Inhaled diesel exhaust alters the allergen-induced bronchial secretome in humans

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Cite this article as: Mookherjee N, Piyadasa H, Ryu MH, *et al.* Inhaled diesel exhaust alters the allergen-induced bronchial secretome in humans. *Eur Respir J* 2018; 51: 1701385 [https://doi.org/10.1183/13993003.01385-2017].

ABSTRACT Diesel exhaust (DE) is a paradigm for traffic-related air pollution. Human adaptation to DE is poorly understood and currently based on oversimplified models. DE promotes allergic responses, but protein expression changes mediated by this interaction have not been systematically investigated. The aim of this study was to define the effect of inhaled DE on allergen-induced proteins in the lung.

We performed a randomised and blinded controlled human crossover exposure study. Participants inhaled filtered air or DE; thereafter, contralateral lung segments were challenged with allergen or saline. Using label-free quantitative proteomics, we comprehensively defined DE-mediated alteration of allergen-driven secreted proteins (secretome) in bronchoalveolar lavage. We further examined expression of proteins selected from the secretome data in independent validation experiments using Western blots, ELISA and immunohistochemistry.

We identified protein changes unique to co-exposure (DE+allergen), undetected with mono-exposures (DE or allergen alone). Validation studies confirmed that specific proteins (*e.g.* the antimicrobial peptide cystatin-SA) were significantly enhanced with DE+allergen compared to either mono-exposure.

This study demonstrates that common environmental co-exposures can uniquely alter protein responses in the lungs, illuminating biology that mono-exposures cannot. This study highlights the value of complex human *in vivo* models in detailing airway responses to inhaled pollution.

This article has supplementary material available from erj.ersjournals.com

Received: July 10 2017 | Accepted after revision: Oct 30 2017

Support statement: Funding for this study was obtained from the British Columbia Lung Association, MITACS, AllerGen NCE and the Canadian Institutes of Health Research (CIHR). N. Mookherjee, M.H. Ryu and C. Carlsten are members of the Canadian Respiratory Research Network (CRRN). Funding information for this article has been deposited with the Crossref Funder Registry.

Conflict of interest: None declared.

Introduction

Diesel exhaust (DE) is a major contributor to traffic-related air pollution, which confers major adverse effects on public health, including decreased lung function [1, 2]. Epidemiological studies suggest that DE promotes asthma [3, 4]. Studies in animal models demonstrate that exposure to DE particles increased allergen-induced airway hyperresponsiveness, pulmonary inflammation, allergen recall responses and lung remodelling, all of which promote asthmatic pathophysiology [5, 6]. There are no studies that define the effects of DE on global protein changes (proteome) in the human lung; existing studies are limited to cell culture *in vitro* studies [7–9] or non-human (rat) models [10]. Moreover, none of these previous studies have examined alteration of the lung proteome in response to DE and allergen co-exposure. Therefore, the aim of this study was to comprehensively define the ability of DE to alter allergen-mediated secreted proteins (secretome) in the bronchoalveolar lavage (BAL), using a human controlled exposure model.

Our Air Pollution Exposure Laboratory (APEL) robustly models real-world conditions, including co-exposures, to determine responses to air pollution in humans [11]. We have recently shown that DE and allergen co-exposure alters gene expression [12] and induces epigenetic changes (DNA methylation [13] and microRNA expression [12]) in the bronchial epithelium. Therefore, we hypothesised that DE could alter the allergen-induced secretome in human BAL. We performed a randomised, blinded and controlled human crossover exposure study [14], to obtain BAL (n=14) from four exposure conditions – FAS (filtered air and saline), FAA (filtered air and allergen), DES (diesel exhaust and saline) and DEA (diesel exhaust and allergen) – in each individual. We used label-free quantitative proteomics to define changes in the composition and abundance of proteins in the BAL, *i.e.* the human bronchial secretome, in response to DE and allergen co-exposure. We demonstrated that inhaled DE and allergen co-exposure uniquely enhances a subset of secreted proteins in human lungs, and further independently validated the expression of individual proteins selected from the proteomic analyses in BAL.

Materials and methods

Study design

This study was approved by the institutional ethics review boards of the University of British Columbia and the Vancouver Coastal Health Research Institute. Details of the demographics of the participants enrolled with informed consent in this study are shown in table S1. We performed an order-randomised, double-blinded crossover study of atopic human volunteers exposed to 2 h of filtered air (FA) or diesel exhaust (DE) (standardised to a $PM_{2.5}$ concentration of 300 $\mu g \cdot m^{-3}$) [14]. Particle size was determined by aerodynamic diameter using a TSI Model 3936 Scanning Mobility Particle Sizer (TSI Inc., Shoreview, MN, USA), which was used to titrate the DE condition to the nominal concentration of particulate matter of 2.5 µm or less per cubic metre (actual average: 302 µg per cubic metre, with a standard deviation of 30.5 µg). These particles were composed of elemental carbon at a ratio four times that of organic carbon, according to thermo-optical transmittance of samples collected on 37-mm tissue-quartz filters. Particle numbers were determined by a TSI Model 3775 Condensation Particle Counter (TSI Inc., Shoreview, MN, USA), which counts particles down to a lower limit of 14 nm; under the DE condition, the average was 540 000 particles per cubic centimetre, while with FA, the average was 1750 particles per cubic centimetre. Briefly, participants (n=14) inhaled either FA or DE for 2 h. Following inhalation, one lung segment was challenged with a standardised (participant-adjusted; based on wheal to skin-prick) concentration of allergen (house dust mite (*D. pteronyssinus*), birch or Pacific grasses) in 5 mL of saline [14], while 5 mL saline alone was administered to the contralateral segment. Bronchoalveolar lavage (BAL) of these segments was obtained 48 h after challenge. The process was repeated identically 4 weeks later, but for opposite inhalation and new segments for the allergen challenge, resulting in four sampled conditions: FAS (filtered air and saline), FAA (filtered air and allergen), DES (diesel exhaust and saline) and DEA (diesel exhaust and allergen). BAL samples were aliquoted and stored at −80°C until processed.

