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Targeted Proteomic Analyses of Nasal Lavage Fluid in Persulfate-Challenged Hairdressers with Bleaching Powder-Associated Rhinitis

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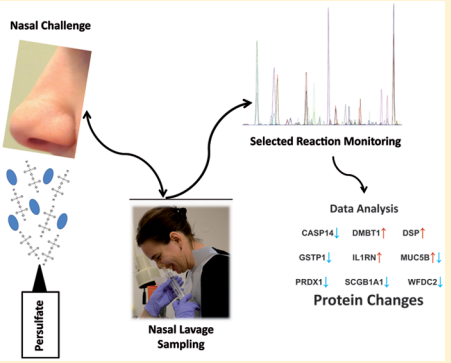
Supporting Information

ABSTRACT: Hairdressers have an increased risk for developing airway symptoms, for example, asthma and rhinitis. Persulfates, which are oxidizing agents in bleaching powder, are considered important causal agents for these symptoms. However, the underlying mechanisms are unclear. The aim was therefore to measure proteomic changes in nasal lavage fluid from persulfate-challenged subjects to identify proteins potentially involved in the pathogenesis of bleaching powder-associated rhinitis or candidate effect biomarkers for persulfate. Also, oxidized peptides were measured to evaluate their usefulness as biomarkers for persulfate exposure or effect, for example, oxidative stress. Samples from hairdressers with and without bleaching powder-associated rhinitis were analyzed with liquid chromatography tandem mass spectrometry using selected reaction monitoring to target 246 proteins and five oxidized peptides. Pathway analysis was applied to obtain a functional overview of the proteins. Several proteins involved in biologically meaningful pathways, functions, or disorders, for example, inflammatory responses, oxidative stress, epithelium integrity, and dermatological disorders, changed after the persulfate challenge. A list with nine proteins that appeared to be affected by the persulfate challenge and should be followed up was defined. An albumin peptide containing oxidized tryptophan increased 2 h and 5 h after the challenge but not after 20 min, which indicates that such peptides may be useful as oxidative stress biomarkers.



KEYWORDS: *nasal lavage fluid, selected reaction monitoring, persulfate, hairdresser, proteomics, oxidation, mass spectrometry, pathway analysis, airways, LC−MS/MS*

INTRODUCTION

Several studies have shown that hairdressers have an increased risk of developing respiratory symptoms from the airways such as asthma and rhinitis.[1−4] Hairdressers are exposed to many reactive chemicals that are capable of irritating the airways and inducing immunological reactions. Those with work-related respiratory symptoms frequently mention hair bleaching products as the cause of their symptoms.[2,5] Hair bleaching products contain persulfates, which are oxidizing agents that act as accelerators in the bleaching process.[6] Even though the association between persulfates and respiratory symptoms has been known for decades, the mechanisms behind these symptoms are unclear, and tools for risk assessment, diagnosis, and prognosis are limited.[7] However, many theories for the pathogenesis of persulfate-associated rhinitis have been suggested. Most studies agree upon the involvement of an immunologic mechanism, but no consensus has been reached regarding the details.[5,7−10] The clinical picture is similar to that of the classical IgE-mediated rhinitis, and an IgE-mediated mechanism has thus been proposed.[8,9,11] Some studies[8,9,11] have been able to detect positive skin prick tests against persulfates in a proportion of the patients, while in other studies,[5,7,10] skin prick tests were negative for all patients. The involvement of T helper 1 cells has also been suggested.[7] The direct action of persulfate salts on cells and biomolecules has also been proposed as a possible mechanism. Persulfates are oxidizing agents, and they have been shown to oxidize proteins *in vitro*[12,13] and induce generation of reactive oxygen species (ROS) from mast cells and basophils.[13]

In the present study, 159 nasal lavage fluid (NLF) samples from persulfate-challenged subjects were analyzed with liquid chromatography−tandem mass spectrometry using selected reaction monitoring (LC-SRM-MS) targeting 246 NLF proteins. This targeted approach was chosen because of the high sensitivity, specificity, and throughput offered by the SRM technique. Hairdressers with bleaching powder-associated rhinitis were compared to hairdressers without any respiratory symptoms and to subjects with rhinitis but without any work-related exposure to persulfates. The aim was to measure

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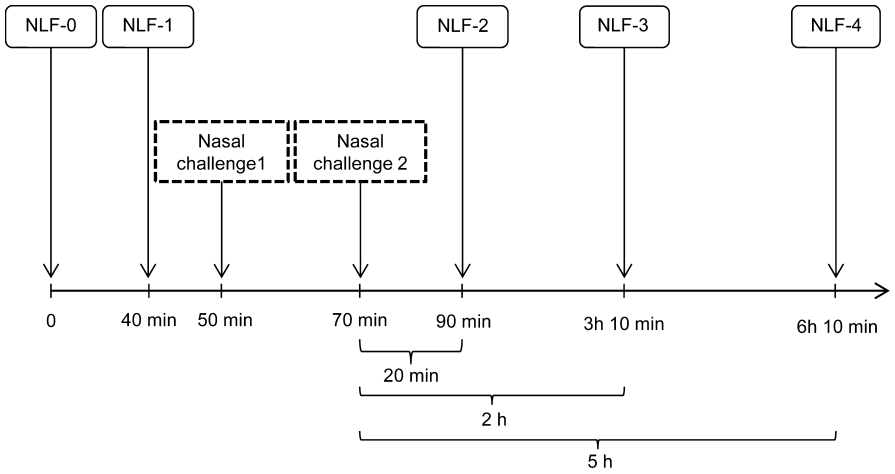
Scheme 1. Experimental Design of Persulfate Challenge.



The nasal challenge was performed in two steps by spraying a potassium persulfate solution into the nasal cavity. NLF samples were collected twice (NLF-0 and NLF-1) before the challenge and then 20 min (NLF-2), 2 h (NLF-3), and 5 h (NLF-4) after the challenge. The second NLF Sample (NLF-1) was used as the baseline sample, and the first NLF sample (NLF-0) was not analyzed.

proteomic changes in NLF after the persulfate challenge to identify proteins that may be involved in the pathogenesis of bleaching powder-associated rhinitis and/or may be used as effect biomarkers for persulfate. Also, because of the oxidizing properties of persulfates and their capabilities to induce ROS generation, oxidized peptides were measured in the samples to investigate whether any of these oxidized peptides could be used as biomarkers of persulfate exposure or effect, for example, oxidative stress.

MATERIALS AND METHODS

Reagents and Chemicals

Amicon Ultra-0.5 centrifugal filters (3000 nominal molecular weight limit) were purchased from Millipore (Bedford, MA, USA). The micro bicinchoninic acid (BCA) protein assay kit was from Thermo Scientific (Rockford, IL, USA). Trypsin (sequencing grade) was purchased from Roche Diagnostics Gmbh (Mannheim, Germany). Calcium chloride, formic acid (FA), hydrochloric acid, ammonium acetate, and potassium persulfate ($K_2S_2O_8$) were purchased from Merck (Darmstadt, Germany). Dithiothreitol (DTT), iodoacetamide, and hydrogen peroxide solution (≥30%; H_2O_2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) was purchased from Lab-scan (Dublin, Ireland).

Study Subjects

The NLF samples were obtained from a previous persulfate-challenge study that examined the effects of persulfates on the nasal mucosa and on the immune cells.[7] The nasal challenge was performed in two steps by spraying a potassium persulfate solution into the nasal cavity (Scheme 1). The subjects were first challenged with 0.001% potassium persulfate and 20 min later with 0.01% potassium persulfate. NLF samples were collected twice before the first challenge (NLF-0 and NLF-1) and then 20 min (NLF-2), 2 h (NLF-3), and 5 h (NLF-4) after the second challenge. The first nasal lavage was performed to wash the nasal cavity, and the second nasal lavage was performed to collect a baseline sample (NLF-1).

Three groups were studied: a target group consisting of female hairdressers with clear bleaching powder-associated nasal symptoms (symptomatic hairdressers (S), N = 14) and two control groups consisting of female hairdressers without nasal symptoms (asymptomatic hairdressers (WS), N = 14) and atopic females (atopy by history) with convincing pollen-associated rhinitis but without work-related exposure to bleaching powder (atopics (A), N = 12 (Enough sample material from the 20 min time-point was lacking for one of the subjects in the A-group. Samples from the other time-points were still analyzed for this subject.)). None of the hairdressers had a history of atopy. The atopics were included as a second control group since they probably have a sensitive nasal mucosa and therefore may nonspecifically react to persulfate. The atopic group can therefore be used to distinguish between specific and nonspecific nasal reactions. Asthmatic subjects were excluded from the study. Before the nasal challenge, a skin prick test against 13 common allergens was performed for all study subjects. Positive skin prick tests were found for two, three, and ten subjects in the WS, S, and A group, respectively. There were three social smokers in each hairdresser group and no smoker in the atopic group. The study subjects, the methods for the nasal challenge, and the nasal lavage are described in detail by Diab *et al.*[7] The study was approved by the Ethical Committee at the Medical Faculty at Lund University, and the participants gave written informed consent.

Preparation of NLF Samples

Samples were desalted using Amicon Ultra-0.5 centrifugal filters according to the manufacturer's instructions. Total protein content was measured before and after desalting using the micro BCA protein assay kit according to the manufacturer's instructions. The samples were evaporated and dissolved in 50 mM ammonium acetate to a concentration of 4.6 mg/mL. Proteins were reduced with DTT (1.25 mg/mg protein) for 1 h at 55 °C, alkylated with iodoacetamide (2.5 mg/mg protein) for 30 min at room temperature in darkness, and trypsin digested (1:50 wt:wt, trypsin:protein) overnight at 37 °C with trypsin dissolved in 0.5 mM hydrochloric acid, 25 mM ammonium acetate, and 1 mM calcium chloride. The digested samples were evaporated to dryness and stored at −20 °C. The samples were prepared in seven series with five to six subjects within each series. To avoid systematic bias, subjects from all three groups were prepared in all series. Since each subject is their own

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control and the samples of a subject were compared to its baseline sample, all samples originating from the same subject but from different time points were prepared and analyzed within the same series.

