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Alterations of the Sputum Proteome and Transcriptome and Serum Lipidome from Smokers and Early-Stage COPD Subjects

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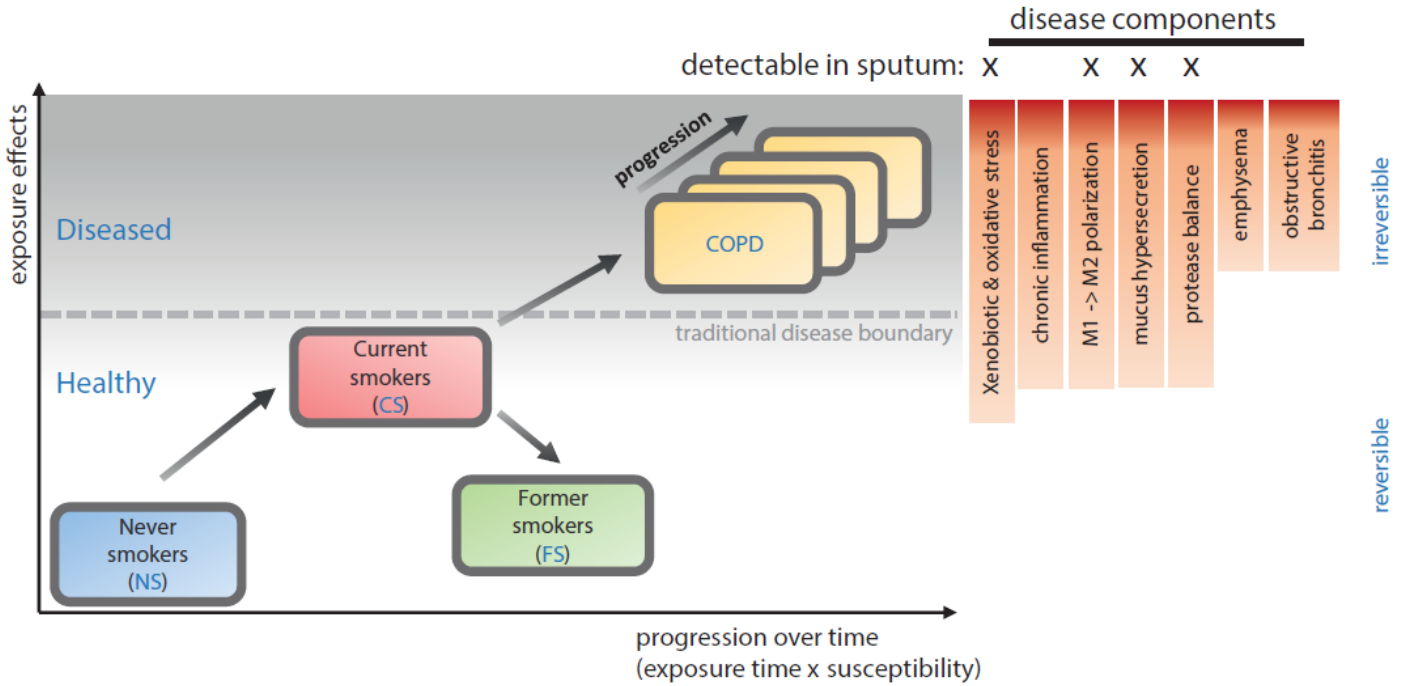
Chronic Obstructive Pulmonary Disease (COPD)

- COPD, a common preventable and treatable disease, is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lungs to noxious particles or gases.
- COPD is a progressive disease, meaning that is worsening with time.
- Cigarette smoking is one of the most common causes of COPD, with factors such as air pollution and genetics play a smaller part.
- Most cases can be prevented by reducing exposure to risk factors.

Global Initiative for Chronic Obstructive Lung Disease (GOLD), http://www.goldcopd.org

Basic Chronic-Exposure-to-Disease-Transition Model of COPD



Question:
How are the (three main) state transitions of this model reflected in the molecular profiles of sputum and serum samples?