Sample processing for proteomics

BAL samples were thawed on ice, centrifuged without brakes at 500×*g* for 5 min at 4°C, to remove cell pellets, followed by an additional centrifugation at 500×*g* for 5 min at 4°C, to obtain cell-free extracts. The two-step centrifugations ensured that the samples were free of cellular components for the measurement of secreted proteins (secretome) in BAL by liquid chromatography—tandem mass spectrometry (LC–MS/MS). Subsequently, the BAL samples were concentrated to 200 µL using an Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-3 kDa membrane (EMD Millipore; Thermo Fisher Scientific, Ottawa, ON, Canada). Protein concentration was determined using a Micro BCA Protein Assay kit (Thermo Fisher Scientific). Samples were pooled and processed using on-filter digestion (10 kDa filter), as previously described [15]. Briefly, the total protein (40 µg) from each condition was pooled (n=5) and treated with 100 mM DTT (dithiothreitol) at 60°C for 60 min. The cooled samples were mixed with 100 µL of 8M urea and 100 mM Tris pH 8.0 (UA buffer), then transferred into an Ultra-0.5 Centrifugal Filter Unit with

Ultracel-10 membrane and spun at 12 000×*g* for 15 min. The retentate was diluted to 400 µL by UA buffer and centrifuged (12 000×*g)* for 15 min to remove excess DTT, twice. 50 mM iodoacetamide (400 µL) freshly prepared in UA buffer was added to the concentrated samples and incubated in the dark for 30 min. The samples were centrifuged at 12 000×*g* for 15 min and washed twice with 400 µL of UA buffer to reduce the residual iodoacetamide. The UA buffer was exchanged with 50 mM Tris pH 8.0, 5 mM $CaCl_2$ (DG buffer), by adding 400 µL of DG buffer and centrifuging for 5 min at 12 000×*g* twice. Sequencing-grade trypsin (Promega) was added at a 1:50 ratio and incubated for 16 h at 37°C with gentle shaking. The digested proteins were mixed with 2M NaCl (final concentration of 500 mM NaCl) and centrifuged at 12 000×*g* for 10 min. The filters were washed once with 400 µL of 50% methanol and once by 400 µL of 15% acetonitrile and 0.1% trifluoroactic acid in water, by centrifugation at 5000×*g* for 5 min. The eluted peptides collected from each wash step were pooled and dried using a SpeedVac concentrator.

LC-MS/MS

The tryptic-digested peptides were analysed using 2D LC-MS/MS, as previously described [16]. Briefly, the peptides were dissolved in 20 mM ammonium formate (800 µL) and separated offline on a C18 reverse phase column at pH 10.0 into eight fractions. Each fraction was analysed on a 5600 Triple TOF mass spectrometer (SCIEX, Canada) in standard MS/MS data dependent acquisition mode. Peptide identification and quantitation was achieved using X!Tandem (cyclone 2012.10.01.1). The source proteome was the June 2015 human database release of SwissProt (20186 proteins). Peptides were identified using the following search settings: constant modification C+57.021 (cystine protection); variable modifications: M, W +15.995 (oxidation) or +31.989 (double oxidation); S, T, Y +79.966 (phosphorylation); N, Q +0.984 (deamidation); parent mass error ±20 PPM; fragment mass error 0.1 Da. Proteins identified with at least two unique peptide sequences and confidence values of log(e)<−3 were selected. The sum of the MS/MS fragment intensities of each member peptide was used for label-free quantitation for each selected protein, and mapped onto a log2 scale for differential analyses.

Western blots

BAL samples (75 µL) were concentrated to ∼30 µL using Amicon Ultra-3 kDa filters. The samples (15 µL) were resolved on 4%–12% Bis-Tris Protein gels (Invitrogen), and stained with Ponceau stain to enumerate whether there was any gross difference in total protein content between different samples for each participant. As the LC-MS/MS studies were performed using equal protein amount across samples, the use of volume to normalise across conditions in validation studies provides a more physiologically relevant second line of evidence for each of the proteins. For Western blot analyses, proteins were transferred to nitrocellulose membranes (EMD Millipore, Thermo Fisher Scientific), blocked with TBST (20 mm Tris-HCl pH 7·5, 150 mm NaCl, 0·1% Tween-20) containing 5% (w/v) milk powder, and probed with antibodies against human CST2 (Origene, TA504268), LCN1 (Abcam, ab76611) and TCN1 (Abcam, ab118386) in TBST containing 1% (w/v) milk powder. Affinity-purified horseradish peroxidase-linked secondary antibodies (Cell Signalling) were used for detection. Membranes were developed with an Amersham ECL detection system (GE Healthcare) according to the manufacturer's instructions. Densitometry for band intensity was determined using an Alpha Innotech Imager with AlphaView software.

ELISA

Bronchial washes (100 µL) obtained after each exposure condition from nine different participants were assessed for C4B abundance using the LSBio human complement C4B ELISA kit (LS-F11162) according to the manufacturer's recommendations.