Mass Spectrometry System

The SRM analyses were conducted using a 5500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer equipped with a TurboIonSpray source (Applied Biosystems/MDS Sciex, Framingham, MA, USA) connected to an LC system (UFLCXR; Shimadzu Corporation, Kyoto, Japan; LC-SRM-MS). The settings of the LC-SRM-MS system are described in the Supporting Information (Data S1).

SRM Assays of NLF Proteins

The SRM assays targeting NLF proteins were developed in a previous study.[14] For the present study, some modifications were made to the original method. To shorten the analysis time, the LC gradient was modified, which led to a reduction of the analysis time from 45 to 32 min. Therefore, the SRM assays of the original method had to be adjusted. The LC-SRM-MS analyses are described in more detail in the Supporting Information (Data S1).

The digested study samples were dissolved in water (1 mg/mL) and analyzed (20 μg injected on column) in replicates of two with LC-SRM-MS. The final SRM method contained 2166 transitions targeting 246 proteins (Table S1, Supporting Information). The SRM method was split into four scheduled LC-SRM-MS methods containing about 540 transitions each (six subjects could not be analyzed twice for all MS/MS methods because of shortage of sample, and two subjects were not analyzed at all for one of the four methods). To minimize between-run variations, all samples of a subject were analyzed in consecutive runs.

SRM Assays of Oxidized Peptides

In vitro-oxidized NLF and HSA samples were used for data acquisition when the SRM assays were developed. Pooled NLF samples were oxidized with $K_2S_2O_8$ (0.04 and 0.1 mg/mg protein) alone or together with H_2O_2 (0.01 and 0.03 mg/mg protein). In addition, HSA was oxidized with $K_2S_2O_8$ (0.04 mg/mg protein). The samples were incubated with the oxidants for 2 h at 37 °C. The samples were desalted, reduced, alkylated, and trypsin digested as described above. SRM assays targeting oxidized peptides were developed using Skyline (Version 1.4.0).[15] NLF proteins and peptides that previously had been shown to be targets for oxidation were selected from the scientific literature.[12,16,17] Both the modified and the corresponding unmodified tryptic peptide were included. Development of SRM assays targeting oxidized peptides is described in the Supporting Information. The study samples (A, 10 subjects; S, 13 subjects; WS, 14 subjects) were analyzed with LC-SRM-MS (10 μg injected) using a scheduled SRM method containing in total 508 transitions (Table S2, Supporting Information). The SRM assays targeted these oxidized peptides as well as 112 peptides from 52 selected proteins reanalyzed to evaluate the between-run variation of the SRM analyses.

Data Analysis of NLF Proteins

The resulting data were reviewed and integrated in Skyline (Version 1.4.0).[15] The transitions were integrated, and mean total peak areas of duplicate analyses were calculated. Peptide fold changes were calculated by relating mean total peak areas for each time-point to the baseline sample. For the majority of proteins, more than one peptide was measured. Protein fold changes were calculated as the median of the peptide fold changes of a protein. Median values were used since they are more robust against outliers than mean values.[18] Finally, normalization was applied to the data by dividing each protein fold change with a correction factor. The median of all protein fold changes for a subject and for each time-point was calculated and used as the correction factor. This implies that for each subject, there were three correction factors: one for each time-point. This normalization accounts for variations in sample handling and instrument operation, and it is based on the assumption that the majority of the proteins do not change in abundance.

Statistical Analysis of NLF Proteins

Linear mixed modeling was used to analyze the data (Scheme 2). The dependent variable was the logarithmic protein ratio (base 2) and the fixed effects in the model were group, time and the interaction, time × group. Subject was set to a random effect. The repeated covariance types chosen were the first-order factor analytic covariance structure (219 proteins), the first-order autoregressive covariance structure with heterogeneous variances (20 proteins), the unstructured correlation metric covariance structure (two proteins), the first-order factor analytic covariance structure with heterogeneous variances (two proteins), and the first-order autoregressive covariance structure with homogeneous variances (one protein). The assumption of normally distributed residuals was checked visually by plotting histograms for each protein. Protein changes were shown by the estimated marginal means of the interaction term. Benjamini and Hochberg correction[19] was used to control for multiple testing (9 tests × 244 proteins = 2196 tests, and FDR-level: 0.05), which resulted in an adjusted significance level of $p < 0.0023$. The significantly changed proteins were selected for a more comprehensive analysis. To evaluate possible confounding effects from smoking, smoking was included in the model, and significant results from the initial analyses were checked for whether they remained significant ($p < 0.0023$). Because of the presence of hairdressers with positive skin prick test and subjects in the atopic group with negative skin prick tests, a sensitivity analysis was conducted. Thus, these individuals were excluded, and the same analysis using the former model was applied again. Data were analyzed with SPSS for Windows 21.0 (SPSS Inc., Chicago, IL, USA). The multiple test adjustment was conducted with the software SGoF+[20] (downloaded from http://webs.uvigo.es/acraaj/SGoF.htm) and with the user-defined significance level set at 0.05.

Data and Statistical Analysis of Oxidized Peptides

The resulting data were reviewed and integrated in Skyline (Version 1.4.0).[15] The oxidation degree of the oxidized peptide was estimated as the ratio between the total peak area of the oxidized and the corresponding unmodified peptide. The data for the oxidized peptides were analyzed with nonparametric statistical tests. Differences in oxidation degree between the groups at each time point (0, 20 min, 2 h, and 5 h) were analyzed using the Kruskal–Wallis H test. Differences between the baseline time point and the other time points (20 min, 2 h, and 5 h) in oxidation degree were analyzed using the Friedman and the Wilcoxon signed-rank tests. The differences between the time points were analyzed for each group separately and also for all subjects without respect to group belonging. Sensitivity analysis was conducted to test the strength of the results. These analyses were conducted in two separate

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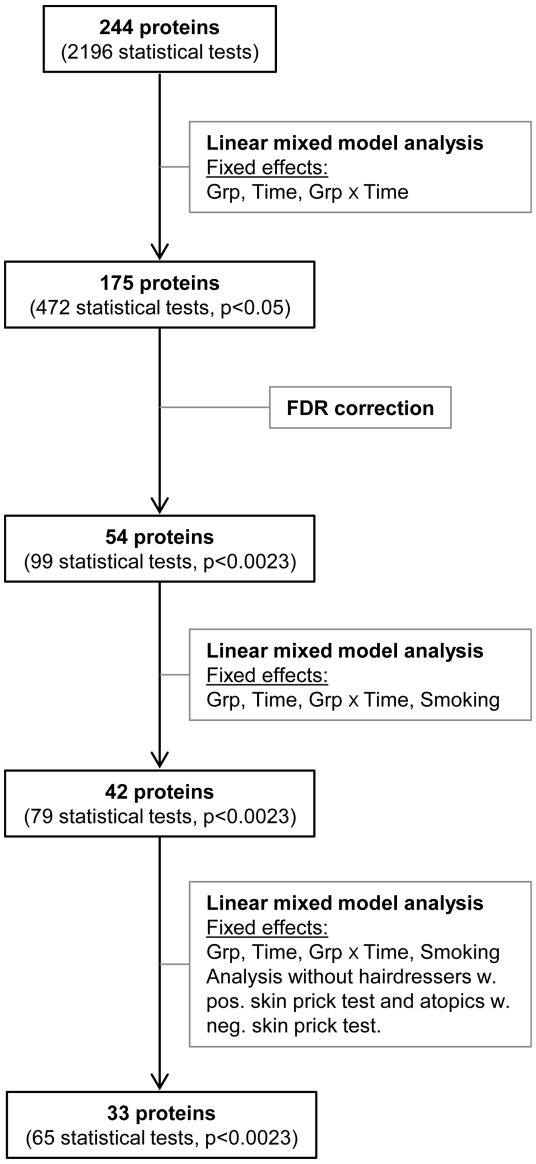
Scheme 2. Statistical Analyses of SRM Data.



The NLF samples were analyzed by SRM, and 244 of the 246 proteins were detected in at least one subject. Data were analyzed by linear mixed modeling, and Benjamini and Hochberg correction (False Discovery Rate, FDR) was used to control for multiple testing. Significantly changed proteins were selected for a more comprehensive analysis to adjust for smoking, and a sensitivity analysis was conducted in which hairdressers with positive skin prick test and subjects in the atopic group with negative skin prick tests were excluded.

analyses: (i) exclusion of smokers and (ii) exclusion of hairdressers with positive skin prick test and subjects in the atopic group with negative skin prick tests. Bonferroni correction was used to control for multiple testing. The significance level was set at 0.05. Data were analyzed with SPSS for Windows 21.0 (SPSS Inc., Chicago, IL, USA).

Pathway, Network, and Functional Analysis of Significantly Changed Proteins

The Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA, www.ingenuity.com) was used to map significantly changed proteins onto known pathways, diseases, and functions. Since many proteins showed similar changes, if any changes, in the three groups, IPA analysis was conducted for all significantly changed proteins and not for each group or time-point separately. Default settings were used except for species, which was set to human, and only experimentally observed relationships were considered. The software was unable to map the two proteins Ig kappa chain V–I region Rei (P01607) and Calmodulin (P62158). Pathways, diseases, and functions with a Benjamini and Hochberg multiple testing correction p-value less than 0.05 were considered statistically significant.

