Using iTRAQ approach to investigate the effect of cigarette smoke-induced COPD and smoking cessation effects in C57BL/6 mice

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Introduction

Chronic obstructive pulmonary disease (COPD) is defined as a lung disease characterized by chronic obstruction of lung airflow that interferes with normal breathing and is associated with narrowing of the small airways, chronic bronchitis, and the development of alveolar emphysema. Cigarette smoke is the primary risk factor in the development and progression of COPD. Following smoking cessation a residual risk remains, although significantly lower than continued smoking. However, the underlying pathogenesis of the disease is not fully understood. In this study, we analyzed the progression of emphysema over a 7 month period of exposure to cigarette smoke (CS, 750 µg/l total particlulate matter from 3R4F cigarettes, University of Kentucky, smoked according to the Health Canada Intense Smoking Regime) or to CS for two months, followed by up to 5 months cessation (fresh air) in C57BL/6 mice. A comprehensive liquid-chromatography mass-spectrometry (LC-MS) based quantitative proteomics approach using isobaric tags for absolute and relative quantification (iTRAQ®) was performed on lung tissues of C57BL/6 mice to detect changes in protein expression levels between control, 3R4F and cessation groups.

The aim of the study was:

(i) To identify regulated proteins in mouse lungs in response to cigarette smoke exposure.
(ii) To determine which biological and molecular function processes are impacted.
(iii) To determine the effect of smoking cessation
(iv) To determine the feasibility of using the iTRAQ approach for product assessment of novel modified risk tobacco products (MRTPs) using *in vivo* model systems.

MATERIALS & METHODS

Eight to 10 week old mice were exposed to fresh air (control) or cigarette smoke (CS, 750 µg/l total particulate matter from 3R4F cigarettes) for 7 months or to CS for 2 months, followed by 5 months cessation (fresh air). Total protein from lung tissue was extracted according to **Figure 1.** The proteomics approach used in this study is summarized in **Figure 2.**

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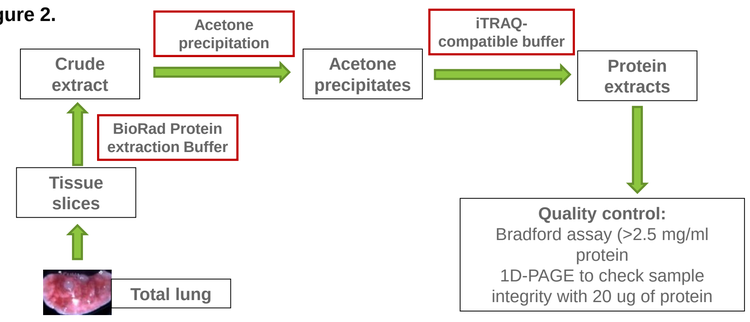

Figure 1: Workflow for the extraction of proteins from mouse lungs

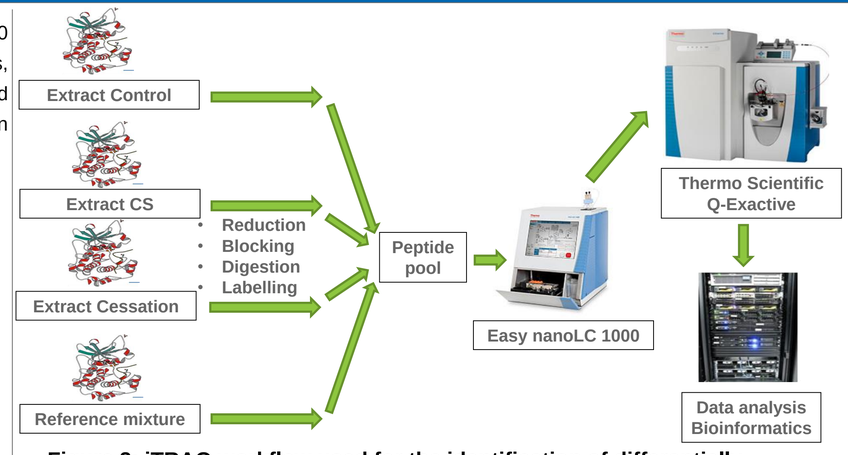

Figure 2: iTRAQ workflow used for the identification of differentially expressed proteins.

LC-MS: Peptide samples were separated on a 50 cm C_{18}-reversed phase column and peptide masses were acquired with a Thermo Scientific Q-Exactive mass-spectrometer.