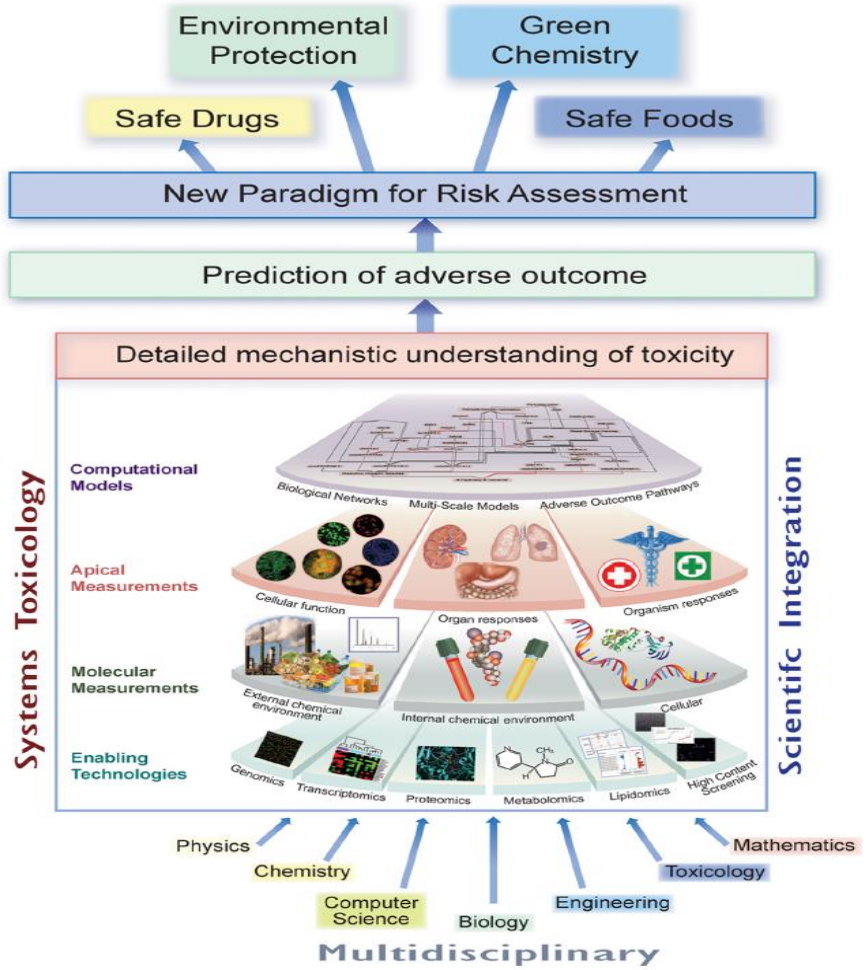
(http://www.sciencedirect.com/science/article/pii/S1874391915301007)

Objectives

- Clinical study on the effects of smoke exposure and early-stage COPD on molecular profiles of sputum and serum samples:
  - To assess how molecular profiles of sputum samples can capture the complex physiological response to cigarette smoke.
  - To identify a biomarker or panel of biomarkers for the differentiation of subjects with COPD, current smokers, former smokers and never-smokers.
  - To assess how serum lipid profiles for serum samples change in response to cigarette smoke.

Titz, et. al. "Alterations in the sputum proteome and transcriptome in smokers and early-stage COPD subjects." 2015 *J Proteomics*. 14;128:306-20
Titz, et. al. "Alterations in Serum Polyunsaturated Fatty Acids and Eicosanoids in Patients with Mild to Moderate Chronic Obstructive Pulmonary Disease (COPD)" 2016 Int J Mol Sci. 20;17(9). pii: E1583.

Systems Toxicology Approach For Product Assessment



"Systems Toxicology is the integration of classical toxicology with quantitative analysis of large networks of molecular and functional changes occurring across multiple levels of biological organization."

- Adds mechanistic insights
- Supports identification of biomarkers for safety assessments
- Adds toward predictive mathematical models of toxicological processes

Sturla et al. Chemical Research in Toxicology (2014)

Design of the study

Study Outline

- We conducted a case-control study designed to identify a biomarker (panel) for the differentiation of subjects with mild and moderate COPD, asymptomatic current, former and never-smokers
- Parallel-group clinical study with 60 age- and gender-matched individuals for each of four study groups (ClinicalTrials.gov identifier NCT01780298)
  - current asymptomatic smokers (CS)
  - current-smoker COPD subjects (COPD)
  - former smokers (FS)
  - never smokers (NS)

- Cell-free sputum supernatant analyzed by quantitative proteomics (n=60)
- Cellular mRNA fraction analyzed by gene expression profiling (n=60)
- Serum samples analyzed by quantitative lipidomics (n=40)