Absolute Analyte Specificity of SRM Assays

The SRM method targeting NLF proteins was developed in a previous study. However, absolute analyte specificity, which was studied using SRM-triggered MS/MS scans, could not be demonstrated for all SRM assays in the original method.[14] Therefore, unconfirmed SRM assays that targeted proteins with statistically significant changes were subjected to SRM-triggered MS/MS analysis. Absolute analyte specificity was demonstrated using SRM-triggered MS/MS scans in accordance with previous work.[14] The resulting MS/MS data was analyzed using the ProteinPilot 4.0 software with the Paragon search algorithm (Applied Biosystems/MDS Sciex). The following settings were used: sample type, identification; cysteine alkylation, iodoacetamide; digestion, trypsin; instrument, 5500 QTRAP ESI; species, *Homo sapiens*; ID focus, "–"; database, the SwissProt all species database (downloaded from uniprot August 13, 2012); search effort, thorough. Peptides with a peptide score >1.3 (confidence >95%) were considered identified.

The SRM assays targeting oxidized peptides were also confirmed for absolute specificity using SRM-triggered MS/MS scans in accordance with previous work.[14] The *in vitro* oxidized samples were used for data acquisition. Peptides were identified using the Proteinpilot 4.0 software with the following settings: sample type, identification; cysteine alkylation, iodoacetamide; digestion, trypsin; instrument, 5500 QTRAP ESI; species, *Homo sapiens*; ID focus, biological modifications, and amino acid substitutions; database, the SwissProt all species database (downloaded from uniprot August 13, 2012); search effort, thorough. Peptides with a peptide score >1.0 (confidence >90%) were considered identified. In addition, the MS/MS spectrum of one peptide, which was not identified by the Proteinpilot 4.0 software, was manually sequenced.

Evaluation of the Nasal Lavage Procedure

To evaluate if the nasal lavage procedure itself induced any protein changes, NLF was collected from six subjects according to the same protocol and time schedule as used in this study but without the persulfate challenge. The samples were prepared as described above and analyzed by LC–MS/MS using the SRM assays targeting the peptides of the proteins that were significantly changed in the present study. Cytokeratin-13, galectin-3-binding protein, and keratin-34 were not monitored, and the two proteins gamma-actin and cofilin-1 were not detected. Protein fold changes were calculated as described above. The protein ratios for the time points (20 min, 2 h, and 5 h) were analyzed with the Wilcoxon signed-rank test using SPSS, and the Benjamini and Hochberg correction[19] was used to control for multiple testing (3 tests × 49 proteins = 147 tests; FDR-level 0.05).

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Table 1. Significantly Changed Proteins in Nasal Lavage Fluid from Subjects Challenged with Persulfate

protein name (accession)	time	protein fold change[a] (95% CI)[b] symptomatics	asymptomatics	atopics	diff.[c]
alpha-1-antichymotrypsin (P01011)	20 min	1.1 (0.8, 1.4)	**1.8*** (1.3, 2.3)**	1.2 (0.9, 1.6)	
	2 h	1.0 (0.7, 1.4)	1.4[T] (1.0, 2.0)	1.3 (0.9, 1.9)	
	5 h	1.4 (1.0, 2.0)	**2.0*** (1.4, 2.9)**	1.7[T] (1.1, 2.4)	
alpha-1-antitrypsin (P01009)	20 min	1.4 (0.8, 2.2)	1.6[T] (1.0, 2.7)	1.1 (0.6, 1.8)	
	2 h	1.7[T] (1.1, 2.8)	**2.2** (1.4, 3.5)**	1.3 (0.8, 2.1)	
	5 h	2.1[T] (1.3, 3.4)	1.7[T] (1.0, 2.8)	1.5 (0.9, 2.5)	
alpha-actin-1 (P68133)	20 min	0.9 (0.5, 1.7)	1.1 (0.6, 1.9)	1.6 (0.9, 2.9)	
	2 h	0.8 (0.5, 1.3)	1.3 (0.8, 2.0)	1.3 (0.9, 2.0)	
	5 h	0.8 (0.5, 1.3)	1.3 (0.9, 2.0)	2.1** (1.4, 3.3)	A > S
annexin A2 (P07355)	20 min	1.1 (0.9, 1.4)	**0.6*** (0.5, 0.8)**	0.9 (0.7, 1.1)	WS < S
	2 h	0.7[T] (0.6, 0.9)	**0.6*** (0.5, 0.8)**	0.7[T] (0.5, 0.9)	
	5 h	0.7[T] (0.5, 0.9)	**0.5*** (0.4, 0.7)**	**0.6*** (0.5, 0.8)**	
antileukoproteinase (P03973)	20 min	0.9 (0.7, 1.2)	0.7[T] (0.5, 0.9)	0.8 (0.6, 1.1)	
	2 h	0.9 (0.7, 1.2)	**0.6** (0.5, 0.8)**	0.8 (0.6, 1.0)	
	5 h	0.8 (0.6, 1.1)	0.7[T] (0.5, 0.9)	0.6[T] (0.5, 0.8)	
arginase-1 (P05089)	20 min	1.2 (0.9, 1.6)	0.8 (0.6, 1.0)	1.0 (0.7, 1.3)	
	2 h	1.1 (0.8, 1.4)	0.8 (0.6, 1.0)	0.9 (0.7, 1.2)	
	5 h	1.1 (0.9, 1.5)	0.7** (0.5, 0.9)	0.9 (0.7, 1.2)	WS < S
beta-2-microglobulin (P61769)	20 min	0.8 (0.7, 1.0)	0.8[T] (0.7, 0.9)	1.0 (0.8, 1.3)	
	2 h	0.9 (0.7, 1.0)	**0.8*** (0.7, 0.9)**	0.9 (0.8, 1.1)	
	5 h	0.9 (0.8, 1.1)	0.8** (0.7, 0.9)	0.9 (0.8, 1.1)	
calmodulin (P62158)	20 min	1.3 (1.0, 1.7)	1.8*** (1.4, 2.4)	1.3 (1.0, 1.8)	
	2 h	1.1 (0.8, 1.5)	1.7** (1.2, 2.3)	1.2 (0.9, 1.7)	
	5 h	1.5[T] (1.1, 2.0)	1.9*** (1.4, 2.6)	1.4 (1.0, 1.9)	
calmodulin-like protein 5 (Q9NZT1)	20 min	0.8[T] (0.7, 1.0)	0.8[T] (0.7, 1.0)	0.8 (0.7, 1.0)	
	2 h	**0.7*** (0.6, 0.8)**	**0.7** (0.6, 0.9)**	0.8[T] (0.6, 0.9)	
	5 h	**0.6*** (0.5, 0.8)**	**0.7*** (0.6, 0.8)**	**0.6*** (0.5, 0.8)**	
caspase-14 (P31944)	20 min	0.8[T] (0.6, 1.0)	**0.5*** (0.4, 0.6)**	0.7[T] (0.5, 0.9)	WS < S
	2 h	**0.6*** (0.4, 0.7)**	**0.6*** (0.4, 0.7)**	0.7[T] (0.5, 0.9)	
	5 h	**0.5*** (0.4, 0.7)**	**0.5*** (0.3, 0.6)**	**0.6** (0.5, 0.8)**	
cofilin-1 (P23528)	20 min	1.0 (0.7, 1.5)	0.7[T] (0.5, 0.9)	0.6[T] (0.4, 0.8)	
	2 h	0.7[T] (0.5, 1.0)	**0.5*** (0.4, 0.7)**	0.8 (0.6, 1.1)	
	5 h	0.6[T] (0.5, 0.9)	**0.5*** (0.4, 0.7)**	0.7[T] (0.5, 0.9)	
complement factor B (P00751)	20 min	0.9 (0.8, 1.2)	0.8[T] (0.6, 1.0)	1.0 (0.8, 1.2)	
	2 h	0.9 (0.8, 1.0)	**0.8*** (0.7, 0.9)**	0.9 (0.7, 1.0)	
	5 h	1.0 (0.8, 1.1)	0.8[T] (0.7, 1.0)	0.9 (0.8, 1.1)	
cystatin-M (Q15828)	20 min	1.1 (0.9, 1.4)	1.5** (1.2, 1.8)	1.4[T] (1.1, 1.8)	
	2 h	1.2 (1.0, 1.5)	1.2 (1.0, 1.6)	1.2 (0.9, 1.5)	
	5 h	1.1 (0.9, 1.5)	1.1 (0.9, 1.4)	1.3[T] (1.0, 1.7)	
cysteine-rich secretory protein 3 (P54108)	20 min	0.8[T] (0.6, 1.0)	**0.7** (0.5, 0.8)**	0.8 (0.6, 1.1)	
	2 h	0.8 (0.6, 1.0)	0.6[T] (0.4, 0.8)	0.8 (0.6, 1.1)	
	5 h	0.6[T] (0.5, 0.8)	**0.5*** (0.4, 0.7)**	0.7[T] (0.5, 0.9)	
cytokeratin-13 (P13646)	20 min	1.0 (0.8, 1.3)	**2.1*** (1.6, 2.7)**	1.6[T] (1.2, 2.2)	WS > S
	2 h	1.0 (0.7, 1.5)	1.7[T] (1.2, 2.6)	1.3 (0.9, 1.9)	
	5 h	1.3 (0.9, 1.9)	**2.0** (1.3, 3.0)**	1.2 (0.8, 1.8)	
cytokeratin-14 (P02533)	20 min	0.9 (0.7, 1.1)	**0.7** (0.5, 0.8)**	0.8 (0.6, 1.1)	
	2 h	0.9 (0.8, 1.1)	0.7[T] (0.6, 0.9)	0.9 (0.7, 1.1)	
	5 h	0.8 (0.7, 1.0)	0.8[T] (0.6, 1.0)	0.7[T] (0.6, 0.9)	
cytokeratin-4 (P19013)	20 min	1.4 (0.9, 2.3)	2.0[T] (1.3, 3.2)	1.7[T] (1.0, 2.8)	
	2 h	0.9 (0.6, 1.4)	1.9[T] (1.3, 2.9)	1.3 (0.9, 2.1)	
	5 h	1.4 (0.9, 2.1)	**2.2*** (1.4, 3.4)**	2.3** (1.4, 3.6)	
cytokeratin-75 (O95678)	20 min	1.1 (0.9, 1.3)	1.2 (1.0, 1.5)	**1.5** (1.2, 1.9)**	
	2 h	1.1 (0.9, 1.4)	1.3[T] (1.1, 1.6)	1.2 (0.9, 1.5)	
	5 h	1.3[T] (1.0, 1.6)	1.4[T] (1.1, 1.8)	**1.7*** (1.3, 2.1)**	
deleted in malignant brain tumors 1 protein (Q9UGM3)	20 min	1.3 (0.9, 1.8)	1.5[T] (1.1, 2.1)	1.3 (0.9, 1.9)	
	2 h	1.3 (0.9, 1.8)	1.7[T] (1.2, 2.5)	1.5[T] (1.0, 2.2)	
	5 h	1.5[T] (1.1, 2.0)	**1.9*** (1.4, 2.7)**	1.7[T] (1.2, 2.4)	
desmoplakin (P15924)	20 min	1.0 (0.8, 1.2)	1.3[T] (1.0, 1.6)	1.3 (1.0, 1.6)	
	2 h	0.8 (0.7, 1.0)	1.5** (1.2, 1.8)	1.3[T] (1.0, 1.7)	WS > S < A