Immunohistochemistry

Endobronchial biopsies were collected 48 h after each exposure and processed (n=6 participants), as previously described [17]. Briefly, biopsies were fixed with cold acetone containing the protease inhibitors iodoacetamide (20 mM) and phenylmethylsulfonylfluoride (PMSF; 2 mM) overnight at −20°C, then embedded in glycol methacrylate acrylic (GMA) resin. Sections (2 µm) were treated with 0.3% H_2O_2 and 0.1% sodium azide, and blocked with 20% fetal calf serum and 1% bovine serum albumin in Dulbecco's modified Eagle's medium. The sections were probed with anti-MUC16 antibody (Abcam, ab110640), followed by secondary antibody (BA-1000 biotinylated goat anti-rabbit IgG (H+L) Vector Z0619). A Vectastain Elite ABC kit (Vector) and AEC substrate solution (Millipore) were used to detect avidin-biotin-peroxidase complex solution, as per the manufacturer's protocol. Sections were counterstained with Mayer's haematoxylin (Dako). Sections were stained in duplicates, and five different areas from each biopsy were photographed at 60× magnification using an EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific). The stained sections were scored by three independent evaluators, blinded to conditions, as follows; 0=negative (with no detectable staining); 1=focal staining (<25% of the

epithelial cells showed positive staining); 2=moderate staining (25%–50% of the epithelial cells were positive); and 3=extensive staining (>50% positivity).

Statistical analysis

Statistical analyses for validation studies examining the individual BAL sample were performed by the Wilcoxon's matched-pairs signed rank test using GraphPad Prism software. A p-value of less than 0.05 was considered statistically significant.

Results

Diesel exhaust alters the allergen-mediated secretome in bronchoalveolar lavage

A subset of BAL samples (n=5) was pooled for each condition and processed by 2D LC-MS/MS [16]. Spectra (in MGF format) and protein quantitation results are available at the University of California, San Diego's MassIVE archive (massive.ucsd.edu) under the accession no. MSV000081110. First, to delineate the effect of DE on allergen-mediated secretome, we compared protein intensities across three conditions (FAS, FAA and DEA). The resultant matrix quantified 1894 proteins with high confidence (proteins with at least two unique peptides and confidence values of log(e)<−3), of which 869 proteins were common in all three conditions (fig. 1a). Protein intensity values for these 869 common proteins were normalised in the FAA and DEA samples, using FAS (control) samples. Subsequent comparison of these normalised protein expression levels demonstrated that DEA co-exposure significantly altered the allergen-mediated secretome (fig. 1b). The difference in the log2 protein expression values between the two exposures was converted into a normalised Z-score. A filter of $|Z|>1.65$ was used to select the outermost 10% population, or $p<0.05$ for a Gaussian distribution, within the 869 common proteins. The Z-score filter demonstrated that the expression of 79 proteins showed a greater than four-fold increase (42 proteins up-regulated and 37 down-regulated) following DEA co-exposure, compared to allergen-alone exposure (table S2).

The most over-represented gene ontology for these 79 proteins, identified using the InnateDB biomolecular database [18], was extracellular vesicular exosome ($p<1.0\times10^{-5}$), confirming that the defined "secretome" was indeed primarily composed of secreted proteins. Further interrogation using the Ingenuity® Pathway Analysis (IPA) bioinformatics tool revealed that 40 out of 79 proteins were connected in a single biological network, employing both a direct and indirect relationship (fig. 2). This network demonstrated that DEA co-exposure alters the expression of allergen-induced proteins in two broad interconnected processes: innate immunity and inflammation (*e.g.* MAPK1, ERK1/2, RAB5C, CD93 and mucin family proteins), and oxidative stress (primarily the protein phosphatase 2A pathway). Biological pathways predicted to be altered by DEA co-exposure using the PANTHER over-representation test were N-acetyl glucosamine metabolism, cytoskeletal regulation by RhoGTPases, Toll-like receptor signalling, FGF (fibroblast growth factor) signalling and T-cell activation, all broadly involved in innate immunity, inflammation and oxidative stress.

