatment did not influence the number of detected proteins (p = 0.716; paired t test). Even though VFF populations originated from distinct individuals, the repertoire of expressed proteins was highly similar, as on average 99.1% of proteins detected in a sample were also present in other samples. Correspondingly, filtering for proteins for which a fold change between CSE and ABC could be confidently calculated resulted in an only slightly reduced dataset, consisting of 1877 unique proteins (Fig. 1B; supplemental Table S3). Unsupervised clustering revealed no overtly distinct protein fingerprint of immortalized hVFF, i.e. dissimilarities between hVFF-E7 cells and the group of near-primary cell populations were less pronounced than dissimilarities of near-primary cell populations among each other (Fig. 1B). Proteins most responsive to CSE are presented in Fig. 1C (top 2%) and Fig. 1D (bottom 2%). Of note, the 4 proteins most upregulated by CSE - Cytochrome P450 1B1, Heme oxygenase 1, sulfiredoxin-1, and Aldo-keto reductase family 1 member C1 - are all well-known mediators of oxidative stress that have previously been described as CSE-responsive in other contexts (29–32). Altogether, a first global analysis for proteins deregulated in hVFF in response to sublethal concentrations of CSE was successfully conducted.

In addition we performed a similar proteomic analysis focusing on immortalized hVFF (hVFF-E7) cells after 4 days of exposure to CSE (see supplemental Table 4). Interestingly, only two out of over 2600 identified and quantified proteins were significantly altered after long term treatment, suggesting that the cells were able to neutralize the cigarette smoke contained radicals responsible for the acute oxidative stress they suffered from at 24 h of exposure. Namely, one of these proteins was the discoidin domain-containing receptor 2, which was slightly upregulated (by about 30%) by CSE, a tyrosine kinase that functions as cell surface receptor for fibrillar collagen and regulates cell differentiation, remodeling of the extracellular matrix via regulation of matrix metalloproteases (MMPs), cell migration and cell proliferation (33–36). However, the two detected MMPs, 2 and 14, were unchanged (supplemental Table 4). The second significantly altered protein was the cathepsin A related protein retinoid-inducible serine carboxypeptidase, which was slightly downregulated by CSE (by about 25%), and has been implicated to play a role in vasoconstriction by cleaving endothelin-1 (37).

**Pathways and Processes Affected by CSE—**We subjected the entire set of robustly detected proteins after 24 h of exposure to CSE to GSEA in order to obtain gene sets (and thus biological processes) which are enriched either among CSE-induced or CSE-depleted proteins. Cellular responses to xenobiotic agents were clearly upregulated by CSE, as indicated by the significant enrichment of proteins annotated to the KEGG pathway “Metabolism of xenobiotics by cytochrome P450” (q = 0.0244), as well as to the hallmark gene set “Xenobiotic metabolism” (q = 0.0165; Table I). Further, the significant enrichment of the hallmark gene set “Oxidative Oxygen Species Pathway” (q = 0.0265) and five gene sets from GO molecular function related to oxidative enzymatic reactions (Table I) confirmed that CSE had confronted VFF with oxidative stress. Also genes assorted to the pentose phosphate pathway were significantly enriched among CSE-upregulated proteins (q = 0.0442), which might reflect the cellular demand for reducing equivalents (i.e. NADPH) that are obligate for many detoxifying redox reactions. Finally, proteins which have previously been shown to be regulated by the transcription factor nuclear respiratory factor 2 (NFE2L2; NRF2) (38) were enriched with high statistical significance among CSE-induced proteins (q = 0.0018). NRF2 has been described in other contexts to be induced in response to oxidative stress and to act as critical mediator of oxidative stress responses (39, 40). Although we did not detect NRF2 in our proteomic screen, we measured a significant upregulation on mRNA level by 5% CSE (Fig. 2A). It has previously been hypothesized that CSE elicits a pro-angiogenic response in VFF by induction of VEGF secretion (14). However, none of the 59 angiogenesis-related gene sets that are part of the Molecular Signatures Database was identified as significantly enriched among CSE-upregulated proteins when we subjected our proteomic dataset to GSEA. We further analyzed the expression of all 4 known VEGF genes in np-hVFF and hVFF-E7 cells but did not find significant changes in VEGF mRNA levels because of CSE treatment (Fig. 2B). In contrast to these results, we could identify a significantly upregulated protein secretion of VEGF-A and a decreased secretion of VEGF-C in hVFF-E7 cells and one np-hVFF donor after CSE treatment (Fig. 2C). As for proteins that were decreased in response to CSE, several significantly enriched gene sets indicate a disruption of the cell cycle and thus proliferation: genes previously described to be upregulated by the KRAS oncogene (41) were globally downregulated in our dataset (q = 0.0535), as were genes promoting DNA replication of melanoma cells (q = 0.0554) (42). Likewise, genes assorted to the GO term “DNA replication” were globally downregulated with high statistical significance (q = 0.0024). In line with
Effects of Cigarette Smoke Extract on the Vocal Fold Fibroblast Proteome