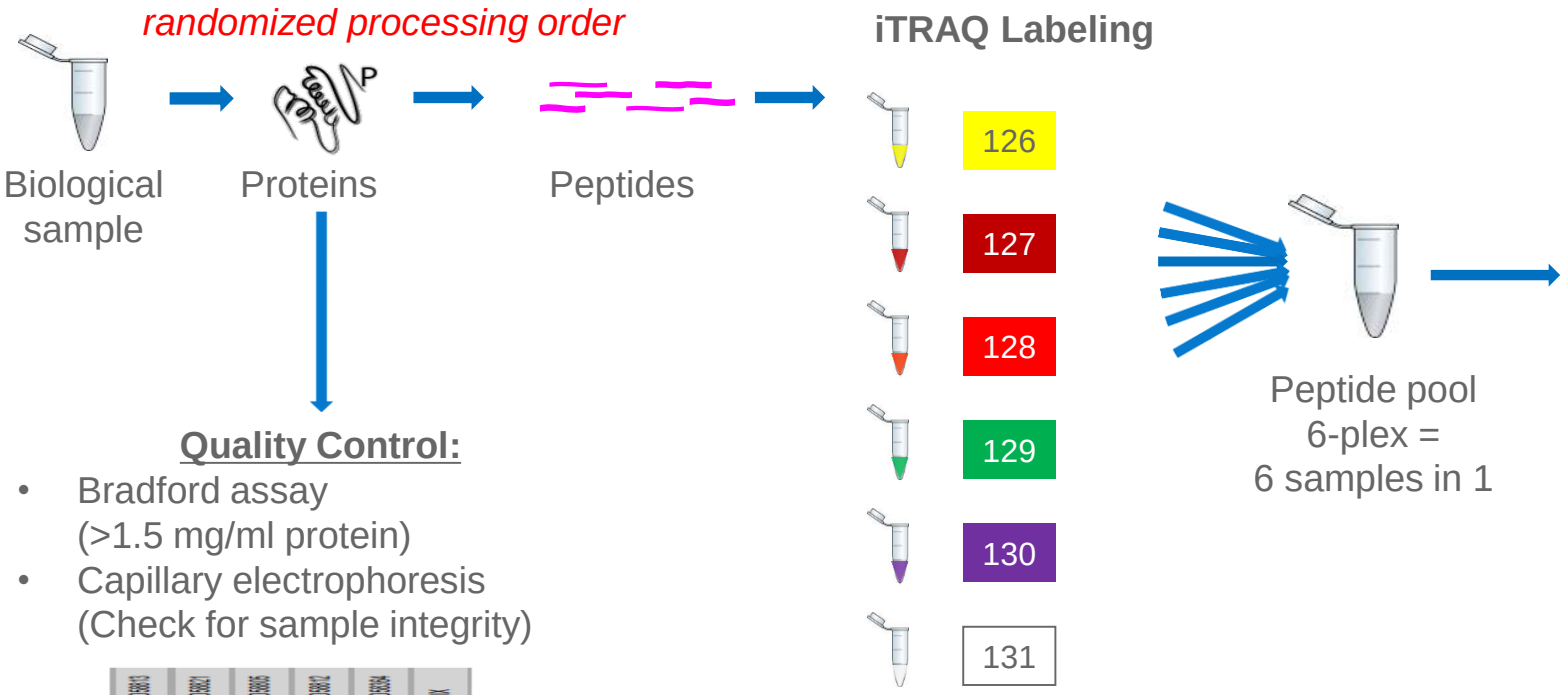
Specifics of the Study Population

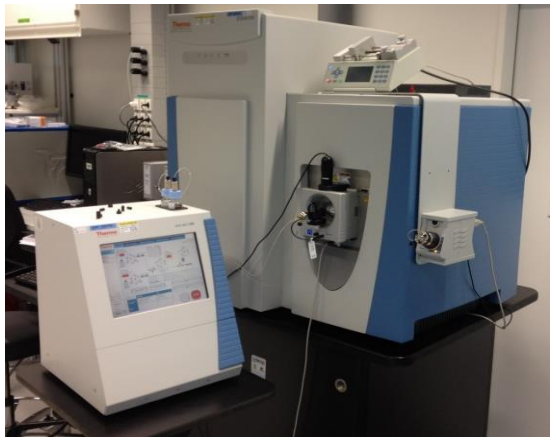
- Each cohort (n=60) was matched closely for age (within 5 years) and gender
- Smoking history (greater than 10-pack years except for never smokers)
- Former smokers had quit for at least 1 year; majority (~78%) had quit for more than five years

Importantly, our selection criteria were different from other related studies such as the ECLIPSE study (www.eclipse-copd.com/): we selected only subjects with mild COPD (29 GOLD stage 1, 31 GOLD stage 2) and subjects with recent infections or a history of exacerbations of COPD were excluded.