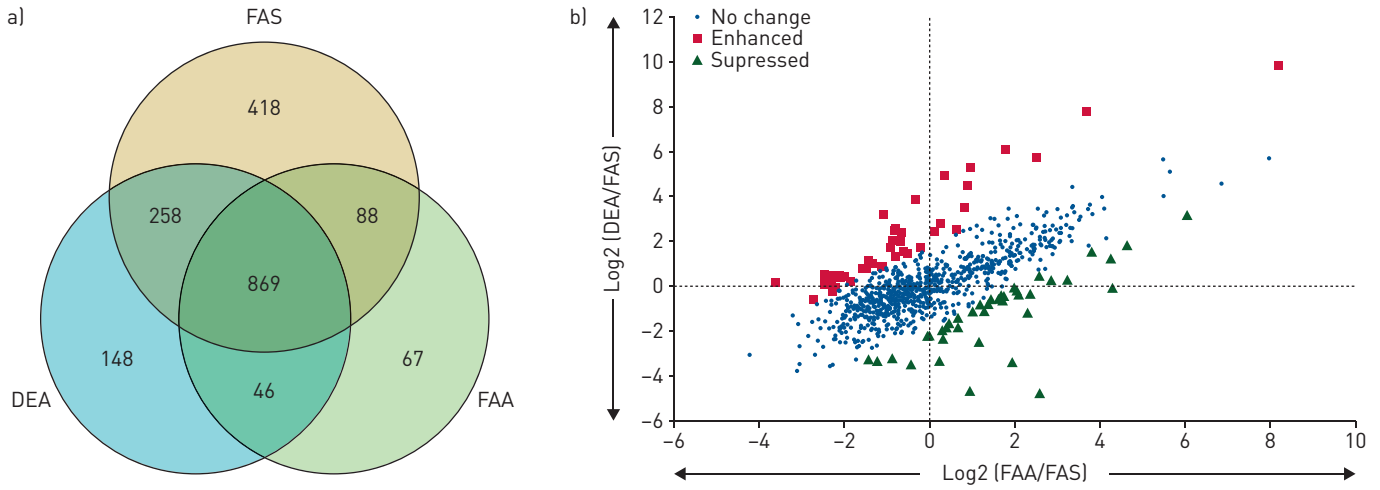

FIGURE 1 Diesel exhaust alters the allergen-mediated secretome in human bronchoalveolar lavage (BAL). Pooled BAL (n=5) was subjected to two-dimensional liquid chromatography–tandem mass spectrometry proteomic analyses. Proteins with at least two unique peptides with high confidence ($\log_e<-3$) were selected. a) 1894 proteins were quantified across three exposure conditions (filtered air and saline (FAS), filtered air and allergen (FAA) and diesel exhaust and allergen (DEA)), of which 869 proteins were quantified in all three conditions. b) Differential analysis was performed by subtracting the $\log_2$ protein intensity values: $x=\log_2(FAA)-\log_2(FAS)$; $y=\log_2(DEA)-\log_2(FAS)$. The normalised differences between these two modes, $Z=\text{norm}(y-x)$, were used to select the outermost 10% population ($|Z|>1.65$).

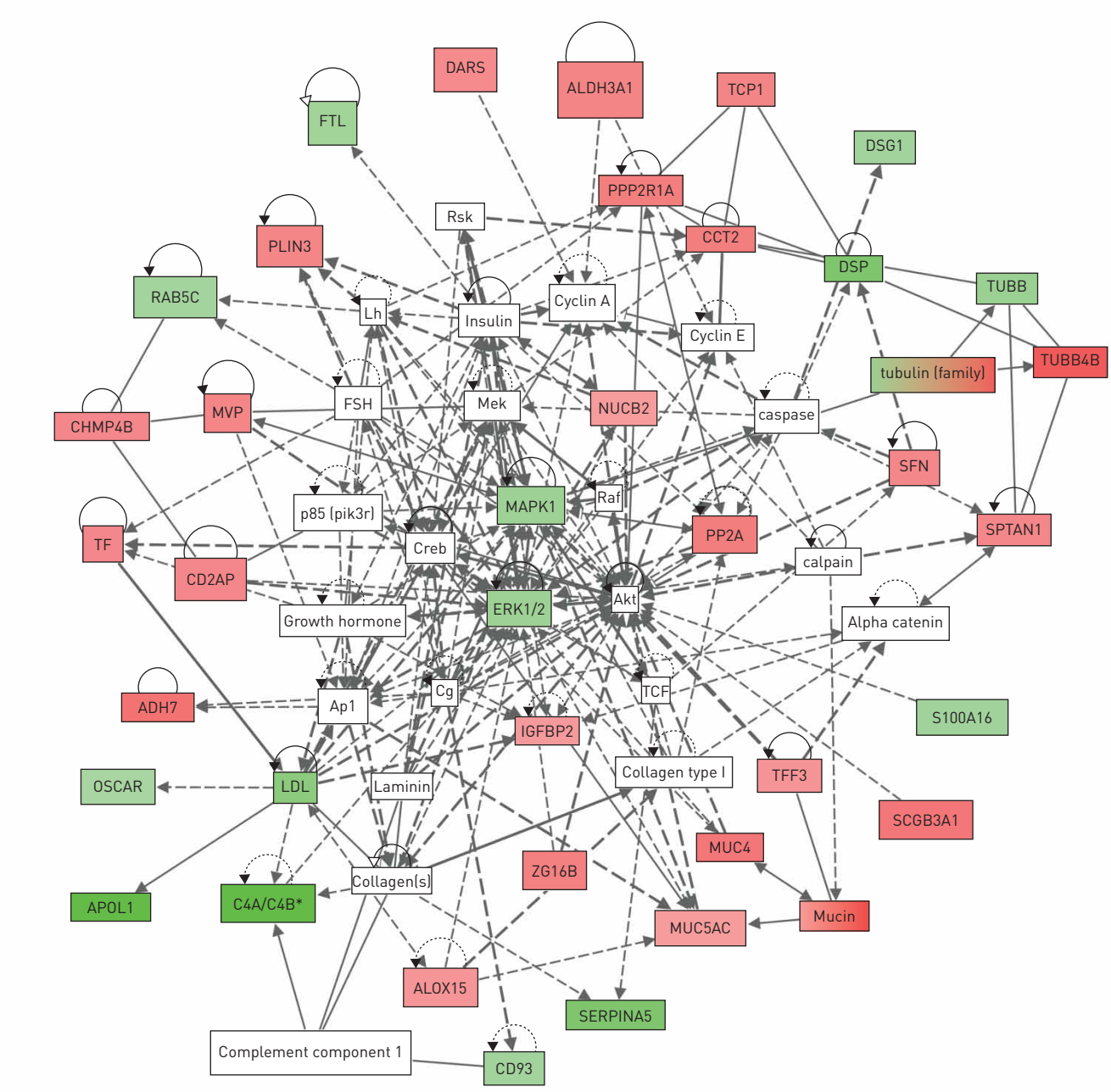

FIGURE 2 Interaction network of proteins altered in response to diesel exhaust and allergen (DEA) co-exposure. Of the 79 allergen-induced proteins altered in response to DEA co-exposure (table S2), 40 proteins connected in an interaction network, using the Ingenuity Pathway Analysis tool. Red nodes represent upregulated proteins and green nodes represent downregulated proteins. The intensity of colour corresponds to the magnitude of change in protein abundance relative to that in allergen-alone bronchoalveolar lavage. Solid lines correspond to direct interactions and dotted lines correspond to indirect interactions.

To further enumerate whether the DEA-mediated protein changes were driven by either allergen or DE alone, we performed K-means clustering for the 869 common proteins, using the signal intensity values from all four conditions (FAS, DES, FAA and DEA). This showed that 40 proteins were uniquely up-regulated in response to DEA, compared to either DE or allergen alone (fig. 3). Of the proteins that were enhanced by DEA, only three were induced by >4-fold by allergen-alone, and only one was induced by >4-fold by DE-alone, and eight proteins were not detected in the DE-alone samples (fig. 3b and table S2).