Bioinformatics: Proteins were identified using a combination of Mascot, Sequest and MS Amanda search engines against the Uniprot mouse reference proteome and inhouse established transcriptome based reference databases. Proteins were quantitated using Proteome Discoverer™ software (ThermoScientific) against a reference sample.

RESULTS

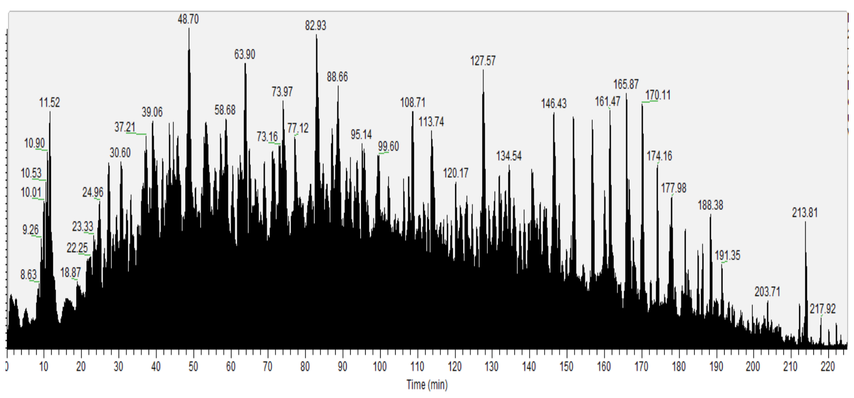

Figure 3: Representative 1D-LC peptide elution profile. ~1.5 µg of peptides were loaded and separated on a 50 cm C_{18} reversed phase column.

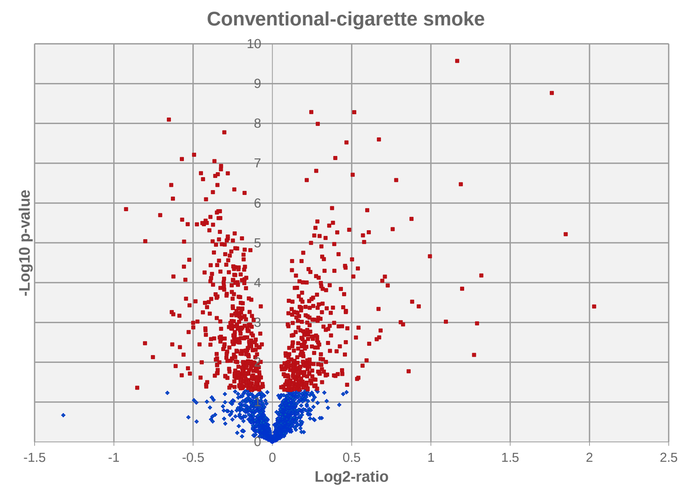

Figure 4: Volcano plot of protein ratios obtained from iTRAQ analysis of lung protein extracts from mice exposed to the conventional cigarette treatment (7 months smoking). Red dots denote proteins with a p-value <0.05 (t-test).

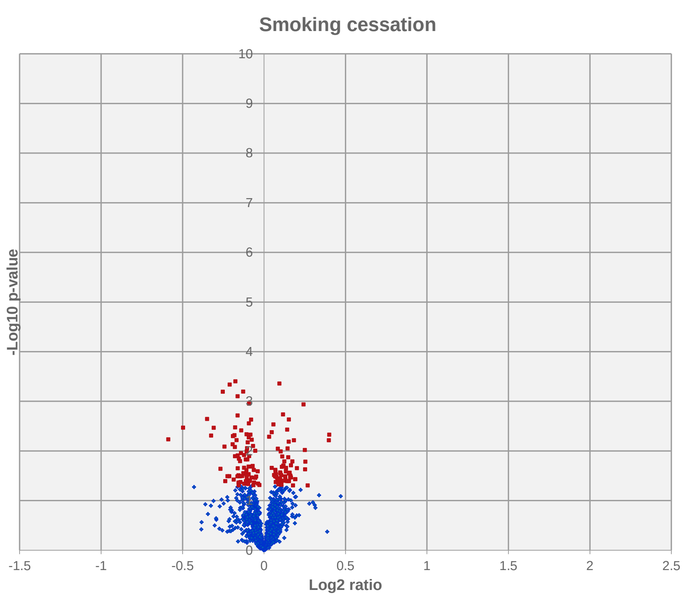

Figure 5: Volcano plot of protein ratios obtained from iTRAQ analysis of lung protein extracts from mice exposed to the cessation treatment (2 months conventional cigarette smoking, five months fresh air). Red dots denote proteins with a p-value <0.05 (t-test).