Methodologies

Quantitative Proteomics – Label based TMT LC-MS/MS Approach

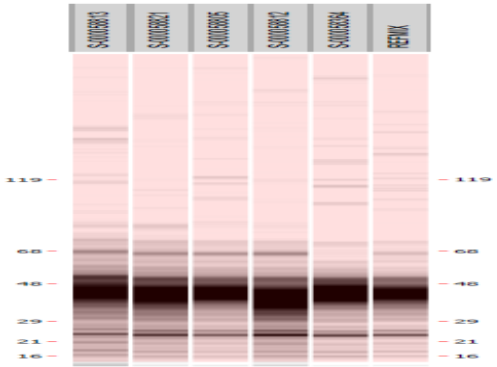




nanoLC connected to a
Q-Exactive (Thermo Scientific)

Quality Control:

- Bradford assay (>1.5 mg/ml protein)
- Capillary electrophoresis (Check for sample integrity)



Titz, B., Schneider, T., Elamin, A., Martin, F., Dijon, S., Ivanov, N.V., Hoeng, J. and Peitsch, M.C., 2015. Analysis of Proteomic Data for Toxicological Applications. *Computational Systems Toxicology*, pp.257-284.

TMT Quantitative Proteomics Pipeline

ACQUISITION

LC-MS/MS Q-Exactive (Thermo)

1. Easy nanoLC 1000 connected to Q-Exactive mass spectrometer
2. Separation on 50-cm C18 reverse phase nanoLC columns (200 min gradient)
3. Each sample analyzed in fast and sensitive acquisition mode (Kelstrup et al., 2012)

.raw files

IDENTIFICATION

Proteome Discoverer (Thermo)

1. Proteome Discoverer software
2. Spectra from fast and sensitive runs merged for each sample
3. Spectra searched against reference databases with Mascot and Sequest HT
4. Percolator tool to calculate peptide probabilities (q-value)
5. Reporter ion quantifier for isobaric tags (no normalization)
6. Export of peptide-to-spectrum matches and raw reporter ion quantities

PSMS file

QUANTIFICATION

R Project for Statistical Computing

1. Filter for “unique” quantification results & q-value of spectrum match < 0.01
2. Global variance stabilizing normalization (VSN) (Huber et al., 2002; Karp et al., 2010).
3. Median scaling and median-collapsing of peptide to protein values (Herbrich et al., 2013).
4. LIMMA model using empirical Bayes approach and FDR-correction for identification of differentially expressed proteins (Gentleman et al., 2004).

result files

Mass-Spectrometry Based Lipidomics Analysis in Serum

Monitored Lipid Classes

Shotgun lipidomics
Cholesterol esters
Phosphatidylcholines
Lysophosphatidylcholines and other lysophospholipids
Ether-linked phosphatidylcholines and other ether-linked phospholipids
Phosphatidylserines
Phosphatidylethanolamines
Phosphatidylglycerols
Phosphatidylinositiols
Phosphatidic acid
Sphingomyelins
Diacylglycerols

Triacylglycerol lipidomics
Triacylglycerols

Ceramide and cerebroside lipidomics
Ceramides
Cerebrosides (Lactosylceramides; Galactosyl- and Glucosylceramides;
Globotriaosylceramides)

Eicosanoid lipidomics
Arachidonic acid
Eicosapentaenoic acid
Docosahexaenoic acid
Prostaglandins
Thromboxanes
Hydroxyeicosapentaenoic acids
Hydroxyeicosatetraenoic acids
Dihydroxyeicosatrienoic acids
Hydroxyoctadecadienoic acids
Hydroxyoctadecatrienoic acids
Leukotrienes
Lipoxines

