



LUND UNIVERSITY

Proteomics in Nasal Lavage Fluids from Persulfate Challenged Hairdressers

Mörtstedt, Harriet

2014

[Link to publication](#)

Citation for published version (APA):

Mörtstedt, H. (2014). *Proteomics in Nasal Lavage Fluids from Persulfate Challenged Hairdressers*. [Doctoral Thesis (compilation), Division of Occupational and Environmental Medicine, Lund University]. Division of Occupational and Environmental Medicine.

Total number of authors:

1

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Proteomics in Nasal Lavage Fluids from Persulfate Challenged Hairdressers

Harriet Mörtstedt

Division of Occupational and Environmental Medicine
Department of Laboratory Medicine
Faculty of Medicine
Lund University



LUNDS
UNIVERSITET

AKADEMISK AVHANDLING
som med vederbörligt tillstånd av Medicinska Fakulteten vid
Lunds universitet för avläggande av doktorexamen i medicinsk
vetenskap kommer att offentligens försvaras i Belfragesalen,
BMC, Lunds Universitet, 21 mars 2014 kl 09:15

Faculty opponent
Professor Jonas Bergquist
Department of Chemistry BMC, Analytical Chemistry, Uppsala University

Copyright © Harriet Mörtstedt

Division of Occupational and Environmental Medicine
Department of Laboratory Medicine
Faculty of Medicine
Lund University

ISBN 978-91-87651-54-0
ISSN 1652-8220

Tryckt i Sverige av Media-Tryck, Lunds universitet
Lund 2013



Med vishet kommer man långt,
med envishet kommer man längre.

(Okänd)

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hårfrisörer utsätts dagligen för en mängd kemikalier på arbetet. Många frisörer drabbas därför av luftvägsbesvär vilka ofta kan kopplas till hårblekningsmedel. Blekningsmedlen innehåller bland annat så kallade persulfater. Dessa tros vara en av huvudorsakerna till luftvägsbesvären. Flera studier har visat att persulfater kan ge symptom från luftvägarna såsom hösnuva eller astma. Det är dock oklart hur dessa symptom uppstår. Det saknas även bra metoder för att fastställa att en person lider av luftvägsbesvär orsakade av persulfater samt bra metoder för att mäta den mängd persulfat som en person inandats. Dessa brister gör att det blir svårt att både diagnostisera luftvägsbesvär orsakade av persulfater samt att arbeta i förebyggande syfte.

Näsan och dess slemhinna utgör det första försvaret mot de bakterier och kemikalier som vi dagligen andas in. Försvaret utgörs till stor del av proteiner. Dessa proteiner har förmåga att binda de ämnen som vi andas in, så att dessa kan transporteras ut ur luftvägarna. Proteinerna kan också, tillsammans med så kallade epitelceller i slemhinnan, bygga upp en barriär som motverkar att bakterier och andra farliga ämnen tar sig in i kroppen. I näsans slemhinna finns också olika sorters immunceller vilka styrs av en viss typ av proteiner.

En av flera mekanismer som tros ligga bakom luftvägsbesvären, är att persulfater oxiderar proteiner och andra strukturer i slemhinnan samt triggar immunceller till att producera så kallade fria syreradikaler, vilka också är oxiderande. Oxidation innebär att en eller flera syremolekyler adderas till proteinet. För många oxiderande processer i kroppen kan ge upphov till ett tillstånd som benämns oxidativ stress, något som kan orsaka vävnadsskada och celledöd.

Proteiner i näsans slemhinna kan samlas upp genom en sköljning av näsan med koksaltlösning. Genom att i denna nässköljvätska mäta mängden av olika proteiner, kan reaktioner i näsans slemhinna studeras efter att den exponerats för ett visst ämne. Mätningar av de oxiderade eller på andra sätt modifierade proteinerna kan ge ett mått på den mängd kemikalie som en person andats in.

Syftet med denna avhandling var att mäta de proteinförändringar som sker i näsan då slemhinnan kommer i kontakt med persulfat. Dessa proteinförändringar skulle

kunna bidra till en ökad förståelse om hur dessa luftvägsbesvär uppstår samt fungera som ett mått på den mängd persulfat en person andats in och/ eller på de reaktioner som satts igång.

I denna avhandling har en metod tagits fram för att mäta över 200 proteiner som finns i nässköljvätska. En metod har även tagits fram för att mäta ett mindre antal proteinoxideringar. Dessa metoder är baserade på så kallad masspektrometri. De framtagna metoderna samt en sedan tidigare befintlig metod användes till att mäta proteinhalter och proteinoxidation i nässköljvätskeprov. Nässköljvätskan erhöles från en tidigare studie där en persulfatlösning sprutades in i näsan på försökspersoner. Nässköljvätskan samlades in före och 20 min, 2h och 5h efter att försökspersonerna utsatts för persulfatlösningen. Frisörer med hörsnuva kopplad till blekmedel jämfördes med frisörer utan luftvägsbesvär samt med individer med hörsnuva men som inte utsattes för blekmedel i sitt arbete.

Resultaten visade att flera proteiner involverade i olika reaktioner i immunförsvaret, samt proteiner som utgör byggstenar i näsans slemhinna, förändrades efter att försökspersonerna utsatts för persulfatlösningen. Den exakta biologiska betydelsen av dessa fynd samt om något av dessa proteiner kan användas som ett mått på en reaktion orsakad av persulfat måste undersökas mer i framtida studier. Det framkom även att persulfater framkallade en viss typ av proteinoxidationer. Dessa typer av proteinoxideringar kan även framkallas av fria syreradikaler som bildas vid oxidativ stress. Denna typ av proteinoxidering skulle därför eventuellt kunna användas som ett mått för oxidativ stress.

LIST OF PAPERS

This thesis is based on the following papers, which are appended at the end of the thesis.

- I. Mörtstedt H, Jeppsson M.C, Ferrari G, Jönsson B.A.G, Kåredal M.H, and Lindh C.H. Strategy for identification and detection of multiple oxidative modifications within proteins applied on persulfate-oxidized hemoglobin and human serum albumin. *Rapid Communications in Mass Spectrometry*. **2011**; 25(2): 327-340.
- II. Kåredal M.H, Mörtstedt H, Jeppsson M.C, Diab K.K, Nielsen J., Jönsson B.A.G, and Lindh C.H. Time-Dependent Proteomic iTRAQ Analysis of Nasal Lavage of Hairdressers Challenged by Persulfate. *Journal of Proteome Research*. **2010**. 9(11): 5620-5628.
- III. Mörtstedt H, Kåredal M.H, Jönsson B.A.G, Lindh C.H. Screening Method Using Selected Reaction Monitoring for Targeted Proteomics Studies of Nasal Lavage Fluid. *Journal of Proteome Research*. **2013**. 12(1):234-47.
- IV. Mörtstedt H, Kåredal M.H, Diab K.K, Jacobsson H, Nielsen J, Jönsson B.A.G, Lindh C.H. Targeted proteomic analyses of nasal lavage fluid in persulfate challenged hairdressers with bleaching powder associated rhinitis. *Manuscript*. **2014**

ABBREVIATIONS

Frequently used abbreviations. For abbreviations of protein names, see Table 2.

DTT	Dithiothreitol
ESI	Electrospray ionization
Hb	Hemoglobin
HSA	Human Serum Albumin
IDA	Information-dependent data acquisition
iTRAQ	Isobaric tags for relative and absolute quantification
LMW	Low-molecular-weight
LC	Liquid chromatography
LOD	Limit of detection
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
NLF	Nasal lavage fluid
OA	Occupational asthma
OR	Occupational rhinitis
ROS	Reactive oxygen species
RP	Reversed phase
SRM	Selected reaction monitoring
TEMPO	2,2,6,6-tetramethylpiperidine 1-oxyl
QqQ/LIT	Triple quadrupole/linear ion trap
QqTOF	Quadrupole time-of-flight

CONTENTS

INTRODUCTION	1
General background	1
Biomarkers	1
Hairdressers and airway diseases	2
Proteins of the Nasal Mucosa	3
Proteomics	4
AIMS	7
METHODS	9
Nasal Lavage	9
<i>In vitro</i> Oxidations	9
Sample Preparation	10
Mass Spectrometry	10
MS-platforms	10
Protein Identification	11
Protein Quantification by iTRAQ-labeling	11
Protein Quantification by SRM	11
MS Analyses of Protein Oxidation	13
Study design	14
Persulfate challenge study	14
NLF Samples	14
Statistical analyses	15
Linear Mixed Model	15
Non-parametric methods	15
RESULTS AND COMMENTS	17
Protein Oxidation	17
Protein Abundances	20
SRM method targeting NLF Proteins	20
Proteomics in NLF from Persulfate Challenged Subjects	21
GENERAL DISCUSSION	25

Key findings	25
Strengths and Weaknesses	26
Strategy to detect and quantify protein oxidation	26
SRM method	26
Study Design	27
Biological Findings	28
CONCLUSIONS	31
FUTURE PERSPECTIVES	33
ACKNOWLEDGEMENT, Tack till...	35
REFERENCES	39

INTRODUCTION

General background

Airway diseases such as asthma and rhinitis are common and it has been estimated that 315 million people suffer from asthma worldwide and the number is even higher for rhinitis [1, 2]. In recent years, work-related asthma and rhinitis have received more attention as a public health concern due to a high prevalence and socioeconomic impact [3]. Many agents present at work are capable of inducing respiratory symptoms and each year new substances are reported [4]. For many low-molecular-weight (LMW) agents the underlying mechanisms are unclear and valuable biomarkers of effect are lacking which limits the possibility for diagnostics. Proteomics, *i.e.* the large-scale study of proteins[5], offer a wide range of powerful techniques that can be applied to almost any disease and/or toxicant to unravel important disease mechanisms and identify potential biomarkers.

Biomarkers

Biomarkers are important tools within research, health care and risk assessment. A biomarker can be defined as “*an indicator signaling an event or condition in a biological system or sample and giving a measure of exposure, effect, or susceptibility*” [6]. A biomarker of exposure is defined as a “*biomarker that relates exposure to a xenobiotic to the levels of the substance or its metabolite, or of the product of an interaction between the substance and some target molecule or cell that can be measured in a compartment within an organism*”, a biomarker of effect is defined as a “*biomarker that, depending on its magnitude, can be recognized as associated with an established or possible health impairment or disease*” and a biomarker of susceptibility is defined as a “*biomarker of an inherent or acquired ability of an organism to respond to exposure to a specific substance*” [6]. Biomarkers of exposure and effect together reflect all events from the initial exposure to the early and late biological changes ultimately resulting in disease, while biomarkers of susceptibility reflect effect-modifying factors that reduce the resistance towards the chemical compound and thereby predisposing

the individual for the disease [7]. A good biomarker should be chemically stable during storage and analysis, not prone to artifactually induced modifications, present at detectable levels, specifically and quantitatively correlated to the event or condition it indicates, have low within-individual variations, and measured with a simple and cost-efficient method that is specific, sensitive, reproducible and non-invasive [7-10].

Hairdressers and airway diseases

Several studies have identified hairdressing as a risk occupation for developing symptoms from the airways such as asthma and rhinitis [11-17]. Two recent studies showed that in women, hairdressing is one of the occupations with highest risks for developing occupational asthma [16, 17]. Occupational rhinitis (OR) and occupational asthma (OA) are inflammatory diseases of the upper and lower airways, respectively, that are caused by agents at the workplace. These diseases are characterized by variable airflow obstruction, hypersecretion and hyperresponsiveness, with airway symptoms present or worsened at work [18, 19].