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Table 1. continued

protein name (accession)	time	protein fold change[a] (95% CI)[b] symptomatics	asymptomatics	atopics	diff.[c]
	5 h	0.9 (0.7, 1.2)	1.6*** (1.3, 2.2)	1.5^{T} (1.1, 2.0)	WS < S
ezrin (P15311)	20 min	0.8 (0.6, 1.2)	0.8 (0.5, 1.1)	**0.5** (0.3, 0.8)**	
	2 h	0.8 (0.6, 1.1)	0.7^{T} (0.5, 0.9)	0.8 (0.6, 1.0)	
	5 h	0.8 (0.6, 1.0)	0.7^{T} (0.6, 0.9)	0.7^{T} (0.5, 0.9)	
fatty acid-binding protein 5 (Q01469)	20 min	0.8^{T} (0.7, 1.0)	0.9 (0.7, 1.0)	0.8^{T} (0.6, 0.9)	
	2 h	**0.6*** (0.5, 0.7)**	**0.7*** (0.6, 0.8)**	0.8^{T} (0.7, 1.0)	S < A
	5 h	**0.6*** (0.5, 0.8)**	0.7^{T} (0.6, 0.9)	0.8^{T} (0.6, 1.0)	
fibrinogen alpha chain (P02671)	20 min	0.7 (0.5, 1.1)	0.6^{T} (0.4, 1.0)	**0.4*** (0.2, 0.6)**	
	2 h	**0.5*** (0.3, 0.7)**	0.6^{T} (0.4, 0.9)	**0.5*** (0.3, 0.7)**	
	5 h	**0.5*** (0.3, 0.7)**	0.7 (0.5, 1.1)	**0.5*** (0.3, 0.7)**	
galectin-3-binding protein (Q08380)	20 min	0.9 (0.7, 1.2)	0.7^{T} (0.5, 0.9)	0.9 (0.6, 1.2)	
	2 h	0.9 (0.7, 1.1)	0.7^{T} (0.6, 0.9)	0.9 (0.7, 1.2)	
	5 h	0.8^{T} (0.6, 0.9)	**0.7*** (0.6, 0.8)**	0.7^{T} (0.6, 0.9)	
gamma-actin (P63261)	20 min	0.9 (0.6, 1.4)	**2.3** (1.4, 3.7)**	1.2 (0.7, 2.0)	WS > S
	2 h	0.9 (0.6, 1.4)	1.6^{T} (1.0, 2.4)	1.0 (0.6, 1.6)	
	5 h	1.0 (0.6, 1.6)	1.1 (0.7, 1.8)	1.0 (0.6, 1.7)	
general transcription factor II−I (P78347)	20 min	1.1 (0.9, 1.5)	1.2 (0.9, 1.5)	1.5^{T} (1.1, 2.1)	
	2 h	1.0 (0.7, 1.2)	1.3 (1.0, 1.6)	1.2 (0.9, 1.5)	
	5 h	1.1 (0.8, 1.4)	**1.6*** (1.3, 2.1)**	1.3^{T} (1.0, 1.7)	
glutathione S-transferase P (P09211)	20 min	0.8 (0.7, 1.1)	**0.7** (0.5, 0.9)**	0.7^{T} (0.6, 1.0)	
	2 h	1.0 (0.8, 1.2)	**0.7** (0.5, 0.8)**	0.8 (0.6, 1.1)	WS < S
	5 h	0.8 (0.6, 1.0)	0.7^{T} (0.5, 0.9)	**0.6** (0.5, 0.8)**	
hemopexin (P02790)	20 min	1.1 (1.0, 1.4)	**1.4*** (1.2, 1.7)**	1.3^{T} (1.1, 1.6)	
	2 h	0.9 (0.8, 1.1)	1.2 (1.0, 1.4)	1.0 (0.9, 1.2)	
	5 h	1.1 (0.9, 1.4)	1.2 (1.0, 1.5)	1.1 (0.9, 1.4)	
Ig alpha-1 chain C region (P01876)	20 min	1.1 (0.8, 1.5)	1.3 (0.9, 1.7)	1.2 (0.8, 1.7)	
	2 h	1.0 (0.7, 1.3)	1.5^{T} (1.1, 2.1)	1.3 (0.9, 1.8)	
	5 h	1.2 (0.9, 1.7)	1.7** (1.3, 2.4)	1.3 (0.9, 1.8)	
Ig gamma-2 chain C region (P01859)	20 min	1.0 (0.8, 1.2)	0.7^{T} (0.6, 0.9)	0.9 (0.7, 1.1)	
	2 h	0.7^{T} (0.6, 1.0)	**0.6*** (0.5, 0.8)**	0.7^{T} (0.5, 0.9)	
	5 h	0.8 (0.6, 1.1)	0.7 (0.5, 1.0)	0.7 (0.5, 1.0)	
Ig kappa chain V−I region Rei (P01607)	20 min	1.2 (0.9, 1.7)	**1.8** (1.3, 2.5)**	1.1 (0.7, 1.5)	
	2 h	1.1 (0.7, 1.7)	1.8^{T} (1.2, 2.7)	1.1 (0.7, 1.7)	
	5 h	1.4^{T} (1.0, 2.0)	**1.9*** (1.3, 2.6)**	1.5^{T} (1.1, 2.2)	
interleukin-1 receptor antagonist protein (P18510)	20 min	0.9 (0.8, 1.2)	1.4** (1.2, 1.8)	1.2 (1.0, 1.5)	WS < S
	2 h	0.8 (0.6, 1.0)	1.5^{T} (1.2, 2.1)	1.2 (0.8, 1.6)	
	5 h	0.9 (0.7, 1.2)	**1.8*** (1.3, 2.3)**	1.1 (0.8, 1.5)	WS > S
keratin-34 (O76011)	20 min	0.8 (0.5, 1.2)	0.8 (0.5, 1.3)	0.5^{T} (0.3, 0.8)	
	2 h	0.9 (0.7, 1.4)	0.7 (0.5, 1.0)	0.7 (0.5, 1.0)	
	5 h	0.9 (0.6, 1.4)	0.7 (0.5, 1.1)	0.5** (0.3, 0.7)	
keratin-35 (Q92764)	20 min	1.1 (0.9, 1.4)	**1.7*** (1.3, 2.1)**	1.0 (0.8, 1.2)	WS > A
	2 h	1.2 (0.9, 1.4)	**1.5*** (1.2, 1.9)**	1.1 (0.9, 1.4)	
	5 h	1.3^{T} (1.0, 1.7)	1.2 (0.9, 1.5)	1.1 (0.8, 1.5)	
lipocalin-15 (Q6UWW0)	20 min	0.9 (0.7, 1.2)	1.0 (0.8, 1.4)	1.1 (0.8, 1.5)	
	2 h	1.0 (0.8, 1.2)	1.2 (0.9, 1.4)	1.1 (0.9, 1.4)	
	5 h	1.0 (0.8, 1.2)	**1.6*** (1.3, 1.9)**	1.3^{T} (1.0, 1.6)	WS > S
moesin (P26038)	20 min	0.9 (0.7, 1.1)	0.8 (0.6, 1.0)	0.9 (0.7, 1.1)	
	2 h	0.8 (0.6, 1.0)	0.7^{T} (0.6, 0.9)	0.8 (0.6, 1.0)	
	5 h	0.8 (0.6, 1.0)	**0.6*** (0.5, 0.8)**	1.0 (0.8, 1.4)	WS < A
mucin-5B (Q9HC84)	20 min	1.5^{T} (1.1, 1.9)	1.1 (0.8, 1.4)	0.9 (0.7, 1.2)	
	2 h	1.5^{T} (1.1, 2.0)	0.9 (0.7, 1.2)	0.8 (0.6, 1.1)	
	5 h	1.1 (0.8, 1.5)	**0.6*** (0.4, 0.8)**	0.7^{T} (0.5, 0.9)	WS < S
neutrophil elastase (P08246)	20 min	1.0 (0.8, 1.3)	0.8^{T} (0.6, 1.0)	0.8 (0.6, 1.0)	
	2 h	0.9 (0.7, 1.2)	0.8^{T} (0.6, 1.0)	0.6*** (0.5, 0.8)	
	5 h	0.9 (0.7, 1.1)	0.7^{T} (0.5, 0.9)	0.6*** (0.4, 0.8)	
PDZ and LIM domain protein 5 (Q96HC4)	20 min	1.0 (0.8, 1.3)	0.8 (0.6, 1.0)	0.9 (0.7, 1.2)	
	2 h	1.0 (0.8, 1.3)	0.8^{T} (0.6, 1.0)	0.9 (0.7, 1.1)	
	5 h	0.9 (0.7, 1.2)	0.7** (0.5, 0.9)	0.8 (0.6, 1.1)	
peroxiredoxin-1 (Q06830)	20 min	0.9 (0.6, 1.3)	0.6^{T} (0.4, 0.9)	0.9 (0.6, 1.4)	