A

B

C

top 2% (37 unique proteins)

D

bottom 2% (37 unique proteins)

1877 unique proteins

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these results, also a hallmark gene set consisting of targets of the E2F family of transcription factors was significantly enriched among proteins downregulated by CSE (q = 0.0523).

Collectively, these analyses reveal that CSE elicits strong antioxidative and anti-xenobiotic responses in VFF while simultaneously stalling cell division (Table II).

**CSE Downregulates Fibrillar Collagens**—We also conducted a manual analysis of those protein groups on which CSE treatment had the greatest impact. Interestingly, both proteins from which mature collagen type I is generated, i.e. collagen alpha-1(I) chain (COL1A1) and collagen alpha-2(I) chain (COL1A2), were among the 2% of proteins most suppressed by CSE (Fig. 1D). Although this downregulation failed to reach statistical significance when correcting for multiple testing (see supplemental Table S3), we noted that LFQ intensities of COL1A1 and COL1A2 were consistently lower for CSE- versus ABC-treated cells across all 6 biological replicates. Because of this result and the fact that fibrillar collagens constitute an elementary component of the VF lamina propria (43), we decided to analyze the relationship between CSE and collagen in more detail. Indeed, 4 days after CSE exposure (i.e. a point of analysis that reflects more a steady state condition, versus more acute effects assayed by proteomics after 24 h), COL1A1 and COL1A2 mRNA levels were significantly reduced in CSE- versus ABC-treated VFF (Fig. 3A). Similarly, the expression of COL3A1, the precursor of type III collagen that is also found in the VF lamina propria (43), was significantly suppressed by CSE. Analysis of fibrillar collagens by protein electrophoresis and silver stain further corroborated these results (Fig. 3B, 3C), whereas proteomics of hVFF-E7 cells did not reveal significant changes in soluble collagen proteins after 4 days of CSE exposure (see supplemental Table S4). Finally, immunofluorescent staining of VFF for type I collagen confirmed the negative impact of CSE on collagen matrix synthesis (Fig. 3D).

**CSE Triggers Hyaluronan Accumulation**—Among the 2% of proteins most induced by CSE after 24 h of exposure, we identified UDP-glucose 6-dehydrogenase (UGDH), which was consistently upregulated in all biological replicates (Fig. 1C, supplemental Table S3). This upregulation was significant after 24 h also after correction for multiple testing (q = 0.0466), whereas proteomics of hVFF-E7 cells did not reveal significant changes in UGDH after 4 days of CSE exposure (see supplemental Table S4). UGDH is an enzyme that converts UDP-glucose into UDP-glucuronate, a molecular building block of glycosaminoglycans (GAGs) such as HA (44). Given the high importance of HA for VF physiology (45), we thus decided to investigate possible effects of CSE on HA biosynthesis in further detail. HA is generated by HA synthases, which perform the repetitive polymerization of UDP-glucuronate and UDP-N-acetyl-glucosamine into a growing GAG chain. Although we did not detect expression of hyaluronan synthase 1 in hVFF, we found hyaluronan synthases 2 (HAS2) and 3 (HAS3) to be expressed and significantly upregulated by CSE 4 days after start of treatment on mRNA level (Fig. 4A). Interestingly, mRNA expression of enzymes involved in HA catabolism, namely hyaluronidase 1 (Hyal1) and 2 (Hyal2), were significantly decreased by CSE exposure. In line with these findings, we indeed found hVFF to secrete HA at significantly higher levels in response to CSE, both 1 and 4 days after start of treatment (Fig. 4B). These results were further corroborated by fluorescent staining of HA (Fig. 4C).