Persulfate salts, which are active ingredients in hair bleaching products, have been recognized as major causal agents for airway symptoms in hairdressers [11, 13, 16, 20] (Figure 1). These products, which can be present at concentrations up to 60% in hair lighteners, are used as oxidizing agents to accelerate the bleaching process of peroxide hair bleaches. There are three types of persulfate salts used in hair bleaching products; ammonium, potassium and sodium persulfate. It is assumed that toxic effects of persulfates will primarily come from the persulfate anion, which is likely to generate hydrogen peroxide and sulfate ion radicals [21].

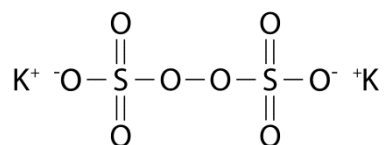


Figure 1. Chemical structure of potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$).

Diagnostic tools for persulfate-induced asthma and rhinitis as well as risk assessment tools for persulfate exposure are limited [20, 22-24]. This makes diagnostic and preventive work challenging and many cases of OA and OR may be overlooked or diagnosed late. Persulfate exposure has been assessed in a few studies by monitoring persulfate air levels [12, 25, 26]. However, no biomarkers

for persulfate exposure are available. A part of the diagnostic work is to establish a causal relationship between persulfate and respiratory symptoms. The golden standard methods for establishing these relationships are currently specific nasal provocation tests or specific bronchial provocation tests [3, 20, 23]. However, specific bronchial provocation tests are relatively expensive, have limited availability and can be risky inducing severe asthmatic reactions. There is also a lack of standardized procedures for nasal provocation tests [3].

Similarly to many other LMW chemicals, the pathogenesis for persulfate-associated airway symptoms is largely unclear. Most studies agree upon the involvement of an immunologic mechanism, but no consensus has been reached regarding the details [20, 24, 27-29]. The clinical picture is similar to the classical IgE-mediated airway diseases and an IgE-mediated mechanism has thus been proposed [27, 28, 30]. Some studies [27, 28, 30] have been able to detect positive skin prick tests against persulfates in a proportion of the patients while, in other studies, skin prick tests were negative for all patients [20, 24, 29]. Other studies have indicated the involvement of T-lymphocytes [24, 31]. Diab *et al* found that persulfates induced a shift towards Th1 cells in hairdressers with bleaching powder associated rhinitis [24].

Also, persulfates can at high concentrations induce airway symptoms through an irritative mechanism [22]. Furthermore, persulfates have been suggested to work as exogenous pro-oxidants, directly acting on cells and proteins and shifting the redox balance towards a state of oxidative stress [32]. The oxidizing salts have been shown to induce histamine release from mast cells [33], induce generation of reactive oxygen species (ROS) from mast cells and basophils and oxidize proteins *in vitro* [32, 34]. Oxidants can induce and/or promote airway inflammation and airway hyperresponsiveness [35, 36]. Oxidative stress is considered to be an important player in the pathogenesis of asthma and rhinitis [35-37].

Proteins of the Nasal Mucosa

The respiratory system is in constant contact with the air and thereby potentially pathogenic microorganisms and noxious substances. The nose and the nasal mucosa are the first line of defense towards potentially harmful environmental agents. In the nose, there is a coarse filter consisting of hair and mucus, which prevents material of larger size to enter the airways [38]. The mechanical barrier, which consists of ciliated columnar epithelial cells linked together with tight junctions and covered with a mucus layer, protects against inhaled pathogens and toxic substances through the actions of the mucociliary clearance system. Thus, pathogens and foreign substances are trapped in the mucus and are then removed

from the airways by the rhythmic beating cilia [39, 40]. The upper layer of the mucus mainly consists of water, ions and cross-linked mucins while the lower layer, which is less viscous, contains proteins with a wide range of properties important to innate immunity *e.g.* antimicrobial, antiprotease/protease, antioxidant, anti-inflammatory and detoxifying properties [41-43]. The mucus also contains immunoglobulins that are key players of adaptive immunity [44]. The mucus proteins are primarily produced by goblet and ciliated cells of the epithelium and by mucus and serous cells of the submucosal glands. Aside from the proteins secreted by epithelial and gland cells, the mucus also contains plasma and salivary proteins as well as proteins produced by immune cells [45].

Collection of nasal lavage fluid (NLF) is a simple and relatively noninvasive technique to obtain proteins originating from the nasal mucosa. Samples can be collected repeatable over relatively short time periods. Therefore the method is especially useful in provocation studies where nasal responses to various stimuli are assessed by analyzing samples collected before and after provocation [46]. Since NLF is obtained by a washing of the nasal epithelium lining it contains secreted proteins from the cells of the nasal mucosa, plasma proteins and proteins that have been cleared from the airway mucosal tissue [45, 47, 48].

Proteomics

Proteins are involved in most biological processes and pathways in the human body. Thus, studies of the proteome can give a better understanding of disease pathogenesis. Several proteomic studies of samples originating from the nasal mucosa have been conducted to map the proteins of the upper airways and assess proteomic changes associated with different exposures and/or airway diseases [48-60]. All of these studies used untargeted approaches, in where the proteins of interest are *a priori* unknown and an attempt is made to measure as many proteins as possible in the sample. An important advantage of such strategies is the potential to discover novel proteins. However, comprehensive proteome discovery experiments using mass spectrometry (MS) often require extensive sample prefractionation and they are therefore often time-consuming and labor-intense limiting the number of samples that can be analyzed [61-63]. Another important weakness of the untargeted approaches is their limited capability to reproducibly monitor proteins in complex samples [61, 62]. On the other hand, targeted MS-based quantification, which is mainly performed by selected reaction monitoring (SRM), can reproducibly quantify proteins in complex samples with high throughput. It has a wider dynamic range and a lower limit of detection (LOD) than the discovery-based quantification [61, 62, 64-66]. Aside from the obvious limitation that no new proteins are detected by this method, the main limitation of

SRM has been the large amount of time, effort, and money required to develop high quality SRM assays [67]. Traditionally, the targeted method has therefore been used to measure smaller sets of proteins. However, in recent years, software tools for building and optimizing SRM assays have emerged to facilitate the development of SRM assays [62, 68, 69] and the interest for applying SRM on a broader scale has rapidly increased [69]. In the last five years, an enormous enhancement in the SRM technology has been made both in terms of accuracy and number of proteins being monitored [70].

AIMS

General aims:

- i) To map proteomic changes in the nasal proteome of hairdressers challenged with persulfates to reveal potential underlying mechanisms and/or identify candidate biomarkers for persulfate exposure and effect.
- ii) To examine if and how persulfates oxidize proteins *in vitro* and to study if these modifications can be detected *in vivo* and possibly be used as biomarkers for persulfate exposure or oxidative stress.

Specific aims:

- i) To develop a strategy for identification and quantification of oxidative modifications and apply the strategy on proteins oxidized *in vitro* with persulfate.
- ii) To investigate if oxidized peptides can be detected in NLF samples from the persulfate challenged subjects and possibly be used as biomarkers for persulfate exposure or oxidative stress.
- iii) To develop a high throughput targeted method for quantification of NLF proteins.
- iv) To measure proteomic changes in NLF from persulfate challenged subjects using an untargeted approach in pooled samples and a targeted high throughput method in individual samples.

METHODS

Nasal Lavage

NLF samples analyzed in the papers were obtained from a previous persulfate challenge study [24] or from volunteers as described by Diab *et al* [24] (Figure 2).



Figure 2. Collection of nasal lavage fluid.

In vitro Oxidations

Hemoglobin (Hb), human serum albumin (HSA; **paper I**) and pooled NLF samples (**paper IV**) were incubated with potassium persulfate at a molar ratio of 1:10 (protein/[K₂S₂O₈]). The oxidants were removed by dialysis (**paper I**) or desalting (**paper II**).

Sample Preparation

Since high concentrations of salt can interfere with enzymatic digestion and electrospray ionization (ESI), all samples were desalted prior to trypsin digestion. Full-length Hb samples were purified prior to MS analysis by the use of C₄ ZipTips (**paper I**).

To identify more proteins, NLF samples were depleted of six high-abundant proteins (HSA, immunoglobulin G and A, transferrin, haptoglobin, and antitrypsin) using multiple affinity removal spin cartridge (**paper II**).

Trypsin, which cleaves the protein C-terminal of arginine and lysine, was used for enzymatic digestion in all papers. In order for trypsin to efficiently cleave the protein into peptides, the three-dimensional structure of the protein has to be interrupted. Therefore, prior to trypsin digestion, proteins were denatured using heat, and disulfide bonds were reduced with dithiothreitol (DTT; **paper I, III and IV**) or tris(2-carboxyethyl)phosphine (TCEP; **paper II**). To prevent disulfide bonds from reforming, the cysteines were alkylated with iodoacetamide.

Offline fractionation (**paper I and III**) by reversed phase liquid chromatography (RPLC) was used in **paper I and III** to reduce sample complexity and thereby ion suppression in MS analysis.

Mass Spectrometry

MS-platforms

Two MS platforms were used for data acquisition. The first MS platform was a QSTAR pulsar hybrid quadrupole time-of-flight mass spectrometer (QqTOF-MS; Applied Biosystems/Sciex) with a nanoelectrospray source connected to an LC system with a capillary and a nano pump (1100 series, Agilent Technologies) and the second MS platform was a 5500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer equipped with a TurboIonSpray source (QqQ/LIT-MS; Applied Biosystems/Sciex) coupled to an online LC system (UFLCXR; Shimadzu Corporation). ESI was used in both MS systems for ionization of peptides. Online RPLC was used for peptide separation in all papers. In **paper II**, to improve peptide separations multidimensional protein identification technology (MudPIT) was applied by combining strong cation-exchange with nanoRPLC.

Protein Identification

In **paper III**, pooled NLF digests were analyzed with nanoLC-QqTOF-MS and LC-QqLIT-MS. Both fractionated and unfractionated samples were analyzed on the QqTOF instrument. Data acquisition was conducted in information-dependent data acquisition (IDA) mode. MS scans were followed by MS/MS scans for the two or three most intense peaks. Proteins were identified by database searching using the Protein Pilot software. Proteins with an unused protein score of >1.3 (confidence 95%) were selected for further analysis.

In **paper III** and **IV**, SRM-triggered MS/MS scans were used to demonstrate absolute analyte specificity of SRM assays. The samples were analyzed with LC-QqQ/LIT-MS in IDA mode. Scheduled SRM acquisition was followed by MS/MS scans for the three most intense peaks. The resulting MS/MS data was analyzed using the Protein Pilot software. Peptides with a peptide score >1.3 (confidence >95%) were considered identified. Oxidized peptides with a peptide score >1.0 (confidence >90%) were considered identified or when manually sequenced, the entire peptide sequence had to be covered by the b- and y-ions together.

Protein Quantification by iTRAQ-labeling

In **paper II**, protein quantification was conducted using isobaric tags for relative and absolute quantification (iTRAQ) labeling. The NLF digests were labeled using 4-plex iTRAQ reagents and the samples were then analyzed with 2D-nanoLC-MS/MS in IDA mode. MS scans were followed by MS/MS scans for the three most intense peaks. Protein identification and quantification was conducted using the Protein Pilot Software. Proteins with an unused protein score of >2.0 (confidence 99%) were considered identified. The 20 min, 2h and 5h time-point were related to the baseline sample and data was normalized dividing each ratio with the bias correction factors (the median value of all ratios). This normalization accounts for variations in sample handling and it is based on the assumption that the majority of the proteins do not change in abundance.

Protein Quantification by SRM

SRM assay development

In **paper III** an SRM method was developed. Protein selection was based on a literature study with proteomic studies of samples originating from the nasal cavity, and on experimentally obtained data. The software Skyline was used to build and optimize the assays.