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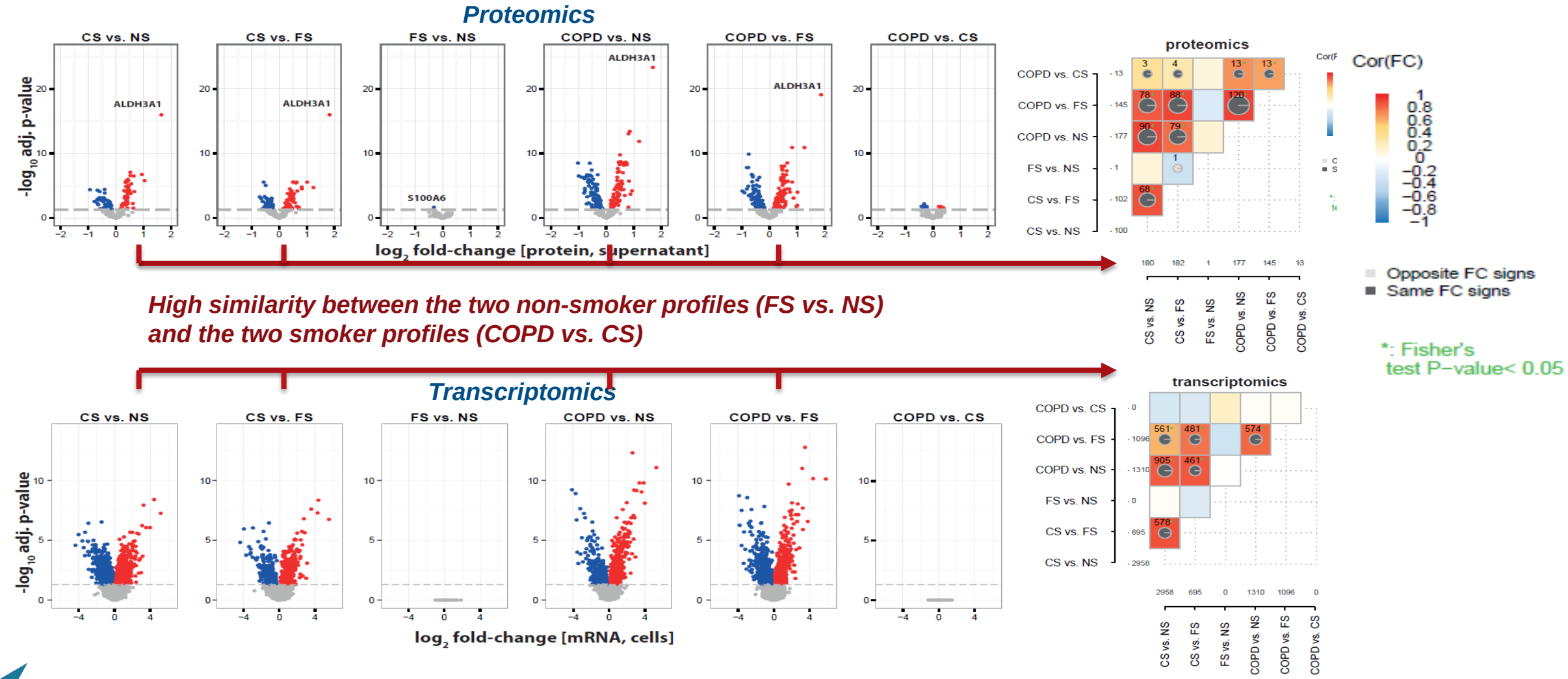
Analyses were performed by Zora Biosciences Oy, Espoo, Finland (www.zora.fi)

Results

Overall Results - Smoke Exposure Effect Dominates the Molecular Profiles of Sputum



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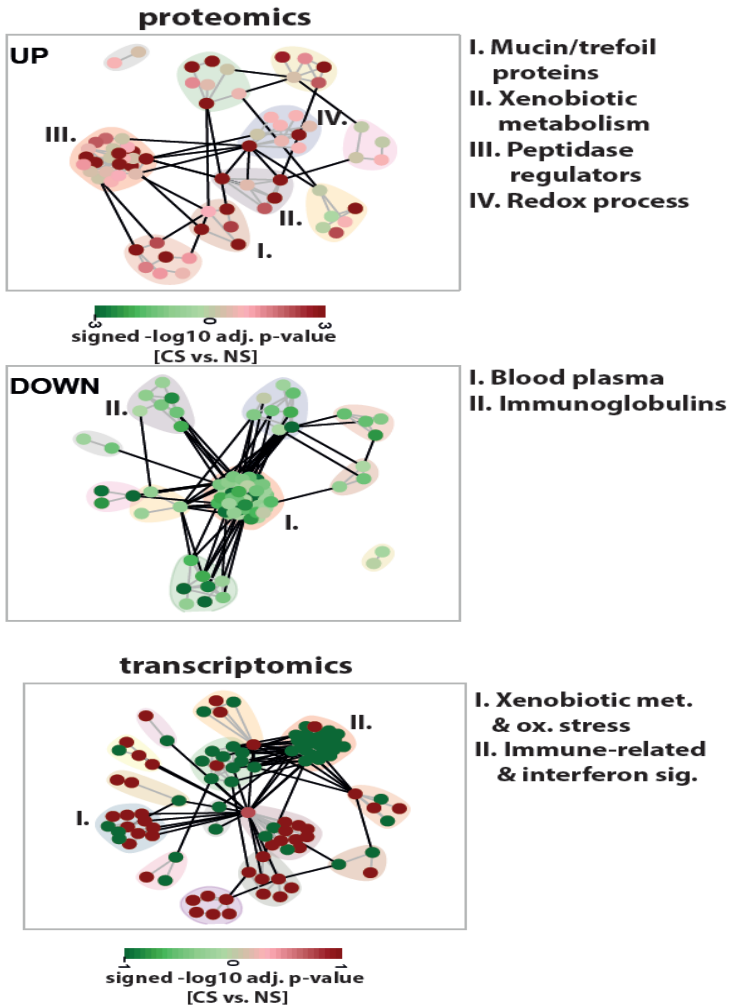
Functional association clustering revealed several biological categories in sputum affected by the smoking status