**CSE Modulates Cytokine Secretion**—Pro-inflammatory cytokines MCP-1 and IL-6 were elevated in supernatants of CSE-treated cells compared with control. No changes in IL-8 levels were found (Fig. 5A). We also measured cytokines known for their involvement in tissue remodeling and found elevated levels of secreted HGF but not FGF basic, in response to CSE exposure (Fig. 5B).

**DISCUSSION**

RE has a multifactorial genesis, with smoking and continuous phonotrauma being evidence-based risk factors. Treatment of choice is a micro-surgical procedure under general anesthesia, where the gelatinous hypertrophied superficial layer of the lamina propria is reduced. Outcomes are highly varying and often come along with unsatisfactory results, such as VF scar or a life-long strained harsh voice (1). In addition, phonosurgery provides only a symptomatic, but not a causal treatment of the pathology.

By proteomic analysis of hVFF cultured in CSE conditioned medium we sought to elucidate the pathophysiology of RE. To our knowledge this is the first time that a large-scale method was employed to explore RE. We could confirm the results of a previous in vitro study (6) that a protein related to the oxidative capacity, heme oxygenase 1, was upregulated in hVFF under 24 h cigarette smoke condensate stimulation. Similarly, Duflo et al. showed in a group of 11 RE specimens that a number of genes involved in protection against oxidative stress and apoptosis were upregulated (10). Smoking is known to induce oxidative stress leading to cell death and
| Gene sets database        | Gene sets significantly (FDR q-value < 0.1) enriched in CSE-treated versus control VFF | FDR q-value | Gene symbols of proteins within core enrichment |
|---------------------------|----------------------------------------------------------------------------------------|-------------|-----------------------------------------------|
| Positional gene sets      | -                                                                                      | -           | -                                             |
| BIOCARTA                  | BIOCARTA_EIF_PATHWAY                                                                    | 0.0970      | EIF1, EIF6, EIF2S1, EIF2S3, EIF4G2, EIF2S2, EIF4A1, EIF4G1 |
| Chemical and genetic      | -                                                                                      | -           | -                                             |
| perturbations             | KEGG                                                                                    | -           | -                                             |
| KEGG                      | KEGG_METABOLISM_OF_XENOBIOTICS_BY_CYTOchrome_P450                                      | 0.0244      | ALDH3A1, AKR1C1, CYP1B1, AKR1C3, MGST1, ADH5 |
| KEGG                      | KEGG_PENTOSE_PHOSPHATE_PATHWAY                                                          | 0.0442      | PGD, TKT, G6PD, TALDO1, PFKP, PRPS1, PGM2, G6P, ALDOA, PKF, PGLS |
| KEGG                      | KEGG_AMINOACYL_TRNA_BIOSYNTHESIS                                                        | 0.0774      | TARS, NARS, YARS, IARS, AARS, LARS, KARS, DAR2, EPRS, FARS, HAR, SARS, MARS |
| REACTOME                  | REACTOME_TRNA_AMINOACYLATION                                                           | 0.0657      | EEF1E1, TARS, NARS, YARS, IARS, AARS, LARS, KARS, DAR2, EPRS, FARS, CAR, HAR, SARS, PPA2, MARS |
| microRNA targets          | -                                                                                      | -           | -                                             |
| Transcription factor      | -                                                                                      | -           | -                                             |