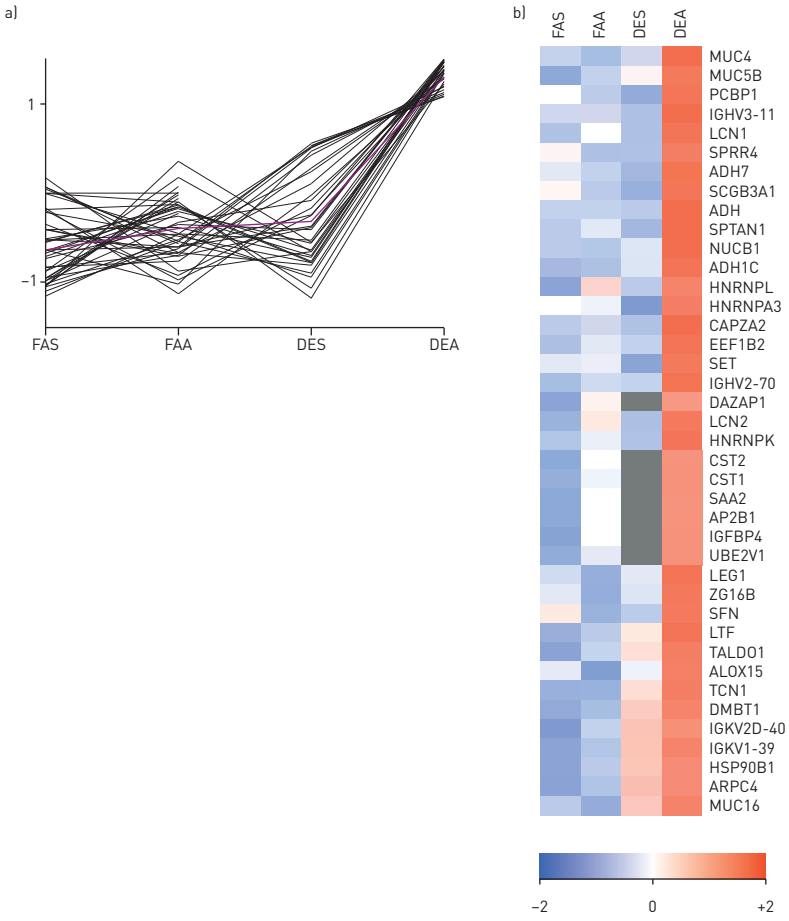

FIGURE 3 Diesel exhaust co-exposure with allergen uniquely enhances a subset of proteins in the bronchoalveolar lavage (BAL) secretome. Pooled BAL (n=5) from each exposure (filtered air and saline (FAS), filtered air and allergen (FAA), diesel exhaust and saline (DES) and diesel exhaust and allergen (DEA)) condition was subjected to two-dimensional liquid chromatography–tandem mass spectrometry proteomics analyses. Proteins with at least two unique peptides with high confidence (log_e<−3) were selected for further analyses. a) Unsupervised K-means clustering of expression profiles across the four conditions (FAS, FAA, DES and DEA) into nine groupings extracted a cluster of 40 proteins that were uniquely upregulated in response to DEA coexposure. The *y*-axis shows sample normalised protein log_2 expression values, additionally normalised for each protein across the four conditions. b) A heat map, generated using a Multiple Experiment Viewer, for the 40 proteins that were uniquely enhanced by DEA co-exposure as identified from (a). Grey indicates no detection.

Allergen-induced cystatin-SA is significantly enhanced by inhaled diesel exhaust

Our secretome analyses demonstrated that, among the top 10 proteins that were increased in BAL in response to DE+allergen co-exposure, only two proteins increased in response to allergen-alone (table S2). These two proteins, CST1 and CST2, both belong to the cystatin family. Cystatin-SA (CST2) abundance was enhanced by allergen-alone (>3.5 log2 fold change compared to FAS), which was further increased by >16-fold (>4 log2 fold change) in response to DE+allergen co-exposure. Therefore, we proceeded to validate the expression of cystatin-SA by probing individual BAL samples obtained from individual participants (n=7) by Western blots (fig. 4). Densitometry analyses of all the individual Western blots demonstrated that exposure to allergen-alone, but not DE-alone, significantly increased the abundance of CST2 (median of enhancement was >5-fold), compared to exposure to FAS. Moreover, allergen-induced CST2 abundance was further enhanced by DE+allergen co-exposure by >20-fold, thus confirming the proteomics data.

Validation of other protein expression selected from the proteomic analyses

As biological variability can confound inferences of omics-based studies, we selected four additional proteins that were enhanced by ⩾16-fold (>4 log2 fold change) by co-exposure to DEA, compared to FAA (table S2) – complement C4B, lipocalin-1 (LCN1), mucin-16 (MUC-16) and transcolbamin-1 (TCN1) – for further validation studies, as described below.