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Table 1. continued

protein name (accession)	time	protein fold change[a] (95% CI)[b] symptomatics	asymptomatics	atopics	diff.[c]
	2 h	0.9 (0.8, 1.1)	**0.7*** (0.5, 0.8)**	0.8 (0.7, 1.0)	WS < S
	5 h	0.9 (0.6, 1.3)	**0.5*** (0.4, 0.8)**	0.6^{T} (0.4, 0.9)	
plasminogen (P00747)	20 min	1.0 (0.8, 1.2)	0.9 (0.7, 1.1)	1.0 (0.8, 1.3)	
	2 h	1.1 (0.9, 1.3)	0.9 (0.8, 1.0)	0.8^{T} (0.7, 1.0)	
	5 h	1.1 (0.9, 1.3)	0.8** (0.6, 0.9)	0.8^{T} (0.7, 1.0)	WS < S
polymeric immunoglobulin receptor (P01833)	20 min	1.1 (0.9, 1.4)	1.1 (0.9, 1.4)	1.3^{T} (1.0, 1.7)	
	2 h	1.0 (0.8, 1.2)	1.1 (0.9, 1.4)	1.1 (0.9, 1.4)	
	5 h	1.1 (0.9, 1.3)	**1.5*** (1.2, 1.8)**	1.2 (1.0, 1.5)	
ribonuclease 4 (P34096)	20 min	1.1 (0.9, 1.4)	**1.5** (1.2, 1.8)**	1.5** (1.2, 1.9)	
	2 h	1.0 (0.8, 1.3)	1.4^{T} (1.1, 1.8)	1.3 (1.0, 1.7)	
	5 h	1.2 (0.9, 1.6)	**1.7*** (1.3, 2.3)**	1.3 (1.0, 1.8)	
sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (O14983)	20 min	1.0 (0.8, 1.2)	1.4** (1.2, 1.7)	1.2 (0.9, 1.5)	WS > S
	2 h	0.9 (0.7, 1.1)	1.2 (0.9, 1.6)	1.0 (0.7, 1.3)	
	5 h	0.9 (0.7, 1.2)	1.4^{T} (1.1, 1.8)	0.8 (0.6, 1.1)	
secretoglobin family 1D member 1 (O95968)	20 min	1.5 (0.7, 2.9)	2.3^{T} (1.2, 4.4)	2.0 (1.0, 4.1)	
	2 h	1.7 (0.7, 3.8)	2.3^{T} (1.1, 4.9)	1.9 (0.8, 4.3)	
	5 h	1.9 (0.8, 4.6)	2.4^{T} (1.1, 5.3)	**5.0*** (2.1, 12.0)**	
SH3 domain-binding protein 1 (Q9H299)	20 min	1.2 (1.0, 1.5)	**1.4** (1.2, 1.7)**	1.2 (1.0, 1.6)	
	2 h	1.2 (0.9, 1.5)	1.3 (1.0, 1.7)	1.1 (0.8, 1.5)	
	5 h	1.2 (0.9, 1.5)	1.2 (0.9, 1.5)	0.9 (0.7, 1.2)	
stratifin (P31947)	20 min	1.2 (1.0, 1.5)	1.1 (0.9, 1.4)	1.5** (1.2, 1.9)	
	2 h	1.1 (0.8, 1.3)	1.2 (1.0, 1.5)	1.1 (0.9, 1.4)	
	5 h	1.1 (0.9, 1.3)	1.3^{T} (1.1, 1.6)	1.1 (0.9, 1.4)	
thymidine phosphorylase (P19971)	20 min	1.1 (0.9, 1.5)	1.4^{T} (1.1, 1.7)	1.4^{T} (1.1, 1.9)	
	2 h	1.2 (0.9, 1.6)	1.5^{T} (1.1, 1.9)	1.3 (1.0, 1.7)	
	5 h	1.4^{T} (1.0, 1.9)	**1.6** (1.2, 2.2)**	1.4^{T} (1.0, 2.0)	
triosephosphate isomerase (P60174)	20 min	1.1 (0.9, 1.3)	0.8 (0.6, 1.0)	0.9 (0.7, 1.2)	
	2 h	0.9 (0.8, 1.1)	0.7** (0.6, 0.9)	0.9 (0.7, 1.1)	
	5 h	0.9 (0.8, 1.1)	0.7*** (0.6, 0.8)	0.7** (0.6, 0.9)	WS < S
unhealthy ribosome biogenesis protein 2 homologue (Q14146)	20 min	0.9 (0.7, 1.1)	0.7^{T} (0.6, 0.9)	0.8 (0.6, 1.0)	
	2 h	1.1 (0.9, 1.4)	0.7^{T} (0.6, 0.9)	0.9 (0.7, 1.2)	
	5 h	0.9 (0.7, 1.2)	0.7^{T} (0.6, 1.0)	**0.6** (0.5, 0.8)**	
uteroglobin (P11684)	20 min	0.6^{T} (0.4, 0.9)	**0.5** (0.4, 0.8)**	0.6^{T} (0.4, 0.9)	
	2 h	**0.5** (0.4, 0.8)**	**0.4*** (0.2, 0.5)**	0.5^{T} (0.3, 0.8)	
	5 h	**0.4*** (0.3, 0.6)**	**0.4*** (0.2, 0.6)**	**0.4*** (0.2, 0.6)**	
WAP four-disulfide core domain protein 2 (Q14508)	20 min	0.9 (0.6, 1.2)	0.7^{T} (0.5, 0.9)	0.8 (0.6, 1.2)	
	2 h	0.7^{T} (0.5, 1.0)	**0.6*** (0.4, 0.8)**	0.8 (0.5, 1.1)	
	5 h	0.6^{T} (0.4, 0.8)	**0.5*** (0.3, 0.7)**	0.7^{T} (0.4, 1.0)	
vimentin (P08670)	20 min	1.2 (0.9, 1.6)	1.3 (0.9, 1.7)	1.6^{T} (1.2, 2.2)	
	2 h	1.2 (0.9, 1.6)	1.5^{T} (1.1, 2.0)	1.4^{T} (1.0, 2.0)	
	5 h	1.3 (0.9, 1.8)	**1.8*** (1.3, 2.5)**	1.7^{T} (1.2, 2.4)	
zymogen granule protein 16 homologue B (Q96DA0)	20 min	1.3 (0.7, 2.4)	1.0 (0.6, 1.8)	1.2 (0.6, 2.2)	
	2 h	2.2^{T} (1.2, 3.9)	1.7 (0.9, 3.2)	1.5 (0.8, 3.0)	
	5 h	**2.9** (1.6, 5.5)**	2.3^{T} (1.2, 4.3)	2.4^{T} (1.2, 4.7)	

[a]Protein fold changes were calculated as the median of the peptide fold changes of a protein. Peptide fold changes were calculated by relating mean total peak areas for each time-point to the baseline sample. Protein fold changes for each group and time-point are presented as estimated marginal means. Statistically significant changes ($p < 0.0023$) are indicated by *; ** ($0.001 < p < 0.0023$); *** ($p < 0.001$). Trends ($0.0023 \leq p < 0.05$) are indicated by ^{T}. Protein ratios that remained statistically significant ($p < 0.0023$) when adjusted for smokers are bold. Protein ratios that remained statistically significant ($p < 0.0023$) when adjusted for smokers and when hairdressers with positive skin prick test and subjects in the atopic group with negative skin prick tests were excluded are bold and underlined. [b]A 95%-confidence interval for estimated marginal means of protein ratios. [c]Differences in protein fold changes between the groups. S, symptomatics; WS, asymptomatics; A, atopics.