| GO biological process     | -                                                                                      | -           | -                                             |
| GO cellular component     | -                                                                                      | -           | -                                             |
| GO molecular function     | GO_OXIDOREDUCTASE_ACTIVITY_ACTING_ON_PAIRED_DONORS_WITH_INCORPORATION_OR_REDUCTION_OF_MOLECULAR_OXYGEN | 0.0713      | HMOX1, AKR1C1, POR, CYP1B1, AKR1C3, MICAL1 |
|                           | GO_OXIDOREDUCTASE_ACTIVITY_ACTING_ON_CH_OH_GROUP_OF_DONORS                              | 0.0882      | ALDH3A1, UGDH, AKR1C1, ALDH3A2 |
|                           | GO_LIGASE_ACTIVITY_FORMING_CARBON_OXYGEN_BONDS                                          | 0.0885      | TARS, NARS, YARS, IARS, AARS, LARS, KARS, DAR2, EPRS, FARS, CAR, SARS, MARS, LRR4C7, SARS, MARS |
|                           | GO_TETRAPYRROLE_BINDING                                                                 | 0.0971      | SRC, MHOX1, CYP1B1, HEBP1 |
|                           | GO_OXIDOREDUCTASE_ACTIVITY_ACTING_ON_THE_CH_OH_GROUP_OF_DONORS_NAD_OR_NADP_AS_ACCEPTOR | 0.0976      | ALDH3A1, UGDH, AKR1C3, AKR1C3, PGD, ME1, CBR3, ADH5, CBR1, HSD1B4, PTGP1, NSDHL, G6PD |
| Oncogenic signatures      | NFE2L2.V2                                                                              | 0.0018      | ALDH3A2, AKR1C3, PGD, SPG20, NUTF2, PHLD1, ME1, MGST1, CBR3, CBR1, TKT, NUMBL, PTGP1, G6PD, TALDO1, PSAT1, EPHX1, EIF2S2, NQO1, GSR |
| Immunologic signatures    | -                                                                                      | -           | -                                             |
| Hallmark gene sets        | HALLMARK_XENOBIOtIC_METABOLISM                                                          | 0.0165      | IGFBP4, ALDH3A1, MHOX1, UGDH, POR, AKR1C3, FBLN1, PGD, DHRS1, ADH5, CBR1, PTGP1, DAK, EPHX1, NQO1, GSR, ALDH9A1, KARS |
|                           | HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY                                                 | 0.0265      | SRXN1, GCLM, NDUF2, TXN, PRDX1, MGST1, PRDX2, G6PD, PFKP, NQO1, GSR |
|                           | HALLMARK_UNFOLDED_PROTEIN_RESPONSE                                                      | 0.0431      | SLC7A5, XOP, SRPRB, E1F2S1, HSP0B1, TARS, PSAT1, PAIP1, HYOU1, IARS, TUBB2A, HSPA5, YWHAZ, SEC31A, FUS, CALR, ERO1L, EIF4A1, HSPA9, E1F4G1, PDI16, ATP5B0D1, SEC11A, PPS14, EEF2 |
|                           | HALLMARK_GLYCOLYSIS                                                                     | 0.0483      | TXN, GNPD1A, ME1, GYS1, NSDHL, G6PD, TALDO1, PFKP, PRPS1, HSPA5, ALDH9A1, PGM2, PSMC4, PMM2, GFT1, FKBP4, ME2, TST3A, ERO1L, ALDOA, MDH1, COP2B, GMAM1, PGLS, EON1, PK1, HK2, IDH1, PLOD1, LDHA, TF1, UGP2, GLRX, AKR1A1, RARS, MDH2 |
carcinogenesis. A high expression of antioxidant proteins with high oxidative capacity may thus be protective. This is also in accordance with existing literature, reporting that the number of malignant transformation is lower in RE patients. It is remarkable that VF malignancy is rarely described in VF with RE despite identical risk factors (46). Zhang et al. exposed human VFF to cigarette smoke condensate under static and dynamic cell culture conditions (47). They described an inhibitory effect of cyclic tensile strain under dynamic conditions on expression of cyclooxygenase 2 (COX-2) and matrix metalloproteinase (MMP-9). COX-2 and MMP-9 expressions were significantly lower in RE- than in paracancerous-tissues. Simultaneously, clinical data showed that RE patients had more extrovert, thus talkative, personality characteristics compared with VF carcinoma patients. Both groups were classified as moderate/heavy smokers, but VF vibration presumably led to lower expression of...
TABLE II