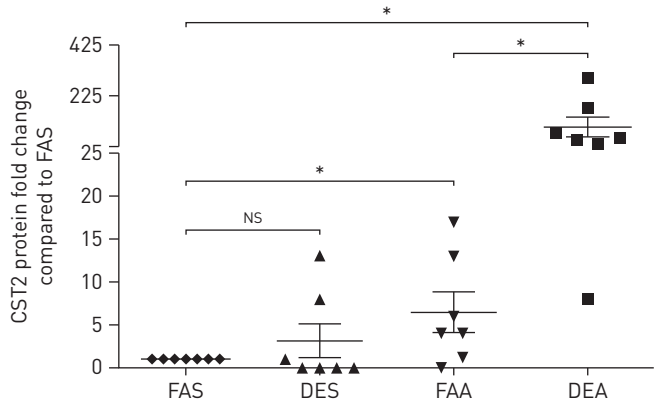

FIGURE 4 Allergen-induced cystatin-SA is significantly enhanced by inhaled diesel exhaust. Bronchoalveolar lavage samples (n=7) were individually probed in Western blots to determine the abundance of cystatin-SA (CST2) following each exposure condition (filtered air and saline (FAS), filtered air and allergen (FAA), diesel exhaust and saline (DES) and diesel exhaust and allergen (DEA)). Protein expression levels were quantified by densitometry. Protein fold change shown in the graphs represents relative band intensity compared to FAS normalised to 1. Each data point in the graphs represents an independent participant, and bars show the mean±SEM. Statistical significance was determined by Wilcoxon's matched-pairs signed rank test. *: p<0.05; NS: nonsignificant).

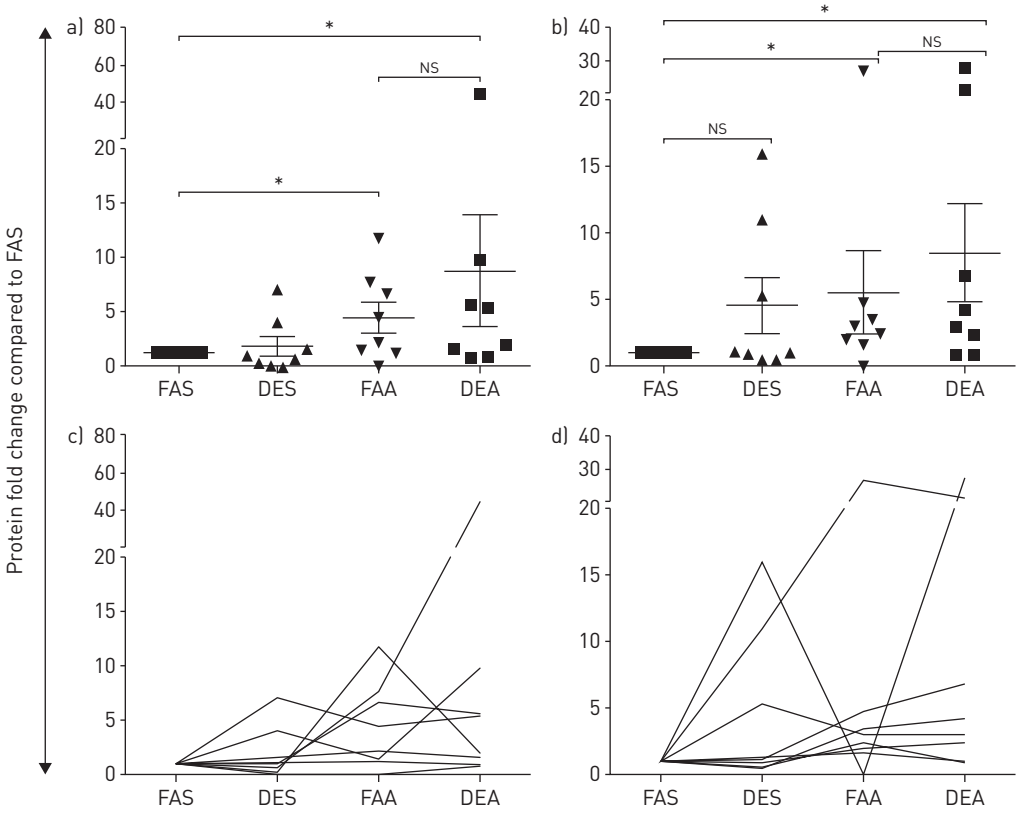

FIGURE 5 Abundance of selected proteins in individual participant bronchial samples. BAL samples (n=8) were individually probed in Western blots to determine the abundance of proteins (a and c) lipocalin-1 and (b and d) transcobalamin-1, following each exposure condition (filtered air and saline (FAS), diesel exhaust and saline (DES), filtered air and allergen (FAA) and diesel exhaust and allergen (DEA)). Protein expression levels were quantified by densitometry. Protein fold change shown in the graphs represents relative band intensity compared to FAS normalised to 1. Each data point in (a) and (b), and each line in (c) and (d), represents an independent participant. Data in (a) and (b) represents mean±SEM. Statistical significance was determined by Wilcoxon's matched-pairs signed rank test. *: p<0.05; NS: nonsignificant.

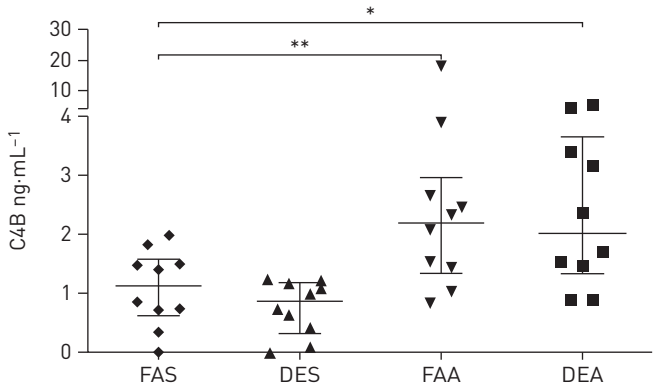

FIGURE 6 Allergen-induced complement C4B is not enhanced by inhaled diesel exhaust. Bronchial wash samples (n=10) were individually examined for complement C4B levels by ELISA. The data are plotted normalised to bronchial wash volume input. Each data point in the graphs represents an independent participant, and bars show the mean±SEM. Statistical significance was determined by Wilcoxon's matched-pairs signed rank test. *: p<0.05; **: p<0.01.