Variations in Sample Preparation and SRM Analyses

Within- and between-run precisions of sample preparation and of the SRM analyses were evaluated in numerous ways. Twelve aliquots of a pooled NLF sample were desalted, and protein abundance was determined before and after the desalting to assess recovery and within-run variations in the desalting procedure. The 12 desalted aliquots were reduced, alkylated, and trypsin digested as described above. The digested samples were analyzed in 12 consecutive runs with LC-SRM-MS targeting 112 peptides from 52 proteins. Within-run precision of desalting, trypsin digestion, and SRM analyses was estimated as the coefficients of variation (CVs) of total peak areas. Between-run variations in the protein concentration analyses were evaluated by comparing protein concentrations assessed when the samples were collected and then again when the

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Table 2. Oxidation Degree of Oxidized Peptides

protein	peptide (modified residue)	oxidation degree[a] median[b] (min, max) 0	20 min	2 h	5 h
Symptomatics					
complement C3	ILLQGTPVAQM (+16) TEDAVDAER (Met968)	0.8 (0.3, 0.9)	0.3 (0.2, 0.3)	0.3 (0.3, 0.7)	0.4 (0.3, 0.8)
fibrinogen α chain	DSHSLTTNIM (+16) EILR (Met91)	2.1 (1.1, 5.6)	1.1 (0.4, 2.8)	2.3 (0.6, 3.2)	1.0 (0.5, 3.5)
serum albumin	AVM (+16) DDFAAFVEK (Met548)	0.1 (0.05, 0.2)	0.08 (0.05, 0.2)	0.1 (0.06, 0.2)	0.1 (0.05, 0.3)
	AW (+32) AVAR (Trp214)	0.008 (0.004, 0.03)	0.008 (0.003, 0.02)	0.01 (0.005, 0.05)	0.01 (0.003, 0.05)
	SHCIAEVENDEM (+16) PADLPSLAADFVESK (Met298)	0.5 (0.2, 1.8)	0.4 (0.1, 2.1)	0.4 (0.2, 5.7)	0.6 (0.2, 1.4)
Asymptomatics					
complement C3	ILLQGTPVAQM (+16) TEDAVDAER (Met968)	0.8 (0.4, 1.5)	0.4 (0.3, 1.3)	0.7 (0.2, 0.8)	0.9 (0.2, 2.0)
fibrinogen α chain	DSHSLTTNIM (+16) EILR (Met91)	4.3 (1.6; 4.7)	2.9 (2.3, 6.7)	3.6 (2.4, 4.7)	3.5 (2.1, 6.0)
serum albumin	AVM (+16) DDFAAFVEK (Met548)	0.1 (0.06, 0.9)	0.1 (0.05, 1.5)	0.1 (0.06, 1.3)	0.2 (0.1, 1.3)
	AW (+32) AVAR (Trp214)	0.009 (0.002, 0.03)	0.01 (0.003, 0.04)	0.01 (0.005, 0.04)	0.02* (0.01, 0.06)
	SHCIAEVENDEM (+16) PADLPSLAADFVESK (Met298)	0.6 (0.2, 3.0)	0.6 (0.2, 2.6)	0.7 (0.1, 5.0)	1.0 (0.2, 6.3)
Atopics					
complement C3	ILLQGTPVAQM (+16) TEDAVDAER (Met968)	0.5 (0.3, 1.2)	0.4 (0.2, 0.8)	0.4 (0.3, 0.7)	0.4 (0.3, 0.8)
fibrinogen α chain	DSHSLTTNIM (+16) EILR (Met91)	1.7 (1.0, 7.7)	1.2 (0.8, 2.3)	2.1 (0.7, 3.6)	1.0 (0.5, 3.5)
serum albumin	AVM (+16) DDFAAFVEK (Met548)	0.2 (0.05, 0.4)	0.1 (0.04, 0.3)	0.2 (0.05, 0.9)	0.1 (0.05, 0.3)
	AW (+32) AVAR (Trp214)	0.007 (0.003, 0.01)	0.008 (0.002, 0.01)	0.02 (0.004, 0.03)	0.01 (0.004, 0.05)
	SHCIAEVENDEM (+16) PADLPSLAADFVESK (Met298)	0.7 (0.2, 8.4)	0.3 (0.1, 2.0)	0.6 (0.2, 4.2)	0.6 (0.2, 1.4)
Total					
complement C3	ILLQGTPVAQM (+16) TEDAVDAER (Met968)	0.6 (0.3, 1.5)	0.4 (0.2, 1.3)	0.4 (0.2, 0.8)	0.4 (0.2, 2.0)
fibrinogen α chain	DSHSLTTNIM (+16) EILR (Met91)	2.0 (1.0, 7.7)	1.6 (0.4, 6.7)	2.3 (0.6, 4.7)	2.1 (0.5, 6.0)
serum albumin	AVM (+16) DDFAAFVEK (Met548)	0.1 (0.05, 0.9)	0.1 (0.04, 1.5)	0.1 (0.05, 1.3)	0.2 (0.05, 1.5)
	AW (+32) AVAR (Trp214)	0.008 (0.002, 0.03)	0.009 (0.002, 0.04)	**0.02*** (0.004, 0.05)**	**0.02*** (0.004, 0.06)**
	SHCIAEVENDEM (+16) PADLPSLAADFVESK (Met298)	0.6 (0.2, 8.4)	0.4 (0.1, 2.6)	0.6 (0.1, 5.7)	0.6 (0.2, 7.3)

[a]The oxidized and the corresponding unmodified peptide were measured by SRM in NLF samples. The oxidation degree was estimated as the ratio between the total peak areas of the oxidized and the corresponding unmodified peptide. Differences between the baseline time-point and the other time-points (20 min, 2 h, and 5 h) were analyzed using Wilcoxon signed rank test. Differences were analyzed for each group separately and for all subjects without respect to group belonging (total). Bonferroni correction was used to control for multiple testing. [b]Bonferroni adjusted p-values less than 0.05 are considered statistically significant. Statistically significant changes are indicated by * ($0.01 < p < 0.05$); ** ($0.001 < p < 0.01$); *** ($p < 0.001$). Ratios that remained statistically significant when smokers were excluded are bold. Ratios that remained statistically significant when smokers and hairdressers with positive skin prick test and subjects in the atopic group with negative skin prick tests were excluded are bold and underlined.

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samples were analyzed with SRM. Between-run variations of the SRM analyses were evaluated by reanalyzing the study samples (A, 10 subjects; S, 13 subjects; WS, 14 subjects) with LC-SRM-MS (10 μg injected) targeting 112 peptides from 52 proteins and comparing the result with the initial analyses. The analyses were conducted six months apart. Protein fold changes were calculated as described above, and the between-run precision was estimated as the geometric CVs of the protein ratios.

RESULTS

Quality of the SRM Data

In total, 244 proteins were detected in the samples, and at least 220 proteins (median (min-max): 236 (220−241)) were detected in each individual. Of the 715 monitored peptides, at least 473 peptides (median (min-max): 602 (473−655)) were detected in each individual. A majority (77%) of the detected peptides had a total peak area of over 10^4 counts. We were not able to analyze all samples in replicates of two because of sample shortage. The detected peptides were measured in replicates of two in 91% of the cases with a total peak area CV of less than 20% for 78% of these peptides. For significantly changed proteins, median total peak area and median CV for detected peptides were 3×10^4 counts (min-max; $2 \times 10^3 - 2 \times 10^7$ counts) and 9% (min-max; 4−19%), respectively (Table S3, Supporting Information). The SRM analyses were run almost nonstop for about 6 weeks with acceptable variation for most peptides. However, some peptides showed large variation in retention times between runs and were therefore missed in some of the samples.

Absolute Analyte Specificity of SRM Assays

In the present study, absolute analyte specificity was demonstrated for two additional peptides, VYIIQACR (caspase-14) and QLGCGWATSAPGNAR (deleted in malignant brain tumors 1 protein (DMBT1)), compared to the previous study.[14] In total, absolute analyte specificity of peptide assays has been demonstrated for 22 of the 54 significantly changed proteins (Table S3, Supporting Information).

Variation in Sample Preparation and SRM Assays

Between- and within-run precisions of the different steps in the sample preparation and in the LC-SRM-MS analyses were evaluated in numerous ways. Mean recovery for the desalting step was 56%, and within-run precision was 8% (CV). Within-run variation in total peak areas was 9% (median CV; min-max: 4%−56%) when desalting, trypsin digestion, and SRM analyses were included. The samples were analyzed twice, six months apart, and between-run variation for protein ratios was 15% (median geometric CV; min-max: 10%−40%). Between-run variation for protein concentration analyses was 7% (median CV; min-max: 0.1%−61%).

Significantly Changed Proteins

In total, 54 proteins were significantly ($p < 0.0023$) changed in at least one of the groups and time-points (Table 1). The largest number of significantly changed proteins was found in the asymptomatic group, 44 proteins (67 statistical tests) compared to six proteins (11 statistical tests) for the symptomatic group, and 17 proteins (21 statistical tests) for the atopic group. However, several of these proteins showed similar trends ($p < 0.05$) in all groups. Of the 54 significantly changed proteins, 33 proteins (65 statistical tests) remained statistically changed ($p < 0.0023$) also when adjusted for smoking and when hairdressers with positive skin prick test and subjects in the atopic group with negative skin prick tests were excluded. An evaluation of the NLF procedure was conducted to examine if the nasal lavage procedure induced any protein changes (Table S4, Supporting Information). After correcting for multiple testing, no proteins were significantly changed in these experiments.

Pathway, Network, and Functional Analysis of Significantly Changed Proteins

Sixteen pathways, for example, acute phase response signaling, RhoA signaling, communication between innate and adaptive immune cells, and nrf2-mediated oxidative stress response, were enriched for the significantly changed proteins (Table S5, Supporting Information). The top three diseases and disorders were dermatological diseases and conditions, inflammatory disease, and inflammatory response, and the top three molecular and cellular functions were cell death and survival, drug metabolism, and lipid metabolism (Table S5, Supporting Information).