Selection of peptides and transitions was based on a public MS/MS library, on experimentally obtained MS/MS data and by systematically testing numerous peptides. Only tryptic peptides, unique to the target protein and with a length of 6-25 amino acids, were selected. To ensure reliable quantification the intention was to include numerous peptides per protein. If possible, five peptides per protein were selected for further analysis. To minimize the risk of monitoring the wrong peptide, numerous co-eluting transitions per peptide were used. Transitions with doubly or triply charged precursor ions and y- and b-ion fragments in m/z range 300-1250 were selected. Unscheduled SRM assays were first acquired to obtain peptide retention times and scheduling was then applied to minimize the number of transitions simultaneously searched for. All precursor ions with signal intensities <500 counts were discarded and three to five transitions per peptide were kept. The ones with the highest intensities were selected. Peptide selection and data acquisition was repeated several times since many peptides were discarded. In the next step, different parameters affecting sensitivity and/or accuracy *e.g.* collision energies, declustering potentials, SRM detection window, and target scan time, were optimized.

Protein Quantification by SRM

The SRM method developed in **paper III**, was slightly modified in **paper IV** and was then applied on the samples from the persulfate challenge study. The final SRM method contained 2166 transitions targeting 715 peptides from 246 proteins. The SRM method was split into four scheduled MS/MS methods containing about 540 transitions each. Each peptide assay contained three to four transitions. The SRM detection window and target scan time were set to 90s and 4s and the total run time per MS/MS method was 32 min. The peaks from the transitions were integrated and mean total peak areas of duplicate analyses were calculated. Peptide ratios 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 ratios were calculated as the median of the peptide ratios of a protein. Median values were used since they are more robust against outliers than mean values[70]. Finally, normalization was applied to the data by dividing each protein ratio with a correction factor. The median of all protein ratios for a subject and for each time-point was calculated and used as the correction factor. 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.

MS Analyses of Protein Oxidation

Detection of Protein Oxidation by MS

In **paper I**, to investigate if any oxidations had been induced, samples of full-length oxidized Hb were analyzed with QqTOF-MS. Full MS scans were collected between m/z 400 to 1400 and the mass spectra with peaks corresponding to the multiply charged Hb ions were converted by a deconvolution algorithm into a mass spectrum containing masses of the Hb molecules. Also, to evaluate whether DTT reduced induced oxidations, full-length Hb, with and without DTT, were analyzed by QqTOF-MS.

Furthermore, tryptic digests of oxidized and non-oxidized samples were analyzed with nanoLC-QqTOF-MS to detect peptides that were potentially modified. The resulting data files were analyzed with two different software packages; MSight [71] and MoDetect, developed in-house. MSight was used to visualize the LC-MS data as two-dimensional (2-D) images where the y-axis represents the retention time and the x-axis represents the m/z values while MoDetect organizes the LC-MS data into a list of three columns containing retention time, m/z , and signal intensity of analyzed peptides. Potentially oxidized peptides were detected by comparing data from oxidized and non-oxidized samples.

Identification of Oxidized Peptides by MS/MS

In **paper I**, the peptides were fractionated offline prior to MS analysis and the fractions were then analyzed by QqTOF-MS. Also, to identify isomeric oxidized peptides, the oxidized trypsin digests were analyzed by nanoLC-QqTOF-MS. Peptides were fragmented by collision induced dissociation (CID) and full fragment ion spectra were then collected. Oxidized peptides were identified by manually sequencing the MS/MS spectra.

Quantification of Oxidized Peptides

In **paper I**, oxidized peptides were quantified by SRM in *in vitro* oxidized samples (Hb and HSA) and in NLF samples (five subjects pooled) obtained from the persulfate challenge study. Furthermore, in **paper IV**, oxidized peptides were quantified by SRM in NLF samples obtained from the same study. Also, in **paper I**, to evaluate if oxidation was artifactually induced, Hb samples were prepared with and without the mild oxidant 2,2,6,6-tetramethylpiperdine 1-oxyl (TEMPO). TEMPO can quickly oxidize transition metals, thereby inhibiting the generation of oxygen radicals[72]. The oxidation degree was then measured using the SRM assays targeting oxidized Hb peptides. Stability of the oxidized peptides from Hb and HSA was evaluated in **paper I** by measuring the oxidized peptides in the oxidized samples before and after 10 months of storage at -20°C .

In **paper I**, each peptide assay contained two transitions. Quantification was based on the peak of highest intensity. In **paper IV**, three transitions were used per peptide assay and total peak areas were used for quantification. In **paper IV**, absolute analyte specificity of the SRM assays was demonstrated by SRM triggered MS/MS. Oxidation degree in each sample was estimated as the ratio between the total peak area of the oxidized and the corresponding unmodified peptide.

Study design

Persulfate challenge study

NLF samples analyzed in **paper I, II** and **IV**, were obtained from a persulfate-challenge study conducted by Diab *et al*[24]. This study examined the effects of persulfate on the nasal mucosa and on immune cells. The nasal challenge was performed in two steps by spraying a potassium persulfate solution into the nasal cavity; first with 0.001% potassium persulfate and then again after 20 min with 0.01% potassium persulfate. A nasal lavage was performed twice before the challenge and then 20 min, 2h and 5h after the challenge. The first nasal lavage was performed to wash the nasal cavity and the second nasal lavage was performed to collect a baseline sample. Nasal symptoms were registered before, 15 min after the first and second challenge and 2h and 5h after the challenge. A total symptom score was then calculated for the different types of nasal symptoms (nasal blockage, secretions and number of sneezes). Three groups were studied; (i) female hairdressers with bleaching powder related nasal symptoms, (symptomatics,(S), N=15), (ii) female hairdressers without nasal symptoms (asymptomatics (WS), N=14) and (iii) atopic females with pollen-related rhinitis but without work-related exposure to bleaching powder (atopics (A), N=12). No asthmatics were included in the study. Before the nasal challenge, a skin prick test against 13 common allergens was performed for all study subjects.

NLF Samples

Samples from the persulfate challenge study were analyzed in **paper I, II** and **IV**. In **paper I**, oxidized peptides were analyzed in pooled samples from five selected individuals. In **paper II**, the iTRAQ technique in combination with 2D-nano LC separations was used, which is labor intensive, and therefore samples were pooled for each group and time point. In **paper II**, more stringent inclusion criteria were

used since the samples were pooled and individual information was thus lost. Also, in **paper IV**, one subject in the symptomatic group was excluded due to sample shortage.

Statistical analyses

Linear Mixed Model

In **paper IV**, linear mixed modeling was used to analyze the protein \log_2 -ratios since this statistical model allows dependent data and since different number of observations per subject can be included [73]. The assumption of normally distributed residuals was checked visually by plotting histograms for each protein. The \log_2 -ratios were analyzed within group (S, WS and A) at each time point (20 min, 2h and 5h) (nine statistical tests per protein). Benjamini and Hochberg correction was used to control for multiple testing since it offers more statistical power compared to traditional approaches e.g. Bonferroni procedures [74]. The proteins that remained statistically significant after the correction were selected for a more comprehensive analysis. Peak integration was reviewed more thoroughly. Three proteins were discarded due to weak signals or shifts in retention time of the peptide assays. The data for the remaining proteins was analyzed again with linear mixed modeling. The \log_2 -ratios were analyzed within group (S, WS and A) and time point (20min, 2h and 5h) as before but with a more careful selection of covariance structure for each protein individually. Differences in \log_2 -ratios between the groups at each time point (20 min, 2h, 5h) were also analyzed and Benjamini and Hochberg correction was used to control for multiple testing with the significance level set at 0.05. Sensitivity analysis was conducted to test the strength of the results. These analyses were conducted in three separate analyses; i) exclusion of smokers, ii) exclusion of hairdressers with positive skin prick test and subjects in the atopic group with negative skin prick tests, iii) exclusion of hairdressers in the symptomatic group with a symptom score change <3 and hairdressers in the asymptomatic group with a symptom score change >1 .

Non-parametric methods

In **paper IV**, the oxidized peptides were analyzed using non-parametric methods. Differences in oxidation degree between the groups at each time point (0, 20min, 2h and 5h) were analyzed using the Kruskal-Wallis H test. Differences between the baseline time point and the other time points (20min, 2h and 5h) were analyzed

using the Friedman and the Wilcoxon signed-rank tests. First data was analyzed within each group and then data was analyzed including all subjects without respect to group belonging. The significance level was set at 0.05 and Bonferroni correction was used to control for multiple testing. Sensitivity analysis was conducted as described above.

RESULTS AND COMMENTS

Protein Oxidation

In **paper I**, a strategy for identification and quantification of multiple oxidative modifications within proteins was developed and applied on persulfate oxidized Hb and HSA.

Full-length analyses of oxidized Hb indicated that persulfate induced oxidations (multiples of +16 Da) in both chains of Hb. Tryptic digest of oxidized proteins showed that persulfate induce oxidations to methionine (+16 Da and +32 Da) and tryptophan (+16 Da and +32 Da) residues. The oxidation of methionine and tryptophan by persulfate is in accordance with results from others [32, 75]. SRM assays targeting the oxidized peptides were developed and the quantitative result showed clear differences in oxidation degree between the *in vitro* oxidized and non-oxidized samples; fold changes up to 500 were seen. In accordance with other studies, Met⁵⁵ in the beta chain [76-79] and Met⁵⁴⁸ in HSA [80] seemed to be especially sensitive for oxidation.

In **paper IV**, new SRM assays targeting some of the oxidized peptides detected in **paper I** and a few additional peptides were developed and measured in the NLF samples from the persulfate challenge study. Assays targeting eleven oxidized peptides and their corresponding unmodified peptide from complement C3, fibrinogen alpha chain, HSA and Hb beta chain were developed. Numerous other proteins *e.g.* 1-antitrypsin, apolipoprotein A-I, and gelsolin, and modification types *e.g.* carbonylations, sulfinic and sulfonic acid, and side chain oxidations of a few amino acid residues, were selected from the literature [81-84] but we were only able to develop assays for peptides with methionine and tryptophan oxidations.

In contrast to Hird *et al* [34], who showed that persulfate oxidized cysteine, we were not able to detect any cysteine oxidations. This may be explained by the use of DTT, which is a strong reducing agent used to reduce disulfide bonds of the proteins. DTT reduces most types of cysteine oxidations except for sulfinic (+32 Da) and sulfonic acid (+48 Da) [85]. The full-length analysis of oxidized Hb, with and without DTT, indicated that some oxidations were reduced by DTT but not all. It is likely that these oxidations were cysteine oxidations.

Methionine and tryptophan have been shown to be susceptible for artificial oxidation, methionine during sample preparation, storage and ESI [86] and tryptophan oxidations during electrophoresis [87]. Therefore artificial oxidation was tested during sample preparation by the addition of the mild oxidant TEMPO. The result for Hb with and without TEMPO added was similar. We therefore concluded that artifactual oxidation by oxygen radicals generated from transition metals was not a major issue during sample preparation of Hb. Also, stability during storage was evaluated for the oxidized peptides. Similar results were obtained before and after 10 months of storage at -20°C suggesting that the modifications are stable.

In total, nine methionine residues and two tryptophan residues in HSA, the alpha- and beta chains of Hb, fibrinogen alpha chain or complement C3 were identified as oxidized (+16 Da and/or +32 Da) (Table 1). These proteins have been shown to be targets for oxidation and most of these oxidations have been shown to be induced by other oxidants as well [76-78, 80, 82, 83, 88, 89]. Thus, the induced modifications do not reflect any specific oxidation pathway by persulfate, which indicates that these modifications are not applicable as biomarkers for persulfate exposure.

In **paper IV**, six oxidized peptides were detected in the NLF samples from the persulfate challenged subjects. Oxidized peptides from all four proteins were detected but it was only HSA peptides that were detected in a majority of the samples. HSA is one of the most abundant proteins in NLF and therefore modifications in HSA are more easily detected. No significant differences in extent of oxidation were found between the groups for any of the six oxidized peptides. The oxidation degree for the HSA peptide AW(+32)AVAR was increased 2h and 5h after the challenge. Similar results were obtained for this peptide in **paper I**, when a subset of individuals from the same study was analyzed.

