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Comparative Proteomics Studies on Mice Lung Proteome for the Assessment of Candidate Modified Risk Tobacco Products.

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Outline of the Presentation

- PMI R&D and heat-not-burn concept
- Introduction to our systems toxicology approach
- Design of the studies
- Proteomics analysis methodology implemented for identification and quantitation
- Results
- Conclusions

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PMI R&D- Background

- Smoking causes serious diseases such as cardiovascular diseases, lung cancer and chronic obstructive pulmonary disease.
- Philip Morris International is developing and commercializing novel products with the potential to reduce individual risk and population harm in comparison to smoking cigarettes.
- To determine whether such potentially reduced-risk products (pRRP), also called modified tobacco risk products (MRTPs) have the potential to reduce individual risk and population harm, we are conducting extensive and rigorous scientific studies comparing their biological impact compared to that of cigarettes

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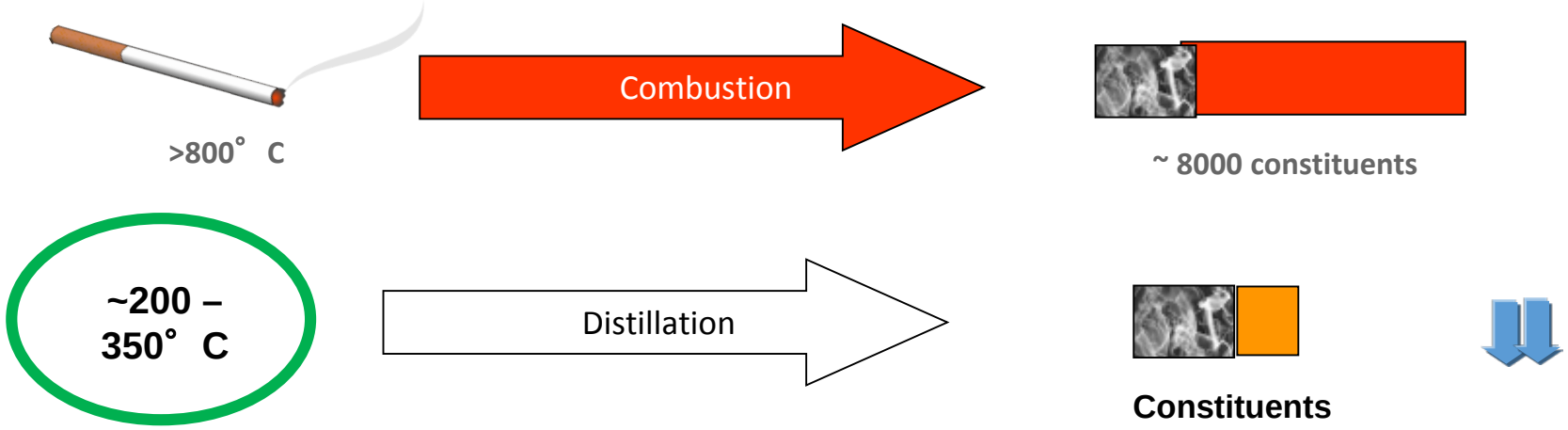
Cigarette Smoke *VS* Heat-Not-Burn

Underlying Principles

- Approximately 8000 constituents identified in cigarette smoke
- About a 100 of which constituents are categorized as harmful and potentially harmful (HPHCs)
- HPHCs are formed during combustion (burning) of the tobacco
- As multiple HPHCs are likely to be responsible for tobacco-related diseases – selective reduction not an effective approach