SRM Assays of Oxidized Peptides

In the present study, five oxidized peptides from complement C3 (P01024), fibrinogen alpha chain (P02671), or albumin (P02768) were detected in the samples (Table 2). Absolute analyte specificity for the assays targeting the oxidized peptides and their corresponding unmodified peptides was confirmed by SRM-triggered MS/MS (Data S2, Supporting Information). Tryptophan and methionine oxidations were identified. Only oxidized peptides from albumin were detected consistently in a majority of the subjects ($N \geq 33$). The oxidation degree was only statistically significantly changed for the albumin peptide AWAVAR containing oxidized (+32 Da) Trp^{214}, and no significant differences in oxidation degree were found between the groups for any of the oxidized peptides. The oxidation degree increased significantly (Bonferroni adjusted $p < 0.05$) for AWAVAR containing oxidized (+32 Da) Trp^{214} 5h after the challenge in the asymptomatic group. The same trend was seen in all groups. When data from all groups were included in the same analysis without respect to group belonging, the oxidation degree for this peptide was significantly increased 2 h and 5 h after the persulfate challenge. This increase remained statistically significant also when smokers and hairdressers with positive skin prick test and subjects in the atopic group with negative skin prick tests were excluded.

DISCUSSION

In this study, we used a targeted screening method to study proteomic changes in NLF to identify proteins that may be involved in the pathogenesis of bleaching powder-associated rhinitis and may be used as effect biomarkers for persulfate. Also, oxidized peptides were measured in the samples to investigate if any of these oxidized peptides could be used as biomarkers of persulfate exposure or effect, for example, oxidative stress. Targeted LC-SRM-MS was used to measure 246 proteins and five oxidized peptides in 159 samples from subjects challenged with persulfate, a probable causal agent to the bleaching powder-associated rhinitis. Hairdressers with bleaching powder-associated rhinitis were compared to asymptomatic hairdressers and atopic subjects without work-related exposure to persulfate. Several proteins involved in biologically meaningful pathways, functions, or disorders, for example, inflammatory responses, oxidative stress, epithelium integrity, and dermatological disorders, were changed after the

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persulfate challenge. A list with nine proteins that appear to be affected by the persulfate challenge and should be followed up was defined. Also, five oxidized peptides were measured in the samples with LC-SRM-MS, and an albumin peptide containing oxidized tryptophan increased 2 h and 5 h after the challenge but not 20 min after the challenge. This oxidation may be the result of an endogenous oxidative stress, which suggests that such peptides might be useful as biomarkers for oxidative stress.

Quality of the SRM Data

Selected reaction monitoring was used to measure 246 proteins and five oxidized peptides in 159 samples. The quality of the SRM data in this study was acceptable with a total peak area CV of less than 20% for the majority of the peptides and even lower CVs (median CV of 9%) for peptides of significantly changed proteins. However, a weakness of the approach used in this study was the global normalization approach, which was applied to control for variations in sample handling and instrument operation. This type of normalization is rather rough, and there is a risk of losing important findings. In future studies, when this many samples are being analyzed, it would be beneficial to include at least a few internal standards, for example, by spiking the samples with labeled peptides, which can be used for normalization. This would improve the precision of the quantification and the comparison between the samples.

Assay Specificity and Variations

A major advantage of SRM is its high specificity due to the two stages of mass filtering. This specificity increases with the number of product ions targeted. In this study, three to four transitions per targeted peptide were used to ensure analyte specificity. Unfortunately, absolute analyte specificity has not been demonstrated for all assays, which probably can be explained by the usage of SRM-triggered MS/MS scans for confirmation. This approach can be problematic since full MS/MS spectrum acquisition is less sensitive than the SRM assay itself. On the other hand, 47 of the significantly changed proteins were represented by at least two peptides, and when these peptides are in line with each other, which was the case for a majority of these proteins (Table S3, Supporting Information), the result can still be reliable. Thus, even though absolute analyte specificity was not confirmed for all SRM assays in this study, the SRM assays and the resulting SRM data had a high degree of reliability.

Furthermore, within- and between-run variations in sample preparation and SRM assays possessed median CVs of ≤15%, which indicated acceptable variation. The main weakness however was the low recovery after the desalting step. This will probably affect the outcome in such a way that some potentially interesting results are missed. Since the within-run variation for desalting, trypsin digestion, and SRM analyses was acceptable, it suggests that the same proteins are lost every time. Thus, we may lose potentially interesting findings due to the desalting procedure, but it would probably not result in the appearance of any false positives.

Study Design and Statistical Analyses

The targeted SRM-approach instead of an untargeted shotgun approach was selected to obtain a high throughput, which was needed for the analyses of the 159 samples. Other studies that have analyzed samples originating from the airways but with an untargeted shotgun approach identify a considerably higher number of proteins compared to the present study.[21−25] However, only about 200−300 proteins were consistently detected in these studies (≥50% of the samples).[21−25] In addition, several studies use depletion or fractionation,[21,22,25] which reduce throughput considerably. Thus, the targeted, label-free quantification strategy applied in this study represents a powerful tool for discovering biomarkers from clinical relevant samples. The assays have low variation, and they detect proteins more consistently combined with a simple sample preparation, which increases the throughput. However, this comes at expense of the potential to discover new proteins.

Studies like this, where biological responses are provoked in humans, are relatively rare within the proteomics field. Since samples are obtained before and after the provocation, each individual is their own control, and thus biological variations are reduced and significant differences are easier to find. In this study, the Benjamini and Hochberg correction was used to control for multiple testing. This type of correction offers more statistical power compared to traditional approaches, for example, Bonferroni procedures.[26] One-hundred seventy-five proteins (472 statistical tests) had p-values less than 0.05, and out of these, 54 proteins (99 statistical tests) were considered statistically significantly changed ($p < 0.0023$ at FDR-level 0.05) (Table 1). It was thus evident that the multiple correction strategy used did remove a high number of findings.

A few social smokers in the hairdresser groups and not full consistency between atopy by history and by skin prick test may indicate some disparity within the groups. However, in the original study, no differences were shown between these subgroups and the main-groups in reactivity to persulfates.[7] In spite of that, a sensitivity analysis was conducted to assess the impact on the result from the subjects in the atopic group with negative skin prick tests and the subjects in the hairdresser groups with positive skin prick tests. Generally, when these individuals were excluded, it was clear that for most proteins, the p-values increased, while the fold change remained nearly unchanged. This suggests that these individuals changed similarly as the other subjects in their respective group. The p-values probably changed because the exclusion of these individuals resulted in smaller study groups, which makes it harder to detect changes. However, we did only check whether the results remained and not if any new appeared. Thus, interesting findings may have been lost by using this strategy, but because of the large amount of SRM data generated from these analyses, there was a need for a rather strict approach in selecting the most prominent findings. To somewhat compensate for this strictness, when the biological meaning was interpreted from these results, trend differences ($p < 0.05$) among the 54 proteins that were selected for further analysis were also taken into account.

Significantly Changed Proteins

To get a functional overview of the 54 significantly changed proteins and to get a general picture over biological processes and possible diseases in which these proteins may be involved, IPA analysis was applied. This analysis showed that many proteins were mapped to inflammatory disorders and responses and to cell death and survival. In addition, several enriched pathways, for example, RhoA signaling, integrin-linked kinase (ILK) signaling, and actin cytoskeleton signaling, were associated with cell migration and cell-adhesion, which are mechanisms important to barrier integrity and tissue remodeling.[27−29] Different adhesive protein complexes play important roles for barrier integrity by connecting epithelial cells to each other and to the surrounding tissue, and chronic

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Table 3. Significantly Changed Proteins Suggested for Follow-up Studies

Protein Name	Protein Fold Change[a]									Possible function/ biological meaning
	Symptomatics			Asymptomatics			Atopics			
	20 min	2h	5h	20 min	2h	5h	20 min	2h	5h	
Caspase-14	↓^{T}	↓***	↓***	↓***	↓***	↓***	↓^{T}	↓^{T}	↓**	Associated with epithelial barrier integrity
DMBT1	→	→	↑^{T}	↑^{T}	↑^{T}	↑***	→	↑^{T}	↑^{T}	Mucosal defense protein Increased expression associated with respiratory inflammation
Desmoplakin	→	→	→	↑^{T}	↑**	↑***	→	↑^{T}	↑^{T}	Associated with epithelial barrier integrity
Glutathione S-transferase P	→	→	→	↓**	↓**	↓^{T}	↓^{T}	→	↓**	Oxidative stress defense
IL1RN	→	→	→	↑**	↑^{T}	↑***	→	→	→	Inhibits IL-1 activity and acute and chronic inflammation
Mucin-5B	↑^{T}	↑^{T}	→	→	→	↓***	→	→	↓^{T}	Mucus secretion
Peroxiredoxin-1	→	→	→	↓^{T}	↓***	↓***	→	→	↓^{T}	Oxidative stress defense
Uteroglobin	↓^{T}	↓**	↓***	↓**	↓***	↓***	↓^{T}	↓^{T}	↓***	Decreased expression associated with respiratory epithelial damage
WFDC2	→	↓^{T}	↓^{T}	↓^{T}	↓***	↓***	→	→	↓^{T}	Decreased expression associated with de-differentiation of bronchial epithelial cells

[a]Statistically significant changes are indicated by ** ($0.001 < p < 0.0023$); *** ($p < 0.001$). Trends ($0.0023 \leq p < 0.05$) are indicated by ^{T}.

exposures to different airway irritants and chronic mucosal inflammation may damage this epithelial barrier.[30] Thus, the results may suggest that the persulfate challenge induce an inflammatory response that possibly resulted in tissue damage and tissue remodeling.