In **paper I**, peptides containing oxidized tryptophan eluting with different retention times but with the same transitions suggests the presence of isomeric structures. Oxidation of tryptophan residues results in a number of possible oxidation products; different isomers of hydroxytryptophan (+16 Da), oxindolylalanine (+16 Da), N-formylkynurenine (+32 Da), dioxindolylalanine (+32 Da) [90, 91]. The presence of different isomeric structures can result in a less accurate and precise quantification.

Table 1. Oxidation degree of oxidized peptides. Oxidized peptides were measured by SRM in *in vitro* oxidized Hb and HSA (**paper I**), and in NLF from a persulfate challenge study (**paper I** and **IV**). Samples were collected before and 20 min, 2h and 5h after the persulfate challenge. Pooled samples from five subjects were analyzed in **paper I**. Median oxidation degree values for all measured samples in **paper IV** are presented. ^anon-detected.

Protein	Peptide	[Control; Ox]	NLF from persulfate challenge study [0; 20min; 2h; 5h]	
		Paper I	Paper I	Paper IV
Complement C3	ILLQGTPVAQM(+16)TEDAVDAER, (Met968)	-	-	[0.6; 0.4; 0.4; 0.4]
Fibrinogen alpha chain	DSHSLTTNIM(+16)EILR, (Met91)	-	-	[2.0; 1.6; 2.3; 2.1]
Serum albumin	AW(+32)AVAR, (Trp214)	[0.004; 0.1]	[0.004; 0.005; 0.02; 0.01]	[0.008; 0.009; 0.02*; 0.02*]
	AW(+16)AVAR, (Trp214)	[0.005; 0.7]	[0.001; 0.002 ; 0.001 ; 0.001]	[nd ^a]
	ETYGEM(+16)ADCCAK, (Met87)	[0.1; 4.0]	[0.04; 0.05; 0.05; 0.05]	-
	LVRPEVDVM(+16)CTAFHDNEETFLK, (Met123)	[0.04; 3.0]	[0.06; 0.05; 0.06; 0.06]	-
	LVRPEVDVM(+32)CTAFHDNEETFLKK, (Met123)	[0.0007; 0.02]	[nd ^a nd ^a ; nd ^a]	-
	SHCIAEVENDEM(+16)PADLPSLAADFVESK, (Met298)	nd ^a	-	[0.6; 0.4*; 0.6; 0.6]
	DVFLGM(+16)FLYEYAR, (Met329)	nd ^a	-	[nd ^a]
	AVM(+16)DDFAAFVEK, (Met548)	[0.1; 50]	[0.05; 0.04; 0.05; 0.05]	[0.1; 0.1; 0.1; 0.2*]
AVM(+32)DDFAAFVEK, (Met548)	[nd ^a ; 0.4]	[nd ^a ; nd ^a ; nd ^a]	-	
Hemoglobin beta chain	LLVVYPW(+16)TQR, (Trp37)	[0.03; 0.2]	-	[nd ^a]
	LLVVYPW(+32)TQR, (Trp37)	[0.003; 0.04]	-	[nd ^a]
	FFESFGDLSTPDAVM(+16)GNPK, (Met55)	[20; 240]	-	[2.3; 1.9; 2.1; 2.3]
Hemoglobin alpha chain	VADALTNAVAHVDDM(+16)PNALSALSDDLHAHKLR, (Met76)	[0.3; 2.0]	-	-

Protein Abundances

SRM method targeting NLF Proteins

In **paper III**, an SRM method targeting 708 tryptic peptides representing 244 proteins was developed. Three to four transitions per peptide assay were used, which resulted in a total number of 2146 transitions. Among the 244 proteins, 207 of the proteins were represented by two to five peptides. The sample analysis run time was 45 min per method. In **paper IV**, to increase throughput, the LC gradient was modified leading to a reduction of the analysis time from 45 min to 32 min. Two additional proteins, identified in NLF in an unpublished untargeted LC-MS/MS experiment, were also added to the SRM method. The final SRM method contained 2166 transitions targeting 246 proteins.

Precision in peak areas for different runs on the same sample for the SRM method has been assessed in numerous ways (**paper III and IV**). The developed SRM method was reproducible and the majority of the peptide assays had within-run and between-run precisions <20%.

Accuracy of the method was evaluated by analyzing three samples and a reference sample prepared with varying and known amounts of HSA, serotransferrin, α -1-acid glycoprotein 1 and apolipoprotein A-I (apo A-I). The proteins were quantified using two to four peptides per protein. The vast majority of peptide and protein ratios were in good agreement with expected ratios, although a majority of the ratios were slightly higher than the expected ratios. Although this test was performed in a less complex matrix it still indicates an acceptable accuracy of the method.

LOD values for two peptides from apo A-I and two from lactotransferrin were in the lower fmol-range (0.3 fmol to 10 fmol on column). The result indicates a high sensitivity even though better sensitivity for SRM based analyses of plasma digests with limit of quantitation values down to amol-levels have been presented by others [92, 93].

Absolute analyte specificity was demonstrated by SRM triggered MS/MS. In total, 135 assays targeting peptides from 71 proteins have at this point been confirmed. In **paper IV**, only two additional peptides were confirmed compared to **paper III**. The concentrations of the other peptides may be too low for SRM triggered MS/MS scans to generate good quality spectra.

In **paper III**, the within-day variation of retention time was acceptable with a median shift of ± 3 sec. However, when the method was applied on the samples from the persulfate challenge study, considerably longer shifts in retention times were seen. The median retention time shift was ± 23 sec between days when the same column was used. Therefore, in **paper IV**, despite close monitoring of retention times a few peptides were missed in some of the samples.

Proteomics in NLF from Persulfate Challenged Subjects

In **paper II** and **IV**, subjects challenged with persulfate were studied. Hairdressers with bleaching powder associated rhinitis were compared to hairdressers without any respiratory symptoms and with atopic individuals with no work related exposure to persulfates.

In total, 99 proteins were detected in **paper II** and 244 proteins were detected in **paper IV**. Twenty-two proteins in **paper II** and 47 proteins in **paper IV** had changes that were statistically significant ($p < 0.05$) in at least one of the groups and time-points. Although, the changes were not statistically significant in both studies, several of the proteins presented similar trends in the ratios *e.g.* uteroglobin, WAP four disulfide core domain protein 2 (WFDC2), lipocalin-15, beta-2-microglobulin, deleted in malignant brain tumors 1 protein and cystatin-S. However, for several proteins the results in **paper II** and **paper IV** were not in line with each other *e.g.* apo A-I and lactotransferrin. Only four proteins presented changes that were statistically significant in both studies; reduced levels 5h after the challenge was seen for neutrophil elastase in the atopic group and beta-2-microglobulin and mucin-5B in the asymptomatic-group while increased levels 5h after the challenge was seen for zymogen granule protein 16 homolog B in the symptomatic group (Table 2).

In **paper IV**, a clear difference was seen between the groups regarding the number of proteins statistically significant changes. Thirty nine proteins in the asymptomatic group had changes that were statistically significant, compared to 13 for the atopic group and five for the symptomatic group. In **paper II**, approximately equally many proteins where changed in the three groups; nine in the symptomatic group, eleven in the asymptomatic group, and 13 in the atopic group.

Few very distinct changes were seen between the symptomatic group and the other groups. In **paper II**, the most pronounced differences were found for lactotransferrin and apo A-I. These findings were not reproduced in **paper IV**. In **paper IV**, distinct differences between the groups, although not statistically significant, were seen for mucin-5B, interleukin-1 receptor antagonist protein

(IL1RA), desmoplakin, Ig alpha-1 chain C region (IGHA1) glutathione S-transferase P (GSTP1), and triosephosphate isomerase (TPIS). Similar protein ratios were seen for mucin-5B in **paper II** and **IV**, except for one ratio. In **paper II** increased levels were seen for this protein in the atopic group after 20 min, while the results in **paper IV** did not indicate any change at all. The other proteins were not detected in **paper II** or as in the case of IGHA1 that was removed in the depletion step and could therefore not be compared.

Table 2. Significantly changed proteins in paper II and paper IV. NLF samples from a persulfate challenge study were analyzed with SRM (paper IV) and with 2D-nanoLC-MS/MS combined with iTRAQ-labeling (paper II). In the iTRAQ-study (paper II) samples were pooled and in the SRM study (paper IV) all samples were analyzed individually. The protein ratios are expressed relative to the baseline sample. *p<0.05. ^anon-detected. ^bEstimated marginal means.

Protein	Time	SRM ^b			iTRAQ		
		S	WS	A	S	WS	A
Apolipoprotein A-I (apo A-I)	20 min	1.0	1.5	1.1	5.1*	1.0	1.1
	2h	0.9	1.5	1.1			
	5h	1.5	1.7	1.8	1.0	1.4	1.2
Desmoplakin	20min	0.9	1.5	1.2	nd ^a	nd ^a	nd ^a
	2h	0.9	1.7*	1.1			
	5h	1.0	1.8*	1.1	nd ^a	nd ^a	nd ^a
Extracellular glycoprotein lacritin	20 min	1.2	1.7	1.1	2.5*	2.2	1.3*
	2h	1.4	1.5	1.2			
	5h	1.6	1.3	2.7	1.3	6.6*	3.2*
Glutathione S-transferase P (GSTP1)	20min	0.8	0.7*	0.7	nd ^a	nd ^a	nd ^a
	2h	1.0	0.6*	0.8			
	5h	0.8	0.7	0.7*	nd ^a	nd ^a	nd ^a
Ig alpha-1 chain C region (IGHA1)	20min	1.0	1.3	1.3	nd ^a	nd ^a	nd ^a
	2h	0.9	1.5	1.3			
	5h	1.2	1.7*	1.3	nd ^a	nd ^a	nd ^a

Protein	Time	SRM ^b			iTRAQ		
		S	WS	A	S	WS	A
Interleukin-1 receptor antagonist protein (IL1RA)	20min	0.9	1.3*	1.1	nd ^a	nd ^a	nd ^a
	2h	0.8	1.3	1.1			
	5h	0.9	1.4*	1.1	nd ^a	nd ^a	nd ^a
Lactotransferrin	20 min	1.2	1.0	1.0	0.5*	0.9	0.9
	2h	1.6	1.2	1.1			
	5h	1.3	1.0	1.1	1.1	1.2*	1.1
Mucin-5B	20min	1.4	1.1	0.9	1.7*	0.9	1.5*
	2h	1.5	0.9	0.8			
	5h	1.1	0.6*	0.7	1.4*	0.6*	0.8
Polymeric immunoglobulin receptor	20min	1.1	1.1	1.3	1.1	1.0	1.0
	2h	1.0	1.1	1.1			
	5h	1.1	1.5*	1.2	1.0	1.1	1.1
Triosephosphate isomerase (TPIS)	20min	1.1	0.8	0.9	nd ^a	nd ^a	nd ^a
	2h	1.0	0.7*	0.9			
	5h	0.9	0.7*	0.6*	nd ^a	nd ^a	nd ^a
Uteroglobin	20min	0.6	0.5*	0.6	0.9	0.6	1.0
	2h	0.5*	0.3*	0.5			
	5h	0.4*	0.3*	0.4*	0.5	0.5	0.8
WAP four-disulfide core domain protein 2 (WFDC2)	20min	0.9	0.7	0.9	0.5	0.2	0.3
	2h	0.7	0.5*	0.8			
	5h	0.6	0.5*	0.7	0.4	0.2	0.2

GENERAL DISCUSSION

Key findings

A powerful, high throughput screening method for quantification of 246 NLF proteins was developed using SRM. The method has repeatedly been shown to hold acceptable within-day precision and between-day precision (CV ~20%) for a majority of the assays. The method presented LOD values in the higher amol to lower fmol-range indicating acceptable sensitivity, a dynamic range around four orders of magnitude and acceptable accuracy.