Abundance of LCN1 and TCN1 were probed in individual BAL samples obtained from independent participants (n=8) from all four conditions (FAS, DES, FAA and DEA) by Western blots. LCN1 (fig. 5a) and TCN1 (fig. 5b) were significantly increased in response to allergen-alone and DEA co-exposure, but not in response to DE-alone (n=8). Allergen-induced LCN1 and TCN1 were enhanced by DEA co-exposure; however, this was not statistically significant. Therefore, we further evaluated the trend of protein expression for LCN1 and TCN1 in individual participants (n=8), where each participant served as his/her own control. Allergen-alone enhanced LCN1 abundance ⩾2-fold in five out of eight individuals (>60%); in three of these participants, LCN1 was further enhanced with DEA co-exposure (fig. 5c and table S3). Similarly, allergen-alone enhanced TCN1 abundance ⩾2-fold in six out of eight participants (∼75%); in three of these, TCN1 was further enhanced by DEA co-exposure (fig. 5d and table S3). It should be noted that in one participant, TCN1 expression was enhanced >20-fold by DEA co-exposure compared to FAS co-exposure, but was not detected in the allergen-alone sample.

The concentration of C4B protein was monitored by ELISA in bronchial wash from independent participants (n=10). C4B abundance was significantly increased by exposure to allergen-alone and DEA co-exposure, but not by exposure to DE-alone (fig. 6). However, allergen-induced C4B was not enhanced by DEA co-exposure. Statistical analyses in the independent validation studies demonstrated statistical significance in several primary *a priori* pair-wise comparisons. However, such significance was no longer present upon formal (Bonferroni) correction for multiple comparisons.

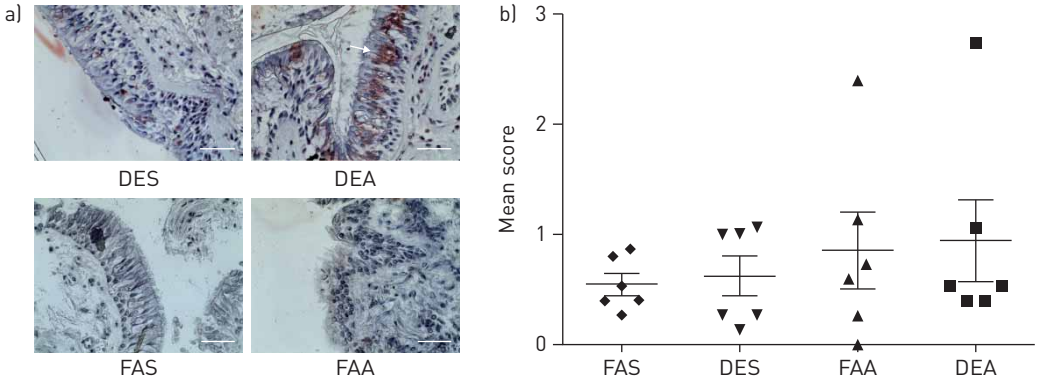

FIGURE 7 Immunohistochemistry to monitor mucin (MUC)-16 abundance in bronchial biopsies. Glycol methacrylate acrylic resin-embedded bronchial biopsies (n=6) obtained for each condition (filtered air and saline (FAS), filtered air and allergen (FAA), diesel exhaust and saline (DES) and diesel exhaust and allergen (DEA)) were independently assessed for the abundance of MUC-16 protein. Sections (2 µm) were probed with anti-MUC-16 antibody and imaged using an EVOS FL Auto Cell Imaging System. a) Representative section for each experimental condition. b) Stained sections were scored by three independent evaluators, blinded to conditions, as follows: 0=negative (no detectable staining), 1=focal staining (<25% of the epithelial cells showing staining), 2=moderate staining (25%–50%), and 3=extensive staining (>50%). Each data point in the graph represents the mean±SEM score (15 independent scores of five sections from each participant).

MUC-16, a component of respiratory tract mucus produced by the epithelium [19], was quantified by immunohistochemistry in bronchial biopsies (n=6). MUC-16 production trended higher in response to DEA co-exposure, relative to FAS and FAA; however, this was not found to be statistically significant (fig. 7).

Discussion

This is the first study to provide a detailed examination of how co-exposure with inhaled DE can alter allergen-mediated secreted global proteins (secretome) in the human lung. Our results suggest that DEA uniquely enhances the abundance of a subset of secreted proteins in BAL, which implies that proteins enhanced by DE+allergen co-exposure would be missed in mono-exposure studies. This study highlights the importance of using complex controlled human co-exposure models, and the strength of proteomic profiling, to better understand the effects of inhaled environmental exposures, such as air pollution, on human health.

Bioinformatics analyses interrogating the proteins that were altered in response to DEA co-exposure relative to allergen-alone demonstrated that more than 50% of the proteins interconnected within a single biological network. A major hub that was suppressed following DEA co-exposure was the MAPK1-ERK1/2 axis, a critical pathway that both positively and negatively regulates inflammation [20]. This is consistent with our previous studies using targeted approaches, demonstrating increase in the expression of specific inflammatory markers both in circulation and in the airways following inhaled DE+allergen co-exposure [14, 21]. We have also shown that expression of miRNAs, such as miR-132-3p, associated with inflammatory gene regulation are altered following DEA co-exposure in the controlled human exposure model [12], as used in this study. Target genes of miR-132-3p, such as CDKN1A, reported in our previous study detailing epigenetic changes modulated by DE+allergen co-exposure, [12] were also observed to be altered at the protein level in this study. Interestingly, the ERK1/2 pathway is known to regulate the miR-132 cluster [22]. Taken together, these studies suggest that mechanisms underlying DE-mediated inflammatory responses may be mediated by the MAPK1-ERK1/2 pathways.