The Nrf2-mediated oxidative stress response pathway was among the enriched pathways, which suggests that the persulfate challenge affects the redox balance in the nasal mucosa. This pathway can be activated by oxidants, and it induces the transcription of genes associated with protection toward oxidants and electrophiles.[31] There is a close connection between oxidative stress and inflammation. Oxidative stress is considered to play an important role in the pathogenesis of asthma and rhinitis.[32−34]

In addition to airway diseases, skin diseases are also common among hairdressers,[35−39] and persulfates are frequently associated with these conditions.[4,5,35,36,38,40] Interestingly, the most enriched category within diseases and disorders was dermatological diseases and conditions with 25 of the significantly changed proteins linked to it (Table S5, Supporting Information). This could indicate that persulfate-associated airway and skin diseases share some of the underlying mechanisms.

The persulfate challenge induced most proteomic effects, measured as number of significantly changed proteins, among asymptomatics and least among symptomatics. However, many of these proteins showed similar trends in all groups, and few distinct differences between the groups were seen. This could indicate nonspecific effects induced by the persulfate challenge in all groups even though a weaker response was seen among symptomatics. Symptomatic hairdressers may initially have had an active inflammation in the nasal mucosa with cells performing at their full capacities. The persulfate challenge may thus lead to an exhaustion of the nasal mucosa cells in this group. The evaluation of the nasal lavage procedure showed that it appears to be well-tolerated, and it does not seem to cause these nonspecific effects. Thus, these effects are most likely connected to the persulfate challenge.

Significantly Changed Proteins on Which To Follow-up

Of the 54 significantly changed proteins, nine were considered to be of special interest and are discussed further below (Table 3). They were selected because they either changed similarly in all three groups or because the protein ratios indicated differences in response in the symptomatic hairdresser group compared to the other groups. They were also selected because of biological reasons, that is, known function or involvement in airway diseases and allergy.

The result pointed toward decreased concentrations of uteroglobin and WAP four-disulfide core domain protein 2 (WFDC2) and increased concentrations of DMBT1 in all three groups. Uteroglobin has been suggested as a biomarker of respiratory epithelial damage in acute and chronic exposures to airway irritants.[41−43] Decreased levels in NLF of this protein have been associated with exposure to tobacco smoke,[44] with chronic rhinosinusitis[45] and with allergen challenge in patients with intermittent allergic rhinitis.[46] Decreased expression of WFDC2 has been linked to dedifferentiation of bronchial epithelial cells,[47] which implies that the cells lose the characteristics of differentiated bronchiolar cells. The mucosal defense protein DMBT1 binds to a broad range of pathogens,[48] and it has been shown to be up-regulated during respiratory inflammation.[49] Thus, the increased levels of DMBT1 could indicate an inflammation in the nasal mucosa, and the decreased levels of uteroglobin and WFDC2 may reflect a damaged respiratory epithelium in all groups.

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The results indicated increased levels of desmoplakin in the asymptomatic hairdresser and atopic groups while unchanged in the symptomatic hairdresser group. Desmoplakin is a component of desmosomes,[50] which are important for epithelial barrier integrity. During epithelial shedding, which has been observed in asthma patients,[51] a disruption occurs at the desmosomes.[50] The increased levels of this protein could reflect an enhanced tissue repair or an enhanced degradation of desmosomes after the persulfate challenge. The exact role of this protein in persulfate-associated rhinitis needs to be further studied.

Decreased levels of Caspase-14 were seen in all groups and at all time-points. This protein is thought to be involved in the degradation of profilaggrin into filaggrin, which is important for hydration of the epidermis and skin barrier function. Mutations in the filaggrin gene are major predisposing factors for atopic dermatitis, but they are also associated with atopic asthma and allergic rhinitis. Atopic diseases of the skin and the airways often coexist, and disruption of epithelial barrier systems seems to be involved in the pathogenesis of immune disorders of both the airways and the skin.[52] The function of Caspase-14 in the airways is unknown but one may speculate that this protein plays a role in epithelium integrity.

Interleukin-1 receptor antagonist protein (IL1RN) increased among asymptomatic hairdressers after the persulfate challenge. This protein inhibits the activity of interleukin-1 (IL-1α and IL-1β), which has been shown to play a crucial role in acute and chronic inflammation.[53] IL1RN has been shown to reduce ozone-induced airway hyperresponsiveness in mice[54] and toluene-2,4-diisocyanate induced allergic rhinitis symptoms in guinea pigs.[55] In addition, this protein has been proposed as a therapeutic target for bronchial asthma.[56] Thus, it is likely that IL1RN has a protective role toward persulfate-induced rhinitis.

Interestingly, IL-1β has been shown to induce expression of mucin-5B in airway epithelial cells through the NF-κB pathway,[57] and the results from this study indicated increased concentrations of mucin-5B 20 min and 2 h after the challenge among symptomatics and decreased concentrations 5 h after the challenge in the other two groups, which suggests differences in mucus secretion between the groups. A balanced mucus production is important for normal lung function, and an overproduction of mucus has been associated with many airway diseases, for example, asthma, chronic bronchitis, cystic fibrosis, chronic obstructive pulmonary disease, and chronic rhinosinusitis.[58,59]

The results also indicated decreased levels of the two antioxidant-related proteins peroxiredoxin-1 and glutathione S-transferase P1 for asymptomatics at all time-points and atopics after 5 h. No change was seen for symptomatics. One may speculate whether this could reflect a more active adaptive response to oxidative stress among symptomatics due to chronic inflammation.

Comparison with Previous Proteomic Study

A subset of the samples analyzed in this study has previously been analyzed using pooled samples by 2D-nanoLC–MS/MS combined with iTRAQ-labeling.[60] Very few proteins had statistically significant changes in both studies. Generally, the agreement in protein ratios between the studies varied with protein. Some proteins presented similar trends in the ratios, for example, uteroglobin, WFDC2, and DMBT1, while the results for others were conflicting. Many of the proteins that differed between the groups in the present study, for example, IL1RN, peroxiredoxin-1, glutathione S-transferase P1, and desmoplakin, were not detected in the iTRAQ-study, and no comparison could thus be made for these proteins. The major findings of the iTRAQ-study were increased levels of apolipoprotein A-I and decreased levels of lactotransferrin exclusively in the symptomatic group. We were not able to reproduce those results even when SRM data originating from the same subset of individuals as analyzed in the iTRAQ-study (data not shown) were statistically analyzed. Of course, a better agreement would have been preferable; however, because of large differences in methodology and statistical design between these two studies, a perfect agreement cannot be expected. Yet, the varying agreement in protein ratios underlines the need for validation studies in general.

SRM Analyses of Oxidized Peptides

Since persulfates are oxidizing agents, it is likely that they shift the redox balance toward a state of oxidative stress. In addition, when these chemicals are inhaled, they may induce an inflammatory response further promoting oxidative stress.[34] It has previously been shown that persulfates oxidize albumin *in vitro*. In these studies, the amino acids tryptophan and methionine were found to be oxidized.[12,13] Therefore, we wanted to measure oxidized peptides in these persulfate-challenged subjects to evaluate whether these oxidized peptides could be used as biomarkers for persulfate exposure or effect, for example, oxidative stress. We presented a simple SRM-based method for quantification of multiple oxidations without the use of any prior enrichment. The oxidation degree for an albumin peptide containing oxidized tryptophan increased after the challenge. Since this increase was not immediate, the result suggests an endogenous oxidative stress rather than an oxidation directly induced by the persulfate challenge. Thus, such oxidized peptides are probably not useful as biomarkers for persulfate exposure. However, they may be of use as biomarkers for oxidative stress.

Tryptophan has been shown to be susceptible for artifactual oxidation, and it could therefore be questioned if this oxidation type is biologically induced. Perdivara *et al.*, who used LC–ESI–MS/MS to detect tryptophan oxidations, showed that these were induced following electrophoresis and in-gel digestion but not following in-solution digestion without the use of electrophoresis.[61] Hence, it is likely that the tryptophan oxidation monitored in the present study represents an oxidation induced *in vivo*. Yet, the usefulness of this oxidation as a marker for oxidative stress remains to be elucidated.

■ CONCLUSIONS

The targeted, label-free quantification strategy applied in this study represents a powerful tool for discovering biomarkers from clinically relevant samples. In general, the IPA analysis of the significantly changed proteins suggested that the persulfate challenge affected proteins associated with oxidative stress and induced an inflammatory response along with a tissue damage and tissue remodeling. Also, persulfate-associated airway and skin diseases may share some of the underlying mechanisms. A list with nine proteins, for example, uteroglobin, IL1RN, and desmoplakin, that appears to be affected by the persulfate challenge was defined. These proteins should be followed-up in a new set of persulfate-exposed hairdressers with fully quantitative SRM assays using stable isotope-labeled peptides. It was also shown that peptides containing oxidized tryptophan

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may be useful as biomarkers for oxidative stress. However, that must be further evaluated.

■ ASSOCIATED CONTENT

Supporting Information

Raw data files have been uploaded to peptide atlas and can be viewed through the data set's official URL: http://www.peptideatlas.org/PASS/PASS00610. Data S1. Settings and development of the LC-SRM-MS system. Data S2. Protein pilot identification scores and annotated MS/MS spectra for oxidized peptides. Table S1. Transitions targeting 246 NLF proteins. Table S2. Transitions targeting oxidized peptides and 52 NLF proteins. Table S3. Quality of SRM assays for significantly changed proteins. Table S4. Protein changes induced by the nasal lavage procedure. Table S5. Canonical pathways and top three diseases and biofunctions mapped to statistically changed proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CV, coefficients of variation; DMBT1, deleted in malignant brain tumors 1 protein; IL-1, Interleukin-1; IL1RN, Interleukin-1 receptor antagonist protein; LC−MS/MS, liquid chromatography−tandem mass spectrometry; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLF, nasal lavage fluid; ROS, reactive oxygen species; SRM, selected reaction monitoring; WFDC2, WAP four-disulfide core domain protein 2

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