Gene sets significantly upregulated in vocal fold fibroblasts (VFF) exposed to control air-bubbled control (ABC) medium versus VFF exposed to cigarette smoke extract (CSE). Proteomic data from VFF that were harvested 24 h after exposure to CSE or air-bubbled control (ABC) medium was subjected to gene set enrichment analysis (GSEA). The analysis was run with distinct gene sets databases (column 1) obtained from the Molecular Signatures Database. Only gene sets (column 2) that are significantly (FDR q-value < 0.1; column 3) enriched among proteins upregulated in ABC- versus CSE-treated cells are depicted. Proteins detected in the samples and having the strongest contribution to the enrichment ("core enrichment") are shown in column 4 and identified by official symbol.

| Gene sets database                                      | Gene sets significantly enriched in control versus CSE-treated VFF | FDR q-value | Gene symbols of proteins within core enrichment                                                                 |
|--------------------------------------------------------|------------------------------------------------------------------|------------|---------------------------------------------------------------------------------------------------------------|
| Positional gene sets                                    |                                                                  |            |                                                                                                                                 |
| BIOCARTA                                               |                                                                  |            |                                                                                                                                 |
| Chemical and genetic perturbations                      |                                                                  |            |                                                                                                                                 |
| LE_EGR2_TARGETS_UP                                     | 0.0376                                                           | LMNB1, RPA3, EZR, HN1, CD44, BZW2, CSR2P, CORO1C, KPNA2, MCM7, HDAC2, MARCKS, MCM4, RRM1, MCM5 |
| PJJANA_BREAST_CANCER_WITH_BRCAl_MUTATED_UP              | 0.0417                                                           | CAD, RPA1, DDX39A, STN1, PNN, MCM4, SRSF11, MCM2, MCM5, MRE11A |
| CHIARADONNA_NEOPLASTIC_TRANSFORMATION_KRAS_UP          | 0.0535                                                           | HMG3B, RASA1, SRRP1, BZW2, ANP3E2, TMSB4X, RANGAP1, UBE2D2, PPA2B, TIMP1, MYO1B, ITGA6, NAP1L1, RRM1 |
| KAUFFMANN_DNA_REPLICATION_GENES                        | 0.0554                                                           | CTBP2, CTBP1, MCM7, MYBBP1A, NFIC, NAP1L1, MCM4, RRM1, MCM2, MCM5 |
| LIM_MAMMARY_LUMINAL_MATURE_DN                          | 0.0556                                                           | EPB41L2, COL6A1, CALD1, FSTL1, FAS, CYGB, RAP2B, RECK, MAPK4 |
| FUJ_IYB1_TARGETS_DN                                    | 0.0730                                                           | NUP62, RAD23B, MCM7, SRR1, MCM4, RRM1, DFFA, MCM2, MCM5, RECK |
| MIKKELSIN_MEF_LCP_WITH_H3K4ME3                         | 0.0736                                                           | TRIM21, BP1, COL6A1, WASA, PDGFB, COL3A1, ECM1 |
| BURTON_ADIPGENESIS_3                                   | 0.0778                                                           | CSR2P, KNA2, MCM7, MCM4, RRM1, MCM2, MCM5 |
| CHANG_CYCLING_GENES                                    | 0.0793                                                           | ANP3E2, RANGAP1, TUBB, TIMP1, MCM4, RRM1, MCM5 |
| BILANGES_SERUM_SENSITIVE_VIA_TSC2                      | 0.0800                                                           | GLG1, NPM3, NUC1A, COL1A2, FM98B, GBF1, COL3A1 |