Accession	Protein Name	Gene Name	Fold change	p-value (t-test)
Q99P91	Transmembrane glycoprotein NMB	Gpnmb	4.1	4.01x10-4
P18242	Cathepsin D	Ctsd	3.6	$6.1x10^{-6}$
A1L314	Macrophage-expressed gene 1 protein	Mpeg1	2.5	6.67x10-5
P09671	Superoxide dismutase, mitochondrial	Sod2	1.4	$4.18x10^{-4}$
P00184	Cytochrome 1A1	Cyt1a1	1.5	$6.5x10^{-6}$
P62835	Ras-related protein Rab-1A	Rab1A	-1.5	$6.27x10^{-4}$
P02301	Histone H3.3C	H3f3c	-1.5	$3.53x10^{-3}$
P16015	Carbonic anhydrase 3	Ca3	1.8	$3.35x10^{-3}$
Q64523	Histone H2A type 2-C	Hist2h2ac	1.8	$4.39x10^{-2}$
P40936	Indolethylamine N-methyltransferase	Inmt	-1.9	$1.43x10^{-6}$

Table 1: Representative candidate proteins that were regulated in response to conventional-cigarette smoking.

	Conventional cigarette smoking	Cessation
Number of identified proteins (≥ 2 peptides)	3389	
Number of quantified proteins (≥ 2 biological replicates)	2647	
Number of significantly regulated proteins (≥ 1.3 fold regulated; p<0.05, t-test)	114	4

Table 2: Number of identified/regulated proteins from comparison of conventional cigarette smoking and cessation to control using the iTRAQ LC MS/MS workflow.

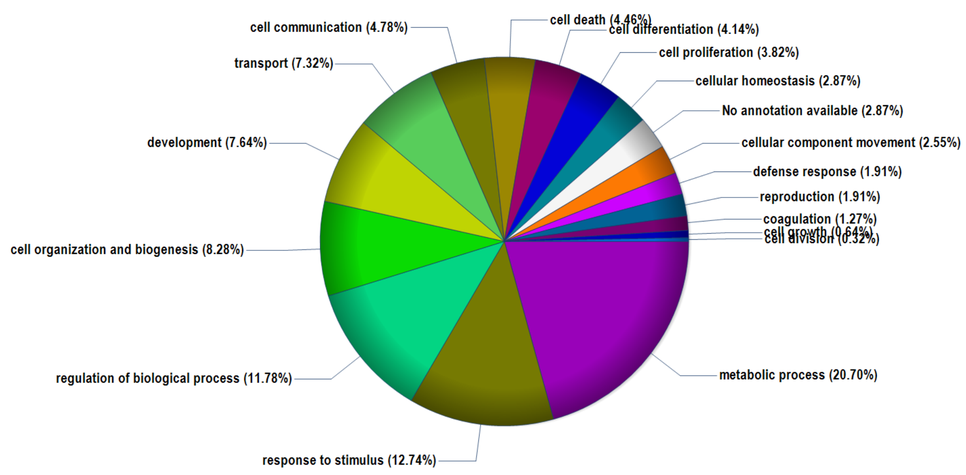

Figure 7: Molecular functions impacted from regulated proteins as a result of exposure to conventional cigarettes.

Figure 6: Biological processes impacted from regulated proteins as a result of exposure to conventional cigarettes.

Conclusions

- The successful implementation of the iTRAQ workflow for identification of differentially expressed proteins as a result of exposure to 3R4F and smoking cessation in mouse lungs.
- The majority of the identified regulated proteins in the iTRAQ approach were verified by a 2D-PAGE analysis that was performed in parallel.
- The majority of the identified regulated proteins impacted the protein binding and catalytic activity of lung tissue as a result of exposure to conventional cigarette smoke.
- System perturbation was significantly reduced after 5 months of smoking cessation as shown in volcano plot analyses.
- The iTRAQ approach is suitable for the future, in order to mechanistically understand if MRTPs are likely to reduce the risk associated with smoking conventional cigarettes.

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