A strategy to detect and quantify multiple protein oxidations was developed and applied on persulfate oxidized proteins. It was shown that persulfate induced oxidations to methionine and tryptophan. SRM assays targeting such peptides were developed and measured in NLF samples from persulfate challenged subjects. The oxidation degree increased after the persulfate challenge for a peptide containing doubly oxidized tryptophan. However, since the reaction *in vivo* was not immediate, it probably does not reflect persulfate exposure but it may be suitable as a biomarker for oxidative stress. These modifications were stable and detectable *in vivo* and can be measured with a high throughput method *i.e.*, non-invasive, reproducible and can measure modifications in a multiplex manner.

In **paper II** and **IV** proteomic methods were applied to study subjects challenged with persulfate. Hairdressers with bleaching powder associated rhinitis were compared to hairdressers without any respiratory symptoms and to atopic subjects without any work-related persulfate exposure. The studies showed that several proteins associated with biologically relevant effects were changed *e.g.* epithelium damage and repair, innate and acquired immunity, inflammation of the mucosa and oxidative stress.

Strengths and Weaknesses

Strategy to detect and quantify protein oxidation

A strategy was developed to detect and identify oxidative modifications in **paper I**. The capability of detecting multiple modifications without any prior knowledge is an important advantage of this method. However, despite a rather high concentration of persulfate used for *in vitro* oxidation, only peptides containing oxidized tryptophan and methionine were identified. Detecting oxidative modifications by MS is challenging since the modifications often are present at very low concentrations and are chemically unstable. Different enrichment strategies and/or derivatization procedures are therefore frequently used when studying oxidative modifications [94]. Thus, it is possible that other types of modifications were induced but were missed due to low-abundance or labile nature. Methionine and tryptophan have been shown to be susceptible for artifactual oxidation [86, 87]. Even if it was shown that artifactual oxidation by oxygen radicals generated from transition metals was not an issue, artifactual oxidation may still be induced by some other pathway. The developed SRM assays showed acceptable precision (<20%) and oxidized peptides were consistently detectable in NLF samples without prior enrichment. Furthermore, they were stable during storage. This method offers simplicity and multiple modifications can be assessed in one analysis.

SRM method

The most important strength of the developed SRM method is that it can be applied to measure a high number of proteins in many samples. It has been shown to be reproducible, accurate and have a wide dynamic range. A main limitation of using SRM to target many proteins is the time and labor demanding process to develop the assays. Also, the SRM analyses conducted in **paper IV**, generated a lot of data, which was manually reviewed. Thus, more efficient software tools for method development and data analysis is desirable.

A shortcoming of this method is the lack of evidence for absolute analyte specificity for many of the assays. On the other side, most proteins are represented by at least two peptide assays and when these assays are in line with each other, the result can still be reliable.

Label free methods usually offer simplicity in terms of fewer sample preparation steps and many samples can be analyzed. However, the need for normalization increases as more samples are analyzed. In **paper IV**, a global normalization

approach was applied to control for variations in sample handling and instrument operation. This type of normalization is rather rough and you risk to lose 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 normalization. To maintain a high throughput and to reduce the sources of variation, sample preparation was kept as simple as possible. This approach was successful when fewer samples were analyzed, but when more samples were analyzed larger variations in retention times appeared. One possible explanation for this may be the lack of a second clean-up step after trypsin digestion and prior to LC-MS/MS analysis.

Study Design

The samples analyzed in **paper II** and **IV**, were obtained from a previous persulfate challenge study conducted by Diab *et al* [24]. In this study, the subjects were nasally provoked with potassium persulfate and NLF samples were collected before and after the challenge. Since each individual is their own control, biological variations are reduced and statistically significant differences are easier to find. The use of two control groups is a clear advantage, one group with hairdressers without respiratory symptoms and one group without any work related exposure to persulfates but with an expected, more sensitive nasal mucosa than healthy individuals. The atopic group may therefore nonspecifically react to persulfate and this group can be used to distinguish between specific and non-specific nasal reactions induced by the persulfate.

The most important limitations in study design were the non-homogenous study groups. Both hairdresser groups contained smokers and subjects with positive skin prick tests, while the atopic group contained subjects with negative skin prick test. Atopy is a well-recognized risk factor for occupational airway diseases when caused by high molecular weight agents but when caused by LMW agents the association is less clear and the result is conflicting. In addition, the relationship between smoking and occupational airway diseases is controversial [95]. We tried to evaluate the strength of the result by conducting a sensitivity analysis but we did only check if the results remained and not if any new appeared. It is likely that some interesting findings were missed but a high number of proteins were analyzed in this study and therefore there was a need for a rather strict approach in selecting the most prominent findings. From a statistical point of view the sample size was rather small. However, as a challenge and proteomics study it is large.

Biological Findings

There were three proteins that had statistically significant changes in one of the studies and with similar trends in the other study. Moreover, they showed the same trend in all three groups. Decreased levels were seen for the two proteins uteroglobin (Clara cell protein) and WFDC2 in all groups. They are produced by different cells in the upper airways *e.g.* epithelial cells, mucous cells and/or cells in the submucosal glands [96, 97]. Uteroglobin has been suggested as a biomarker of respiratory epithelial damage after exposures to airway irritants [98] and decreased levels of the WFDC2 have been associated with de-differentiation of bronchial epithelial cells [96]. The changes in these proteins may suggest that an epithelial damage was induced by the challenge. In addition, increased levels were seen for extracellular glycoprotein lacritin. This protein, which is secreted from lacrimal and salivary glands [99], has been suggested to promote epithelial proliferation [100] and may thus promote tissue repair.

Only a few distinct changes were seen between the symptomatic hairdressers and the other two groups. In **paper II**, increased levels of apo A-I and decreased levels of lactotransferrin were seen exclusively in the symptomatic group 20 min after the challenge. We were not able to reproduce these findings in **paper IV**. Apo A-I, which is the major protein of high density lipoprotein, has been shown to have antioxidant and anti-inflammatory properties [101]. It has been shown to be positively associated with allergic rhinitis and the increased levels seen among subjects with allergic rhinitis has been suggested to reflect an inflammation in the nasal mucosa [102]. Lactotransferrin is considered to be a part of host defense due to its antibacterial capabilities [103]. Even though conflicting results were obtained for these proteins in our two studies, they are biologically relevant and should be further analyzed in future studies.

In **paper IV**, differences between the groups were found for mucin-5B, IL1RA, desmoplakin, IGHA1, GSTP1, and TPIS. Unfortunately, among these proteins it was only mucin-5B that was detected in **paper II**.

Both papers presented increased levels of mucin-5B in the symptomatic hairdresser group while unchanged or decreased levels were seen in the asymptomatic hairdresser group. The result was contradictory for the atopic group at the 20 min time point; in **paper II** the protein was significantly increased and in **paper IV** the result indicated unchanged concentrations, but at the 5h time point ratios indicated decreased levels. Mucin-5B is produced by mucous cells in the submucosal glands. It has been suggested to be involved in the clearance of specific pathogens or airway irritants [41]. A balanced mucus production is important for a normal lung function and an overproduction of mucus has been associated with many airway diseases such as asthma, chronic bronchitis, cystic

fibrosis, chronic obstructive pulmonary disease and chronic rhinosinusitis [41, 104]. The changed levels in this protein probably reflect the nasal symptoms induced by the challenge in the different groups.

Increased levels of IL1RA was seen in the asymptomatic hairdresser group. This protein inhibits the activity of Interleukin-1 (IL-1 and IL-1), which has been shown to play crucial roles in acute and chronic inflammation and is fundamental to innate immunity [105]. In addition, this protein has been proposed as therapeutic target for bronchial asthma [106]. Thus, it is likely that IL1RA has a protective role towards persulfate induced rhinitis.

Another protein that had a statistically significant increase in the asymptomatic hairdresser group but was unchanged in the other two groups was desmoplakin. Different adhesive protein complexes, such as tight junctions and desmosomes, connect the epithelial cells to each other and to the surrounding tissue. These protein complexes play vital roles in maintaining the structural integrity of the epithelium [107]. Desmoplakin is one of the components of the desmosomes [108]. The results may reflect an enhanced tissue repair among asymptomatic hairdressers compared to the other two groups.

Statistically significant increased levels of IGHA1 and the closely related polymeric immunoglobulin receptor (PIGR) were seen in the asymptomatic hairdresser group after the challenge. The adaptive immune mechanisms of the upper airway mucosa are mainly mediated by antibodies belonging to the secretory immunoglobulin A (IgA) [44]. IgA in complex with PIGR is secreted into the nasal lumen where it binds to infectious agents and toxic substances thereby preventing them from penetrating the mucosa [44]. Decreased levels of IgA and PIGR have been associated with allergic airway diseases [109, 110]. Thus, the increased levels of IgA and PIGR may indicate a protection against persulfate-induced rhinitis.

When persulfates are inhaled they may induce an inflammatory response leading to oxidative stress. It is also generally recognized that oxidative stress is associated with allergic diseases such as rhinitis and asthma [37]. Decreased levels of proteins linked to resistance towards oxidative stress were seen in asymptomatic and atopic groups, but not among symptomatics. GSTP1 is an antioxidant enzyme catalyzing the conjugation of reduced glutathione with various electrophilic compounds [111]. The decreased levels of TPIS probably reflect a metabolic reconfiguration, which is a known adaptive response to oxidative stress. It has been shown that a decreased activity of this glycolytic enzyme results in resistance against oxidative stress [112]. Thus, the decreased levels of TPIS in these groups may be linked to a protection towards oxidation mediated by persulfates or inflammation. In **paper IV**, the oxidation degree of the albumin peptide

AW(+32)AVAR increased 2h and 5h after the challenge indicating an endogenous oxidative stress induced by the persulfate challenge.

CONCLUSIONS

From this thesis the following conclusions can be drawn:

- i) A strategy for identification of multiple oxidative modifications within proteins was developed and SRM was used for quantification of oxidized peptides in NLF samples. This approach demonstrates the usefulness of SRM as a method for targeting multiple oxidative modifications simultaneously.
- ii) A high throughput screening method for quantification of 246 NLF proteins was developed using SRM. The method is reproducible and it can be applied to analyze many samples. However, some improvements are needed *e.g.* internal standards, sample clean-up and softwares for data analysis.
- iii) Persulfates induced oxidizations to tryptophan and methionine residues *in vitro*. Both types of modifications were detectable *in vivo* but only tryptophan oxidations are stable enough *in vivo* to be suitable as a biomarker. Peptides containing oxidized tryptophan are probably not suitable as biomarkers for persulfate exposure due to poor specificity. Instead they may be useful as biomarkers of oxidative stress.
- iv) Several proteins with biologically relevant functions were significantly changed after the persulfate challenge in one of the studies or in both. The significantly changed proteins were associated with epithelium damage and repair, innate and acquired immunity, inflammation of the mucosa and oxidative stress. Some of them seem to be involved in mechanisms of toxicity and may be useful as early or late effect biomarkers while others may have a protective function. The exact role of these proteins in relation to persulfate induced respiratory symptoms needs to be further studied as well as the usefulness of them as effect biomarkers.

FUTURE PERSPECTIVES

A group of proteins were defined that appears to be affected by the persulfate challenge. The exact role of these proteins in relation to persulfate induced respiratory symptoms needs to be further studied as well as the usefulness of them as biomarkers of effect, in a new group of subjects challenged with persulfate.