| CROONQUIST_IL6_DERIVATION_DN                           | 0.0820                                                           | STN1, ANP3E2, SNRPA1, KPNA2, MCM7, MCM4, MCM2 |
| MISSAGLIA_REGULATED_BY_METHYLATION_DN                   | 0.0947                                                           | RANBP1, PRKDC, LMNB1, CAD, RBMX, RPA3, ACT2, TMPO, CAV1, H2AFV, HMG3B, STN1, HNRNP4L, PSP1, SNRP40, RRM1, SRSF3, MCM5 |
| KEGG                                                   |                                                                  |            |                                                                                                                                 |
| REACTOME                                               |                                                                  |            |                                                                                                                                 |
| microRNA targets                                       |                                                                  |            |                                                                                                                                 |
| Transcription factor targets                           |                                                                  |            |                                                                                                                                 |
| GO biological process                                   |                                                                  | 0.0024     | MCM7, PARP1, NFIC, NUP98, NAP1L1, MCM4, RRM1, MCM2, MCM5, MRE11A |
| GO CELLULAR COMPONENT                                   |                                                                  | 0.0964     | RAD23B, MCM7, PARP1, MCM4, MCM2, MCM5, MRE11A |
| GO CELLULAR COMPONENT                                   |                                                                  | 0.0984     | SUN2, MCM7, PARP1, MCM4, MCM2, MCM5, MRE11A |
| GO CELLULAR COMPONENT                                   |                                                                  | 0.0990     | SUN2, MCM7, PARP1, MCM4, MCM2, MCM5, MRE11A |
| GO ACTOMYOSIN                                          |                                                                  | 0.0826     | ACTN1, ACTN4, ACTA1, MYO1C, PG5M, DBN1, PDLIM2, MYO1A8, PNX, ZYX, LMA1, PDLIM7, MYL9, CNIN2, TPM4 |
| GO CHROMOSOMAL REGION                                   |                                                                  | 0.0912     | RANGAP1, SUN2, TPR, MCM7, PARP1, NUP98, MCM4, MCM2, MCM5, MRE11A |
| GO molecular function                                   |                                                                  |            |                                                                                                                                 |
| Oncogenic signatures                                   |                                                                  | 0.0135     | GBP1, MKV, LUC7L3, VWA5A, ANX3A, MYL9, CIRBP, TAGLN |
| Immunologic signatures                                 |                                                                  | 0.0168     | SERpine1, FHL2, CAV1, CSR2P, CD44, ALCAM, TGFB111, ANX3A, COL1A2, VAC14, CALD1, COL1A2, AHNAK, ANX3A, TAGLN, COL3A1 |
| Hallmark gene sets                                      |                                                                  | 0.0523     | PRKDC, LMNB1, NOP56, AK2, RPA1, RPA3, TMPO, DDX39A, HMG3B, HN1, SRRP1, TRA2B, STN1, RBBP7, PNN, ANP3E2, LUC7L3, PIP1, TUBB, KPNA2, MCM7, NAP1L1, MCM4, MCM2, MCM5, MRE11A |
| HALLMARK_E2F_TARGETS                                   |                                                                  | 0.0690     | ATP2B4, ADD3, SERpine1, FHL2, CAV1, PRKCA, TJ1P, PDLIM5, ANX3A, COL1A1, MGLL, NFKB1, COL1A2, PPA2B, RBM5, PDGFB, COL3A1 |
proinflammatory mediators protecting RE patients from VF cancer.