Functional clusters from String-database (string-db.org)



Up-regulated categories:

- Mucin/trefoil proteins (e.g., MUC5AC and TFF1/3)
  - Detected in proteomics approach
- Xenobiotic metabolism enzymes (e.g., ALDH3A1, NQO1, and GSTA1)
  - detected proteomics and transcriptomics
- Peptidase regulators (e.g., TIMP1 and SERPINB1)
  - detected in proteomics approach

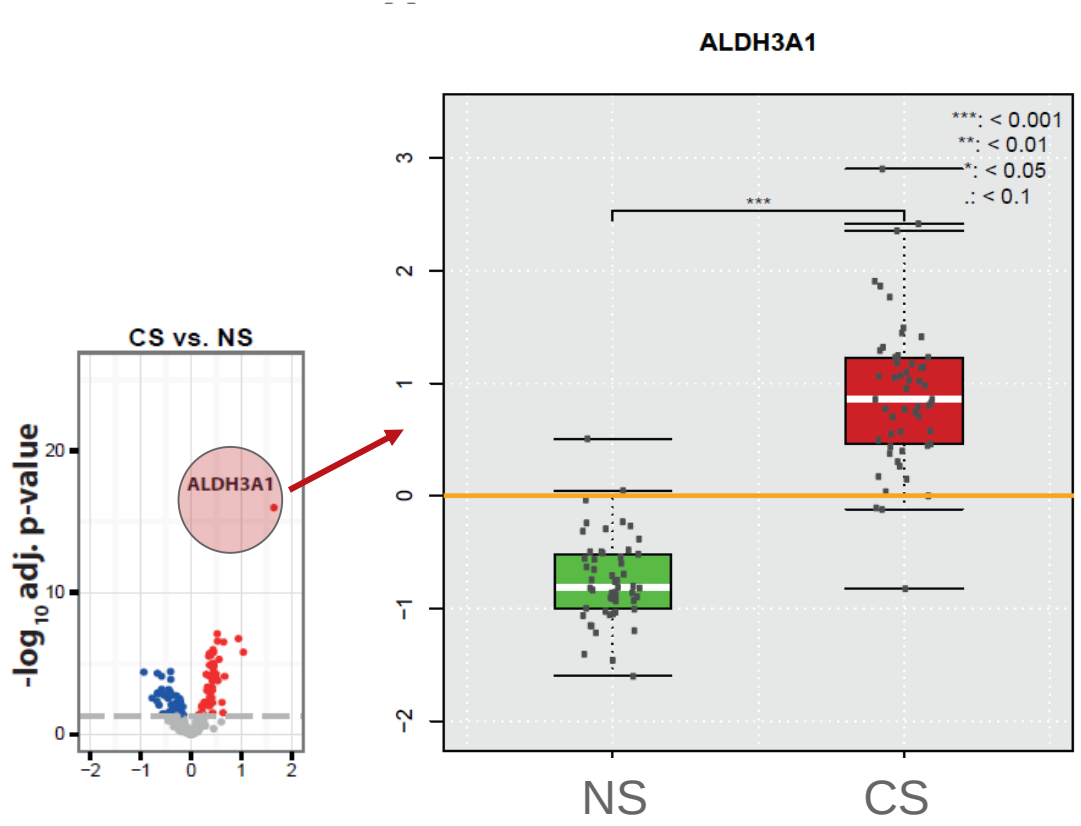
Down-regulated categories:

- Plasma-derived proteins (e.g., ALB, APOA1, and TF)
  - Detected in proteomics approach
- Immunoglobulins (e.g., IGHG1-4 and IGKC) [proteomics]
  - Detected in proteomics approach
- Immune-related cluster enriched for interferon signaling pathway components
  - Detected in transcriptomics approach

REMINDER: Different sputum fractions for proteomics (supernatant) and transcriptomics (cells)