In addition, different LMW agents may induce airway symptoms by similar mechanisms and therefore these proteins should also be measured in subjects exposed to other LMW agents.

The SRM method can be applied to other proteomic studies of the upper and potentially also the lower airways. Some of the assays in this method can undergo a more rigorous validation and can then be further developed into fully quantitative assays.

The peptide containing oxidized tryptophan should be further evaluated as an oxidative stress biomarker.

Other oxidized peptides that can be used to study oxidative stress should be investigated using similar methods as in the present thesis.

ACKNOWLEDGEMENT, Tack till...

Mycket har hänt sedan jag kom ner till Skåne en höstdag i september. Jag kom från ett trevligt labb till ett annat, från en trevlig dialekt till en annan, från en fin stad till en annan och framförallt, från en massa fina människor till en massa nya fina människor. Jag vill rikta ett stort TACK till er som under dessa år har funnits runt omkring mig och bidragit med skratt, kunskap och stöttande ord:

Christian Lindh för att du låtit mig jobba självständigt men ändå funnits där när jag har behövt hjälp. För att du har uppmuntrat mig och fått mig att känna det som om jag gjort ett bra jobb. För att du har varit otroligt stöttande när jag har drabbats av tidsbrist och paniken lyst i mina ögon. För att du inte bara har sett till prestation utan även visat omtanke när det ibland har känts hopplöst.

Bo Jönsson för att du har hjälpt mig otroligt mycket med att strukturera mina tankar och idéer och få ner det i text. För att du hjälpt mig att sålla bort alla detaljer som jag gillar att läsa och skriva om. För att du liksom jag gillar helgarbete. För att du en gång i tiden sa åt mig att gå och ta en öl istället för att vara besviken över en halvtidskontroll som inte gick som jag ville.

Monica Kåredal för att du alltid tar dig tid till att lyssna. För att du alltid har ett så öppet och välkomnande sätt som gör att man gärna kommer in på ditt rum och slår sig ner för en diskussion. För att du ställer frågor som får en att tänka till. För att du bjuder hem mig på spaghetti och köttfärssås när jag inte har haft tid till att äta.

Helene Jacobsson för en massa hjälp med statistik och för att du har svarat på alla mina frågor med stort tålamod.

Kerstin Kronholm Diab och **Jörn Nielsen** för er stora hjälpsamhet med allt som rör frisörstudien.

Eva Assarsson, Pia Tallving och **Else Åkerberg** för all hjälp med nässköljningar.

Anna Axmon för hjälp med statistik och för att du har förklarat statistiska begrepp på ett lättillgängligt sätt.

Yulia Lindholm för all administrativ hjälp, speciellt i samband med en resa som varade lite längre än planerat p.g.a. snökaos i England.

Heléne Åkesson för en massa hjälp med beställningar och felanmälan av instrument.

Giovanni Ferrari för ett fint samarbete i början av min doktorandtid och för hjälp med krånglande datorer.

Ralf Rittner för hjälp med krånglande datorer.

Till alla ni som är med i **limporna** och gör tisdagarna lite mer speciella.

Till alla på **labbet** som gör det till en så trevlig plats att vara på.

Thanks to **Ayman Alhamdow, Shegufta Ameer, Moosa Faniband, Bakhtiar Hossain, Huiqi Li** and **Gerda Rentschler** for bringing the world and a very nice atmosphere to the lab.

Helen Karlsson, Mats Lindahl, Bijar Ghafouri, Louise Fornander, och resten av gänget på labbet i Linköping för alla roliga stunder som jag hade med er före min doktorandtid. Tack speciellt **Helen Karlsson** och **Mats Lindahl** för att ni introducerade masspektrometrins spännande värld för mig.

Neserin Ali, Julia Broström, Eva Ekman, Karin Engström, Mats Gustavsson and **Hanna Landberg** för alla roliga stunder på och utanför labbet. Jag har haft fantastiskt roligt med er på alla fikapauserna, på våra vandringar, drinkprovningar, schlagerkvällar, after works, practical jokes, vårruslopp i Malmö och resor. Tack även Mats för att du en gång hjälpte mig att röja undan bevismaterial från en liten "olycka" som drabbade en viss byrå.

Marina Jeppsson och **Katarina Friman** för alla roliga stunder på och utanför jobb. Tack för att ni tog så väl hand om mig när jag var ny på labbet och ny i stan.

Gabriella Johansson för alla fina och roliga stunder med vandring, fest, klättring och samtal om meningen med livet. Tack för alla kramar och stöttande ord som jag fått under dessa år.

Alla lagkamrater i **IBK Finn** som jag har haft många roliga och dramatiska ögonblick med. Vad vore livet utan sport och tävling? Jag vill speciellt tacka mitt fina innebandy-vandringsgäng **Johanna Aspenberg, Linnea Ingemansson, Annika Hammar** och **Helena Sääf** för våra roliga stunder i Skånes fina natur.

Kompis- och rese­gänget med **Ann, Lars, Louise, Pernilla, Therése, Elena, Sandra, Sara, KG** och det senaste nytillskottet **Johan**. Jag har alltid lika roligt i ert sällskap och jag får en massa energi när vi ses. Tack **Pernilla** för alla roliga äventyr vi har haft tillsammans, som när vi kämpade i vågorna i Lacanau, hoppade i de stora guppen i Argenti­ere och gjorde det berömda Dirty Dancing lyftet. Tack **Louise** för att du står ut med att vi släpar ut dig på Åreskutan i regn och rusk och för att du vidgar mina vyer och ser till att jag får lite mer kultur i tillvaron. Tack **Ann** för fin kartläsning i skid- och downhill spåret och för att vi alltid har så mycket att prata om. Tack **Therése** för att du är den mest godhjärtade och omtänksamma människa jag känner. En vän som alltid finns där i vått och torrt. Speciellt tack till **dr Tepp-Tau** och **dr Tox** för att ni åkte ner till Skåne en lördagskväll i februari för att hjälpa en vän i nöd. Ni betyder otroligt mycket för mig. Bättre vänner får man leta efter.

Min kära familj som jag tycker så otroligt mycket om men som har fått komma lite väl mycket i andra hand de senaste åren. **Mamma** och **pappa** för att ni är otroligt stöttande och det alltid känns så skönt att komma hem. Min fina syster **Tine** och bästa **Andreas** som alltid tar med mig på äventyr med familjen kaos. Tack för alla fina stunder **Max, Felix, Jonathan** och **Julian**. Ni gör att tillvaron blir lite mer spännande. Jag blir varm inombords när jag tänker på er. Snart kommer jag att ha mer tid att leka mer er! Tack **Morris** för att du alltid välkomnar mig så glatt!

REFERENCES

1. Weinberg, E.G., *The WAO white book on allergy 2011-2012*. Current Allergy and Clinical Immunology, 2011. **24**(3):p. 156-157.
2. To, T., Stanojevic, S., Moores, G., Gershon, A.S., Bateman, E.D., Cruz, A.A., and Boulet, L.P., *Global asthma prevalence in adults: findings from the cross-sectional world health survey*. BMC Public Health, 2012. **12**:p. 204.
3. Vandenas, O., *Asthma and Rhinitis in the Workplace*. Current Allergy and Asthma Reports, 2010. **10**(5):p. 373-380.
4. Malo, J.L. and Chan-Yeung, M., *Agents causing occupational asthma*. J Allergy Clin Immunol, 2009. **123**(3):p. 545-50.
5. Pandey, A. and Mann, M., *Proteomics to study genes and genomes*. Nature, 2000. **405**(6788):p. 837-46.
6. Nordberg, M., Duffus, J.H., and Templeton, D.M., *Explanatory dictionary of key terms in toxicology (IUPAC Recommendations 2007)*. Pure and applied chemistry, 2007. **79**(9):p. 1583-1633.
7. Manini, P., De Palma, G., and Mutti, A., *Exposure assessment at the workplace: implications of biological variability*. Toxicol Lett, 2007. **168**(3):p. 210-8.
8. Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D., and Milzani, A., *Biomarkers of oxidative damage in human disease*. Clinical Chemistry, 2006. **52**(4):p. 601-623.
9. Giustarini, D., Dalle-Donne, I., Tsikas, D., and Rossi, R., *Oxidative stress and human diseases: Origin, link, measurement, mechanisms, and biomarkers*. Crit Rev Clin Lab Sci, 2009. **46**(5-6):p. 241-81.
10. Editorial, *Biomarkers on a roll*. Nat Biotech, 2010. **28**(5):p. 431-431.
11. Leino, T., Tammilehto, L., Hytonen, M., Sala, E., Paakkulainen, H., and Kanerva, L., *Occupational skin and respiratory diseases among hairdressers*. Scand J Work Environ Health, 1998. **24**(5):p. 398-406.
12. Albin, M., Rylander, L., Mikoczy, Z., Lillienberg, L., Hoglund, A.D., Brisman, J., Toren, K., Meding, B., Diab, K.K., and Nielsen, J., *Incidence of asthma in female Swedish hairdressers*. Occupational and Environmental Medicine, 2002. **59**(2):p. 119-123.
13. Ameille, J., Pauli, G., Calastreng-Crinquand, A., Vervloët, D., Iwatsubo, Y., Popin, E., Bayeux-Dunglas, M.C., and Kopferschmitt-Kubler, M.C., *Reported incidence of occupational asthma in France, 1996-99: the ONAP programme*. Occupational & Environmental Medicine, 2003. **60**(2):p. 136-141.
14. Brisman, J., Albin, M., Rylander, L., Mikoczy, Z., Lillienberg, L., Hoglund, A.D., Toren, K., Meding, B., Diab, K.K., and Nielsen, J., *The incidence of respiratory symptoms in female Swedish hairdressers*. American Journal of Industrial Medicine, 2003. **44**(6):p. 673-678.

15. Espuga, M., Muñoz, X., Plana, E., Ramón, M.A., Morell, F., Sunyer, J., and Cruz, M.J., *Prevalence of Possible Occupational Asthma in Hairdressers Working in Hair Salons for Women*. International Archives of Allergy & Immunology, 2011. **155**(4):p. 379-388.
16. Ameille, J., Hamelin, K., Andujar, P., Bensefa-Colas, L., Bonnetterre, V., Dupas, D., Garnier, R., Loddé, B.A., Rinaldo, M., Descatha, A., Lasfargues, G., and Pairon, J.-C., *Occupational asthma and occupational rhinitis: the united airways disease model revisited*. Occupational & Environmental Medicine, 2013. **70**(7):p. 471-475.
17. Lillienberg, L., Andersson, E., Janson, C., Dahlman-Hoglund, A., Forsberg, B., Holm, M., Gislason, T., Jogi, R., Omenaas, E., Schlunssen, V., Sigsgaard, T., Svanes, C., and Toren, K., *Occupational exposure and new-onset asthma in a population-based study in Northern Europe (RHINE)*. Ann Occup Hyg, 2013. **57**(4):p. 482-92.
18. Moscato, G., Vandenplas, O., Van Wijk, R.G., Malo, J.L., Quirce, S., Walusiak, J., Castano, R., De Groot, H., Folletti, I., Gautrin, D., Yacoub, M.R., Perfetti, L., and Siracusa, A., *Occupational rhinitis*. Allergy, 2008. **63**(8):p. 969-980.
19. Gautrin, D. and Malo, J.-L., *Risk Factors, Predictors, and Markers for Work-related Asthma and Rhinitis*. Current Allergy and Asthma Reports, 2010. **10**(5):p. 365-372.
20. Moscato, G., Pignatti, P., Yacoub, M.R., Romano, C., Spezia, S., and Perfetti, L., *Occupational asthma and occupational rhinitis in hairdressers*. Chest, 2005. **128**(5):p. 3590-8.
21. Pang, S. and Fiume, M.Z., *Final report on the safety assessment of Ammonium, Potassium, and Sodium Persulfate*. Int J Toxicol, 2001. **20 Suppl 3**:p. 7-21.
22. Munoz, X., Cruz, M.J., Orriols, R., Torres, F., Espuga, M., and Morell, F., *Validation of specific inhalation challenge for the diagnosis of occupational asthma due to persulphate salts*. Occupational and Environmental Medicine, 2004. **61**(10):p. 861-866.
23. Figueiredo, J.P., Pomiecinski, F., Yang, A.C., Castro, F.F., Kalil, J., and Galvao, C.E., *Diagnostic assessment of occupational asthma due to persulfate salts in a professional hairdresser: a case report*. Clinics (Sao Paulo), 2008. **63**(1):p. 149-50.
24. Diab, K.K., Truedsson, L., Albin, M., and Nielsen, J., *Persulfate challenge in female hairdressers with nasal hyperreactivity suggests immune cell, but no IgE reaction*. International Archives of Occupational and Environmental Health, 2009. **82**(6):p. 771-777.
25. Leino, T., Kahkonen, E., Saarinen, L., Henriks-Eckerman, M.L., and Paakkulainen, H., *Working conditions and health in hairdressing salons*. Appl Occup Environ Hyg, 1999. **14**(1):p. 26-33.
26. Mounier-Geyssant, E., Oury, V., Mouchot, L., Paris, C., and Zmirou-Navier, D., *Exposure of hairdressing apprentices to airborne hazardous substances*. Environ Health, 2006. **5**:p. 23.
27. Wrbitzky, R., Drexler, H., and Letzel, S., *Early reaction type allergies and diseases of the respiratory passages in employees from persulphate production*. International Archives Of Occupational And Environmental Health, 1995. **67**(6):p. 413-417.