We found that CSE stimulation significantly increased the secretion of IL-6 and MCP-1. Interleukin-6 is a pro-inflammatory cytokine known to induce MCP-1 (48, 49), which in turn plays an important role in the monocyte/macrophage chemotaxis involved in acute and chronic inflammation (50). This is in line with a previous study where inflammatory cells (mostly macrophages and mastocytes) were found in the lamina propria of RE specimens (8). Interestingly, CSE induced significantly higher levels of secreted HGF, a pleiotropic cytokine and growth factor. Animal studies showed that HGF reduces collagen I synthesis while stimulating HA production (51, 52).

Our results also revealed a shift in the production of several important extracellular matrix components. UPD glucose 6-dehydrogenase (UGDH), a protein involved in HA synthesis, was found to be significantly upregulated under 24 h CSE...
stimulation. HA is a major constituent of the superficial layer of the VF lamina propria, important to maintain the pliability required for VF vibration (53). Subsequent experiments showed that not only the production of HA was upregulated but that also the genes encoding the proteins degrading HA, hyaluronidases (HYAL1 and −2), were downregulated. This suggests a gain in the net amounts of HA in the VF. This is in accordance with a recently published preliminary clinical case series, including RE patients, where the injection of hyaluronidase led to a marked decrement in the VF mass accompanied by an increase in voice quality (54). However, the results contrast with histological data from Dikkers, who could not describe elevated levels of HA in the lamina propria of RE (55).

Our results are nonetheless in line with in vivo studies where the effects of cigarette smoke was investigated in the context of lower airway diseases. For instance, lung tissues of mice exposed to cigarette smoke from 4 weeks to six months revealed increased peribronchial HA deposition (56) as well as increased amounts of HA in lavage fluids (57).

The expression of several fibrillar collagen types was also altered by CSE stimulation. Lower levels of collagen might reduce the stiffness of the tissue easing the development of edema especially under the conditions of vibratory stress. Of course, this hypothesis requires further experimental investigation, as the present study was designed to acquire short-term proteomic changes induced by cigarette smoke in only one VF cell type, without employing vibration stimuli. It is however remarkable that congruent results were described in a previous study by Dufl et al. when comparing the gene expression profiles of 11 human RE specimens and 17 VF polypoid tissue (10). However, this study did not include a healthy control group.

Proliferated and elongated vessels in the VF of RE are a common endoscopic finding. Sato et al. examined blood vessels in Reinke space immunohistochemically and by electron microscopy (8). They described teleangiectasia-like vessels with thin endothelium and many fenestrae, most likely leading to an increased permeability and exuded plasma in the surrounding tissue. The cytoplasm of interstitial cells and inflammatory cells in Reinke space stained positive for vascular endothelial growth factor. This is also in line with a previous study describing increased VEGF secretion by immortalized
VFF because of cigarette smoke exposure (14). Although we found no increase of VEGFs in the cell lysates (proteomic analysis and RT-qPCR), we could identify a significantly upregulated secretion of VEGF-A in the supernatants of VFF 24 h after CSE exposure. VEGF-C secretion was significantly decreased at this time point. In addition to VEGFs, the increased HA levels might evoke a pro-angiogenic effect: First, it is known that cigarette smoke constituents (presumably reactive oxygen species) lead to a non-enzymatic breakdown of long HA chains into oligomers (58). Second, HA oligomers have repeatedly been demonstrated to possess pro-angiogenic activity (59–61).

However, our study protocol has limitations too: As mentioned in the introduction, RE occurs far more frequently in females with a predominance of 70–90%. Therefore, we focused our study protocol on females, as specimens from male donors are even harder to obtain. The underlying reasons for this imbalance are still unknown but will need to be addressed in future studies.

Collectively, our study provides the first comprehensive description of immediate cigarette smoke effects on VFF. Although we could confirm the previously described acute upregulation of oxidative stress responses, we also newly discovered a shift in the synthesis of extracellular matrix components, i.e., a net gain of HA and a loss of fibrillar collagen. Future studies are needed to clarify whether this shift might be a critical component in the development of RE, as only the faithful exploration of disease pathogenesis will ultimately lead to better, i.e., causal treatments.

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DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (24) partner repository and can be retrieved via the dataset identifier PXD010640.

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