The ERK1/2 pathway is also integrally connected to the oxidative stress response controlled by the hypoxia-induced protein phosphatase 2A (PP2A), which was enhanced following DEA co-exposure in the network analyses. As expression of PP2A is known to be directly enhanced by mitochondrial reactive oxygen species (ROS), our analyses suggest that DE exacerbates the allergen-induced oxidative stress response. This is consistent with the findings of our previous studies linking inhaled DE-related airway responsiveness with variants in genes of oxidative stress metabolism [23, 24]. This is also corroborated by our recent study demonstrating that DE particles activate transient receptor potential channels to initiate respiratory reflexes in a ROS-dependent mechanism [25]. DE particles are known to induce ROS, and various epidemiological studies have suggested an association of DE with the oxidative stress response [26]. This study details oxidative stress related proteins in BAL that are altered following inhaled DE+allergen co-exposure. Overall, the bioinformatics analyses indicate that proteins altered by DE+allergen co-exposure are primarily those involved in inflammatory and oxidative stress pathways.

We have demonstrated – using both a broad omics-based approach and with further targeted independent validation studies – that allergen-induced CST2 is significantly increased in response to inhaled DE+allergen co-exposure. This is the first study to describe CST2 expression in BAL. CST2 is an extracellular antimicrobial peptide with protease inhibitory activity previously described as originating from saliva [27]. There is limited information regarding the role of cystatins in immune responses. CST2 is known to induce the production of pro-inflammatory cytokines such as IFN-γ and IL-6, and promote NF-κB activity [28, 29]. These studies suggest that CST2 can facilitate inflammatory responses. Results from this study, demonstrating DE-mediated increase in allergen-induced inflammatory protein CST2, align with those of our recent study, showing that DE can enhance lower airway inflammation in allergic individuals [14]. This is also corroborated by a previous study demonstrating that the expression of CST2 is increased in the sputum from patients with uncontrolled asthma [30]. A related peptide from the cystatin family, cystatin-SN (CST1), is associated with upper airway inflammation and described to be a biomarker in nasal lavage for inhaled metal toxins [31]. CST1 was also found to be increased >3-fold by DEA co-exposure, compared to allergen-alone exposure, in our proteomic analyses. Therefore, results from this study give impetus for investigating the role of cystatins in response to air pollution.

Results from our independent validation studies probing individual BAL samples for abundance of some of the proteins selected from the secretome data were either not statistically significant or did not corroborate the proteomic analyses. For example, examining LCN1 and TCN1 in individual BAL samples from the different conditions demonstrated considerable biological variability between samples, and validation studies for C4B did not correlate with the secretome analyses. These validation studies emphasise the importance of independent confirmation using different methodologies to reconcile biological variability of human biomarkers selected from omics-based analysis approaches. These results

suggest that while pooling body fluids for secretome profiling is a valid approach to overcoming constraints of sample size and volume [32], this does not optimally capture intra-individual dynamics and may skew quantitative estimates to represent samples with highest changes in the pool.

Despite observed biological variabilities, our results suggest that DEA co-exposure increases LCN1 and TCN1 abundance in BAL. TCN1 has not yet been described in the context of either airway inflammation or respiratory diseases. LCN1 belongs to the family of proteins known as lipocalins. LCN1 is most highly expressed in tears, but is also found in the upper and lower airways, BAL and sputum [33]. Similar to the results of this study, LCN1 is up-regulated in BAL from smokers [34]. LCN1 has previously been associated with respiratory diseases in humans, *e.g.* in COPD (chronic obstructive pulmonary disease) [35] and cystic fibrosis [36]. Animal lipocalins are known allergens promoting Th2 responses and inflammation [37]. Thus, it is probable that LCN1 may also contribute to allergen-mediated Th2-skewed inflammation following inhaled DE exposure.

Our proteomic analyses identified several mucins (MUC-4, MUC-5B and MUC-16) that were enhanced in the BAL secretome in response to DE+allergen co-exposure, compared to allergen-alone exposure. However, we focused on MUC-16 in our validation studies as this was increased >16-fold in DE+allergen co-exposure, compared to allergen-alone exposure. MUC-16 is a component of respiratory tract mucus produced by the epithelium [19]. We showed that MUC-16 production trended higher in response to DEA co-exposure, relative to both saline-alone and allergen-alone exposure in bronchial biopsies; however, this was not statistically significant. This raises the possibility that MUC-16 secretion, but not production, is enhanced by DEA co-exposure. Nevertheless, the BAL secretome defined in this study in response to inhaled DE+allergen provides the rationale for examining the effects of air pollution on allergen-induced mucins.

Overall, we have demonstrated that inhaled DE+allergen co-exposure can enhance the abundance of secreted proteins in human lungs. Using a comprehensive proteomic approach, we have identified new protein candidates that are enhanced by DE+allergen co-exposure in humans. Some of these proteins are inflammatory mediators and associated with uncontrolled asthma, such as cystatin-SA. Our results suggest that atopic patients already sensitised to an allergen may be susceptible following exposure to air pollution. Given the high global prevalence of atopy [38], and commonality of these exposures, our findings have considerable relevance to public health internationally.

Acknowledgements

The authors gratefully acknowledge Jeremy Hirota, Oleg Krokhin and John Wilkins for extensive discussion and their intellectual input into this study.

Author contributions: CC and NM conceived the study. NM and PE performed the LC/MS-MS experiments, VS performed the bioinformatics analyses, HP performed the Western blots, MH performed the immunohistochemistry, and CR performed the ELISA assays. NM wrote the manuscript and was the lead for the proteomics team. CC was the senior lead for this study, undertook the bronchoscopy and sample collection, provided extensive intellectual input, and extensively edited the manuscript. All authors reviewed and edited the final manuscript.

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