➔ Molecular sputum profiles reflect several of the main known effects of cigarette smoke exposure including the xenobiotic and oxidative stress response, changes in mucin production, and alterations in the protease balance

ALDH3A1 Protein Expression Predicts the Current Smoking Status for Subjects of the Four Study Groups



Cross−validation confusion table (k=10,L=5)

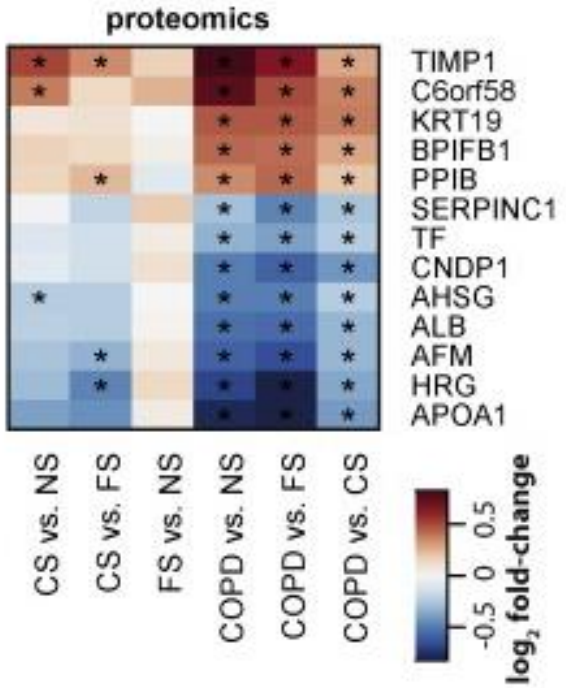
	NS	CS	TR
NS	53	1	0.98
CS	5	49	0.91

		Prediction	
		NS	CS
Group	COPD	2	52
	FS	52	2
	NS	53	1
	CS	5	49

Robust enough across independent studies?

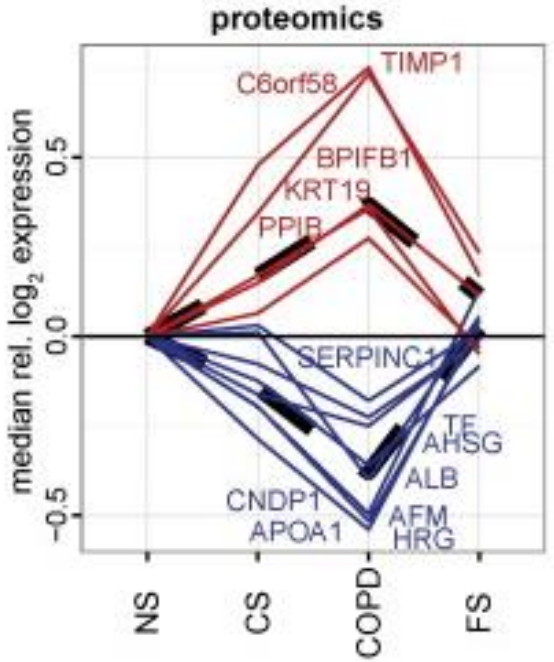
A forward selection linear discriminant analysis (LDA) model was trained on the CS vs. NS proteomics data. ALDH3A1 was selected as the protein which provided the best separation of these groups in a cross-validation.