28. Aalto-Korte, K. and Makinen-Kiljunen, S., *Specific immunoglobulin E in patients with immediate persulfate hypersensitivity*. Contact Dermatitis, 2003. **49**(1):p. 22-25.
29. Moscato, G., Pala, G., Perfetti, L., Frascaroli, M., and Pignatti, P., *Clinical and inflammatory features of occupational asthma caused by persulphate salts in comparison with asthma associated with occupational rhinitis*. Allergy, 2010. **65**:p. 784-790.
30. Munoz, X., Cruz, M.J., Orriols, R., Bravo, C., Espuga, M., and Morell, F., *Occupational asthma due to persulfate salts: diagnosis and follow-up*. Chest, 2003. **123**(6):p. 2124-9.
31. Yawalkar, N., Helbling, A., Pichler, C.E., Zala, L., and Pichler, W.J., *T cell involvement in persulfate triggered occupational contact dermatitis and asthma*. Annals of Allergy Asthma & Immunology, 1999. **82**(4):p. 401-404.
32. Pignatti, P., Frossi, B., Pala, G., Negri, S., Oman, H., Perfetti, L., Pucillo, C., Imbriani, M., and Moscato, G., *Oxidative activity of ammonium persulfate salt on mast cells and basophils: implication in hairdressers' asthma*. Int Arch Allergy Immunol, 2013. **160**(4):p. 409-19.
33. Parsons, J.F., Goodwin, B.F., and Safford, R.J., *Studies on the action of histamine release by persulphates*. Food And Cosmetics Toxicology, 1979. **17**(2):p. 129-135.
34. Hird, F.J. and Yates, J.R., *The oxidation of protein thiol groups by iodate, bromate and persulphate*. Biochem J, 1961. **80**:p. 612-6.
35. Ciencewicki, J., Trivedi, S., and Kleeberger, S.R., *Oxidants and the pathogenesis of lung diseases*. Journal of Allergy and Clinical Immunology, 2008. **122**(3):p. 456-468.
36. Zuo, L., Otenbaker, N.P., Rose, B.A., and Salisbury, K.S., *Molecular mechanisms of reactive oxygen species-related pulmonary inflammation and asthma*. Molecular Immunology, 2013. **56**(1-2):p. 57-63.
37. Bowler, R.P. and Crapo, J.D., *Oxidative stress in allergic respiratory diseases*. The Journal Of Allergy And Clinical Immunology, 2002. **110**(3):p. 349-356.
38. Hasenberg, M., Stegemann-Koniszewski, S., and Gunzer, M., *Cellular immune reactions in the lung*. Immunological Reviews, 2013. **251**:p. 189-214.
39. Grzela, K., Zagorska, W., and Grzela, T., *Mechanisms of the innate immunity in the respiratory system*. Central European Journal of Immunology, 2012. **37**(3):p. 280-285.
40. Mayer, A.K. and Dalpke, A.H., *Regulation of local immunity by airway epithelial cells*. Arch Immunol Ther Exp (Warsz), 2007. **55**(6):p. 353-62.
41. Lillehoj, E.P., Kato, K., Lu, W., and Kim, K.C., *Cellular and molecular biology of airway mucins*. Int Rev Cell Mol Biol, 2013. **303**:p. 139-202.
42. Shusterman, D., *The effects of air pollutants and irritants on the upper airway*. Proceedings of the American Thoracic Society, 2011. **8**(1):p. 101-105.
43. Fokkens, W.J. and Scheeren, R.A., *Upper airway defence mechanisms*. Paediatr Respir Rev, 2000. **1**(4):p. 336-41.
44. Brandtzaeg, P., *Mucosal immunity in infectious disease and allergy*. International Congress Series, 2003. **1257**:p. 11-20.
45. Kesimer, M., Kirkham, S., Pickles, R.J., Henderson, A.G., Alexis, N.E., Demaria, G., Knight, D., Thornton, D.J., and Sheehan, J.K., *Tracheobronchial air-liquid*

- interface cell culture: a model for innate mucosal defense of the upper airways?* Am J Physiol Lung Cell Mol Physiol, 2009. **296**(1):p. L92-L100.
46. Quirce, S., Lemièrre, C., De Blay, F., Del Pozo, V., Gerth Van Wijk, R., Maestrelli, P., Pauli, G., Pignatti, P., Raulf-Heimsoth, M., Sastre, J., Storaas, T., and Moscato, G., *Noninvasive methods for assessment of airway inflammation in occupational settings*. Allergy, 2010. **65**(4):p. 445-458.
47. Howarth, P.H., Persson, C.G.A., Meltzer, E.O., Jacobson, M.R., Durham, S.R., and Silkoff, P.E., *Objective monitoring of nasal airway inflammation in rhinitis*. The Journal of allergy and clinical immunology, 2005. **115**(3):p. S414-S441.
48. Casado, B., Pannell, L.K., Iadarola, P., and Baraniuk, J.N., *Identification of human nasal mucous proteins using proteomics*. Proteomics, 2005. **5**(11):p. 2949-2959.
49. Wang, H., Chavali, S., Mobini, R., Muraro, A., Barbon, F., Boldrin, D., Aberg, N., and Benson, M., *A pathway-based approach to find novel markers of local glucocorticoid treatment in intermittent allergic rhinitis*. Allergy, 2011. **66**(1):p. 132-140.
50. Wang, H., Gottfries, J., Barrenas, F., and Benson, M., *Identification of Novel Biomarkers in Seasonal Allergic Rhinitis by Combining Proteomic, Multivariate and Pathway Analysis*. Plos One, 2011. **6**(8):p. e23563.
51. Lindahl, M., Stahlbom, B., and Tagesson, C., *Two-dimensional ge-electrophoresis of nasal and bronchoalveolar lavage fluids after occupational exposure*. Electrophoresis, 1995. **16**(7):p. 1199-1204.
52. Benson, L.M., Mason, C.J., Friedman, O., Kita, H., Bergen, H.R., and Plager, D.A., *Extensive fractionation and identification of proteins within nasal lavage fluids from allergic rhinitis and asthmatic chronic rhinosinusitis patients*. Journal of Separation Science, 2009. **32**(1):p. 44-56.
53. Bryborn, M., Adner, M., and Cardell, L.O., *Psoriasin, one of several new proteins identified in nasal lavage fluid from allergic and non-allergic individuals using 2-dimensional gel electrophoresis and mass spectrometry*. Respiratory Research, 2005. **6**:p. 118.
54. Casado, B., Pannell, L.K., Viglio, S., Iadarola, P., and Baraniuk, J.N., *Analysis of the sinusitis nasal lavage fluid proteome using capillary liquid chromatography interfaced to electrospray ionization-quadrupole time of flight-tandem mass spectrometry*. Electrophoresis, 2004. **25**(9):p. 1386-1393.
55. Ghafouri, B., Irander, K., Lindbom, J., Tagesson, C., and Lindahl, M., *Comparative proteomics of nasal fluid in seasonal allergic rhinitis*. Journal of Proteome Research, 2006. **5**(2):p. 330-338.
56. Ghafouri, B., Stahlbom, B., Tagesson, C., and Lindahl, M., *Newly identified proteins in human nasal lavage fluid from non-smokers and smokers using two-dimensional gel electrophoresis and peptide mass fingerprinting*. Proteomics, 2002. **2**(1):p. 112-120.
57. Lindahl, M., Irander, K., Tagesson, C., and Stahlbom, B., *Nasal lavage fluid and proteomics as means to identify the effects of the irritating epoxy chemical dimethylbenzylamine*. Biomarkers, 2004. **9**(1):p. 56-70.
58. Lindahl, M., Stahlbom, B., Svartz, J., and Tagesson, C., *Protein patterns of human nasal and bronchoalveolar lavage fluids analyzed with two-dimensional gel electrophoresis*. Electrophoresis, 1998. **19**(18):p. 3222-3229.

59. Lindahl, M., Stahlbom, B., and Tagesson, C., *Newly identified proteins in human nasal and bronchoalveolar lavage fluids: Potential biomedical and clinical applications*. Electrophoresis, 1999. **20**(18):p. 3670-3676.
60. Lindahl, M., Stahlbom, B., and Tagesson, C., *Identification of a new potential airway irritation marker, palate lung nasal epithelial clone protein, in human nasal lavage fluid with two-dimensional electrophoresis and matrix-assisted laser desorption/ionization-time of flight*. Electrophoresis, 2001. **22**(9):p. 1795-1800.
61. Maiolica, A., Junger, M.A., Ezkurdia, I., and Aebersold, R., *Targeted proteome investigation via selected reaction monitoring mass spectrometry*. J Proteomics, 2012. **75**(12):p. 3495-513.
62. Domon, B. and Aebersold, R., *Options and considerations when selecting a quantitative proteomics strategy*. Nature Biotechnology, 2010. **28**(7):p. 710-721.
63. Meng, Z. and Veenstra, T.D., *Targeted mass spectrometry approaches for protein biomarker verification*. Journal of Proteomics, 2011. **74**(12):p. 2650-2659.
64. Walsh, G.M., Rogalski, J.C., Klockenbusch, C., and Kast, J., *Mass spectrometry-based proteomics in biomedical research: emerging technologies and future strategies*. Expert Reviews in Molecular Medicine, 2010. **12**:p. e30.
65. Lange, V., Picotti, P., Domon, B., and Aebersold, R., *Selected reaction monitoring for quantitative proteomics: a tutorial*. Molecular Systems Biology, 2008. **4**:p. 1-14.
66. Boja, E.S. and Rodriguez, H., *Mass spectrometry-based targeted quantitative proteomics: Achieving sensitive and reproducible detection of proteins*. Proteomics, 2012. **12**(8):p. 1093-1110.
67. Picotti, P., Rinner, O., Stallmach, R., Dautel, F., Farrah, T., Domon, B., Wenschuh, H., and Aebersold, R., *High-throughput generation of selected reaction-monitoring assays for proteins and proteomes*. Nat Meth, 2010. **7**(1):p. 43-46.
68. Cham, J.A., Bianco, L., and Bessant, C., *Free computational resources for designing selected reaction monitoring transitions*. Proteomics, 2010. **10**(6):p. 1106-1126.
69. Editorial, *Method of the Year 2012*. Nat Methods, 2013. **10**(1):p. 1.
70. Bantscheff, M., Lemeer, S., Savitski, M., and Kuster, B., *Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present*. Analytical and Bioanalytical Chemistry, 2012. **404**(4):p. 939-965.
71. Palagi, P.M., Walther, D., Quadroni, M., Catherinet, S., Burgess, J., Zimmermann-Ivol, C.G., Sanchez, J.C., Binz, P.A., Hochstrasser, D.F., and Appel, R.D., *MSight: An image analysis software for liquid chromatography-mass spectrometry*. Proteomics, 2005. **5**(9):p. 2381-2384.
72. Hofer, T. and Moller, L., *Reduction of oxidation during the preparation of DNA and analysis of 8-hydroxy-2'-deoxyguanosine*. Chemical Research in Toxicology, 1998. **11**(8):p. 882-887.
73. Ranstam, J., *Problems in orthopedic research: dependent observations*. Acta Orthop Scand, 2002. **73**(4):p. 447-50.
74. Verhoeven, K.J.F., Simonsen, K.L., and McIntyre, L.M., *Implementing false discovery rate control: increasing your power*. Oikos, 2005. **108**(3):p. 643-647.
75. Boyland, E., Sims, P., and Williams, D.C., *The oxidation of tryptophan and some related compounds with persulphate*. Biochem J, 1956. **62**(4):p. 546-50.

76. Jia, Y.P., Buehler, P.W., Boykins, R.A., Venable, R.M., and Alayash, A.I., *Structural basis of peroxide-mediated changes in human hemoglobin - A novel oxidative pathway*. Journal of Biological Chemistry, 2007. **282**(7):p. 4894-4907.
77. Vallelian, F., Pimenova, T., Pereira, C.P., Abraham, B., Mikolajczyk, M.G., Schoedon, G., Zenobi, R., Alayash, A.I., Buehler, P.W., and Schaer, D.J., *The reaction of hydrogen peroxide with hemoglobin induces extensive alpha-globin crosslinking and impairs the interaction of hemoglobin with endogenous scavenger pathways*. Free Radical Biology and Medicine, 2008. **45**(8):p. 1150-1158.
78. Xiang, W., Weisbach, V., Sticht, H., Seebahn, A., Bussmann, J., Zimmermann, R., and Becker, C.M., *Oxidative stress-induced posttranslational modifications of human hemoglobin in erythrocytes*. Arch Biochem Biophys, 2013. **529**(1):p. 34-44.
79. Chen, H.J. and Chen, Y.C., *Reactive nitrogen oxide species-induced post-translational modifications in human hemoglobin and the association with cigarette smoking*. Anal Chem, 2012. **84**(18):p. 7881-90.
80. Finch, J.W., Crouch, R.K., Knapp, D.R., and Schey, K.L., *Mass-spectrometric identification of modifications to human serum-albumin treated with hydrogen-peroxide*. Archives of Biochemistry and Biophysics, 1993. **305**(2):p. 595-599.
81. Madian, A.G. and Regnier, F.E., *Profiling Carbonylated Proteins in Human Plasma*. Journal of Proteome Research, 2010. **9**(3):p. 1330-1343.
82. Madian, A.G., Diaz-Maldonado, N., Gao, Q., and Regnier, F.E., *Oxidative stress induced carbonylation in human plasma*. Journal of Proteomics, 2011. **74**(11):p. 2395-2416.
83. Salzano, A.M., Renzone, G., Scaloni, A., Torreggiani, A., Ferreri, C., and Chatgililoglu, C., *Human serum albumin modifications associated with reductive radical stress*. Molecular BioSystems, 2011. **7**(3):p. 889-898.
84. Kiselar, J.G., Janmey, P.A., Almo, S.C., and Chance, M.R., *Structural Analysis of Gelsolin Using Synchrotron Protein Footprinting*. Molecular & Cellular Proteomics, 2003. **2**(10):p. 1120-1132.
85. Held, J.M. and Gibson, B.W., *Regulatory Control or Oxidative Damage? Proteomic Approaches to Interrogate the Role of Cysteine Oxidation Status in Biological Processes*. Molecular & Cellular Proteomics, 2012. **11**:p. 1-14.
86. Chen, M. and Cook, K.D., *Oxidation artifacts in the electrospray mass spectrometry of Abeta Peptide*. Anal Chem, 2007. **79**(5):p. 2031-6.
87. Perdivara, I., Deterding, L.J., Przybylski, M., and Tomer, K.B., *Mass spectrometric identification of oxidative modifications of tryptophan residues in proteins: chemical artifact or post-translational modification?* J Am Soc Mass Spectrom, 2010. **21**(7):p. 1114-7.
88. Naskalski, J.W. and Bartosz, G., *Oxidative modifications of protein structures*. Advances In Clinical Chemistry, 2000. **35**:p. 161-253.
89. Candiano, G., Petretto, A., Bruschi, M., Santucci, L., Dimuccio, V., Prunotto, M., Gusmano, R., Urbani, A., and Ghiggeri, G.M., *The oxido-redox potential of albumin methodological approach and relevance to human diseases*. J Proteomics, 2009. **73**(2):p. 188-95.

90. van de Weert, M., Lagerwerf, F.M., Haverkamp, J., and Heerma, W., *Mass spectrometric analysis of oxidized tryptophan*. Journal of Mass Spectrometry, 1998. **33**(9):p. 884-891.
91. Stadtman, E.R. and Levine, R.L., *Free radical-mediated oxidation of free amino acids and amino acid residues in proteins*. Amino Acids, 2003. **25**(3-4):p. 207-218.
92. Domanski, D., Percy, A.J., Yang, J.C., Chambers, A.G., Hill, J.S., Freue, G.V.C., and Borchers, C.H., *MRM-based multiplexed quantitation of 67 putative cardiovascular disease biomarkers in human plasma*. Proteomics, 2012. **12**(8):p. 1222-1243.
93. Kuzyk, M.A., Smith, D., Yang, J., Cross, T.J., Jackson, A.M., Hardie, D.B., Anderson, N.L., and Borchers, C.H., *Multiple Reaction Monitoring-based, Multiplexed, Absolute Quantitation of 45 Proteins in Human Plasma*. Molecular & Cellular Proteomics, 2009. **8**(8):p. 1860-1877.
94. Silva, A.M.N., Vitorino, R., Domingues, M.R.M., Spickett, C.M., and Domingues, P., *Post-translational Modifications and Mass Spectrometry Detection*. Free Radical Biology and Medicine, 2013. **65**(0):p. 925-941.
95. Moscato, G., Vandenplas, O., Van Wijck, R.G., Malo, J.L., Perfetti, L., Quirce, S., Walusiak, J., Castano, R., Pala, G., Gautrin, D., De Groot, H., Folletti, I., Yacoub, M.R., and Siracusa, A., *EAACI position paper on occupational rhinitis*. Respir Res, 2009. **10**(16):p. 1-20.
96. Bingle, L., Cross, S.S., High, A.S., Wallace, W.A., Rassl, D., Yuan, G., Hellstrom, I., Campos, M.A., and Bingle, C.D., *WFDC2 (HE4): a potential role in the innate immunity of the oral cavity and respiratory tract and the development of adenocarcinomas of the lung*. Respir Res, 2006. **7**:p. 61.
97. Lakind, J.S., Holgate, S.T., Ownby, D.R., Mansur, A.H., Helms, P.J., Pyatt, D., and Hays, S.M., *A critical review of the use of Clara cell secretory protein (CC16) as a biomarker of acute or chronic pulmonary effects*. Biomarkers, 2007. **12**(5):p. 445-67.
98. Broeckaert, F. and Bernard, A., *Clara cell secretory protein (CC16): characteristics and perspectives as lung peripheral biomarker*. Clin Exp Allergy, 2000. **30**(4):p. 469-75.
99. Sanghi, S., Kumar, R., Lumsden, A., Dickinson, D., Klepeis, V., Trinkaus-Randall, V., Frierson, H.F., Jr., and Laurie, G.W., *cDNA and genomic cloning of lacritin, a novel secretion enhancing factor from the human lacrimal gland*. J Mol Biol, 2001. **310**(1):p. 127-39.
100. Wang, J., Wang, N., Xie, J., Walton, S.C., McKown, R.L., Raab, R.W., Ma, P., Beck, S.L., Coffman, G.L., Hussaini, I.M., and Laurie, G.W., *Restricted epithelial proliferation by lacritin via PKC alpha-dependent NFAT and mTOR pathways*. Journal of Cell Biology, 2006. **174**(5):p. 689-700.
101. Ansell, B.J., Watson, K.E., Fogelman, A.M., Navab, M., and Fonarow, G.C., *High-density lipoprotein function - Recent advances*. Journal of the American College of Cardiology, 2005. **46**(10):p. 1792-1798.
102. Tomazic, P.V., Leitner, A., Lang-Loidolt, D., Birner-Gruenberger, R., Obrist, B., and Spoerk, S., *Nasal mucus proteomic changes reflect altered immune responses and epithelial permeability in patients with allergic rhinitis*. Journal of Allergy and Clinical Immunology, 2013.

103. Raphael, G.D., Jeney, E.V., Baraniuk, J.N., Kim, I., Meredith, S.D., and Kaliner, M.A., *Patho-physiology of rhinitis - lactoferrin and lysozyme in nasal secretions*. Journal of Clinical Investigation, 1989. **84**(5):p. 1528-1535.
104. Kim, D., Chu, H., Lee, J., Hwang, S., Lee, S., and Lee, H., *UP-regulation of muc5ac and muc5b mucin genes in chronic rhinosinusitis*. Archives of Otolaryngology–Head & Neck Surgery, 2004. **130**(6):p. 747-752.
105. Dinarello, C.A., *Interleukin-1 in the pathogenesis and treatment of inflammatory diseases*. Blood, 2011. **117**(14):p. 3720-32.
106. Jyh-Hong, L., Li-Chieh, W., Hsin-Hui, Y., Yu-Tsan, L., Yao-Hsu, Y., and Bor-Luen, C., *Type 1 IL-1 Receptor (IL-1RI) as Potential New Therapeutic Target for Bronchial Asthma*. Mediators of Inflammation, 2010:p. 1-7.
107. Ivanov, A.I., Parkos, C.A., and Nusrat, A., *Cytoskeletal Regulation of Epithelial Barrier Function During Inflammation*. The American Journal of Pathology, 2010. **177**:p. 512-524.
108. Huber, O., *Structure and function of desmosomal proteins and their role in development and disease*. Cell Mol Life Sci, 2003. **60**(9):p. 1872-90.
109. Gloudemans, A.K., Lambrecht, B.N., and Smits, H.H., *Potential of Immunoglobulin A to Prevent Allergic Asthma*. Clinical & Developmental Immunology, 2013:p. 1-12.
110. Hupin, C., Rombaux, P., Bowen, H., Gould, H., Lecocq, M., and Pilette, C., *Downregulation of polymeric immunoglobulin receptor and secretory IgA antibodies in eosinophilic upper airway diseases*. Allergy, 2013. **68**(12):p. 1589-1597.
111. Ishii, T., Ouchi, Y., Matsuse, T., Igarashi, H., Masuda, M., and Teramoto, S., *Tobacco smoke reduces viability in human lung fibroblasts: Protective effect of glutathione S-transferase P1*. American Journal of Physiology - Lung Cellular and Molecular Physiology, 2001. **280**(6 24-6):p. L1189-L1195.
112. Grant, C., *Metabolic reconfiguration is a regulated response to oxidative stress*. Journal of Biology, 2008. **7**(1).