Differentially Abundant Proteins for COPD vs. CS



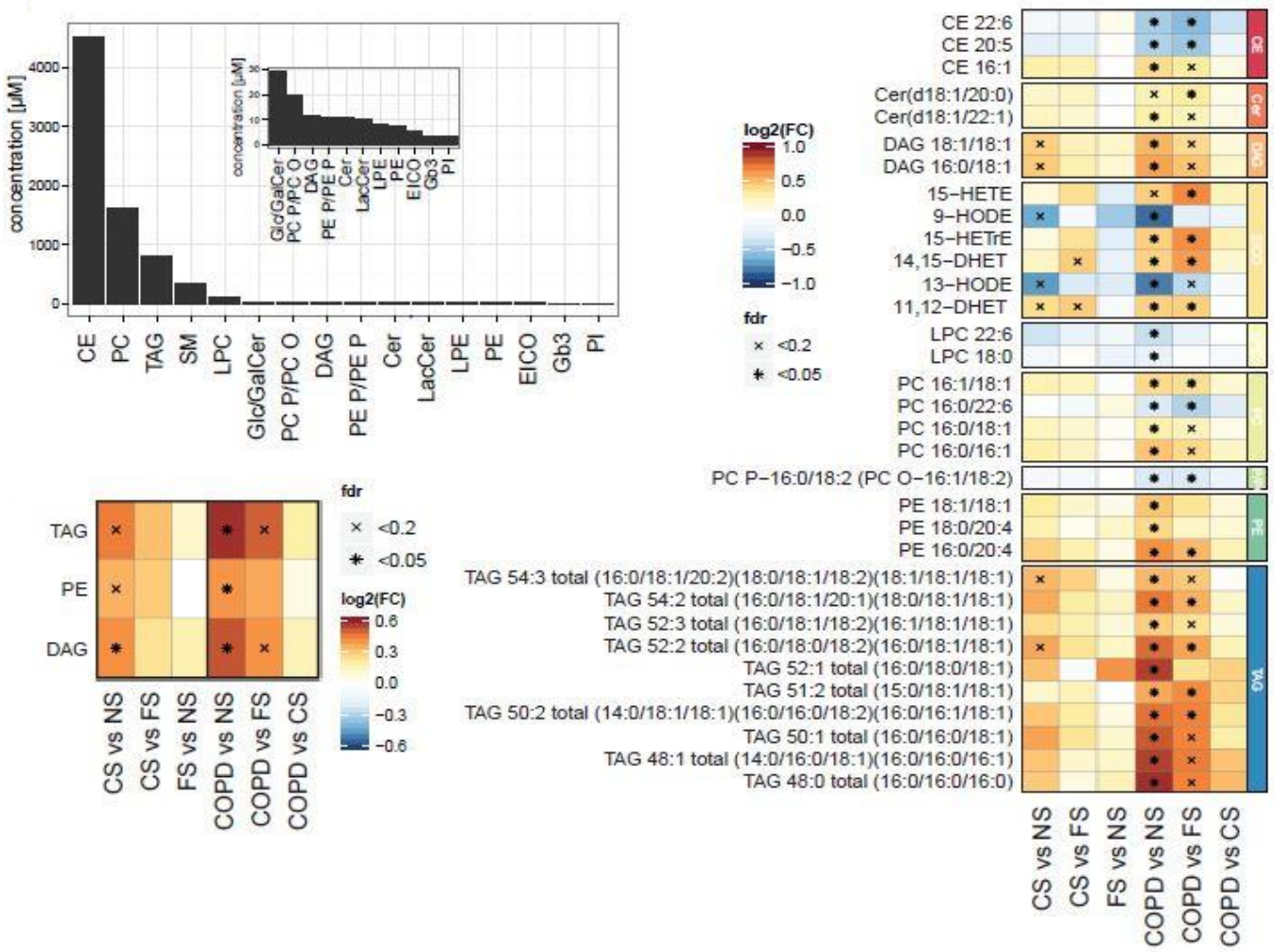
13 differentially abundant proteins were detected for COPD vs. CS; mostly "amplification" of trends already observed for CS vs. NS

For approx. 50% of these proteins, their levels have been previously reported to be affected in COPD, e.g. TIMP1, BPIFB1, AHSG, and APOA1.



- Differentially abundant proteins for COPD vs. CS mostly further amplify trends observed for CS vs. NS

Smoke Exposure Effect Dominates the Lipidome Profiles in Serum



Three main trends were identified when comparing smokers, (especially those with COPD), with non-smokers:

- A general increase in glycero(phospho)lipids, including triglycerols
- Changes in fatty acid desaturation (decrease in ω-3 polyunsaturated fatty acids, and an increase in monounsaturated fatty acids);
- An imbalance in eicosanoids (increase in 11,12- and 14,15-DHETs (dihydroxyeicosatrienoic acids), and a decrease in 9- and 13-HODEs (hydroxyoctadecadienoic acids

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Summary & Conclusions

- Overall, our study demonstrates that sputum and serum profiling can capture the complex physiological response and reversibility to cigarette smoke exposure. Thus, the sputum proteome and transcriptome could – in principle – be used to assess several relevant smoke exposure effects in human studies.
- The sputum proteome of current smokers clearly reflected the common physiological responses to smoke exposure, including alterations in mucin/trefoil proteins and a prominent xenobiotic/oxidative stress response, while ex-smoker proteome showed reversal of the effects
- Thirteen differentially abundant proteins between the COPD and the asymptomatic smoker group were identified including TIMP1, APOA1, C6orf58, and BPIFB1 (LPLUNC1)
- Three main trends were identified in the lipidomics analyses of serum when comparing smokers, especially those with COPD, with non-smokers: a general increase in glycero(phospho)lipids, changes in fatty acid desaturation and an imbalance in eicosanoids

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