Lower temperatures reduce constituents in the aerosol

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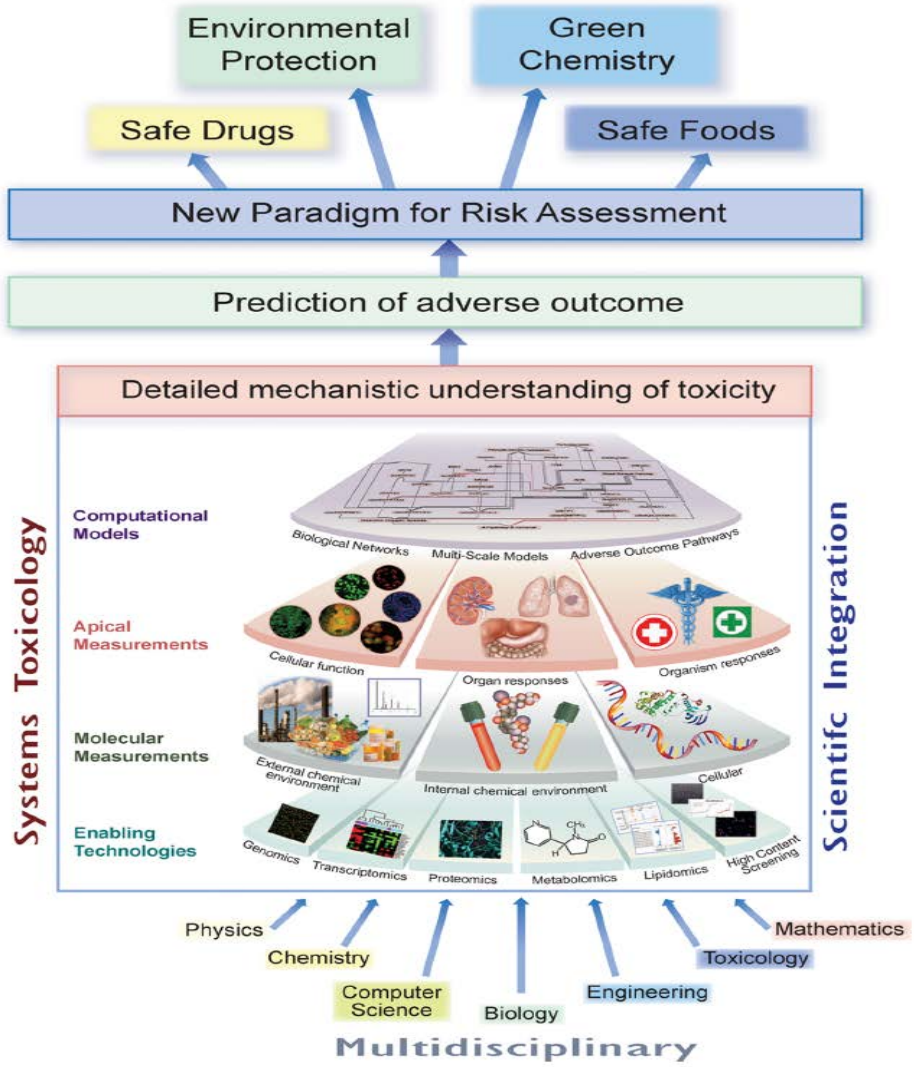
Systems Toxicology Approach For Product Assessment



"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
→ Can supports identification of biomarkers for safety assessments
→ Toward predictive mathematical models of toxicological processes

Sturla et al. Chemical Research in Toxicology (2014)

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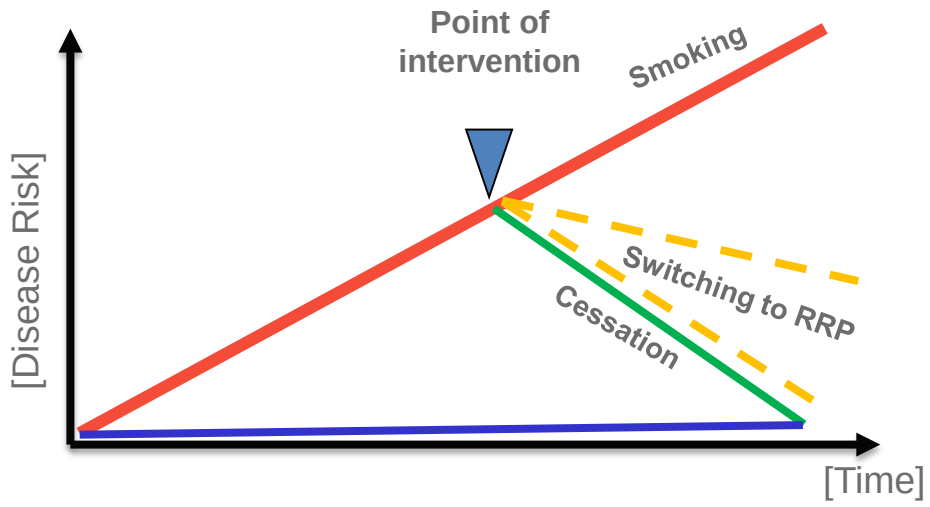
Design of Studies

Why Animal Models Switching Studies?

Comparing switching to MRTPs with ongoing smoking and benchmarking against cessation



- Main objective:
  Do switching from 3R4F cigarettes to THS2.2, a candidate MRTP (cMRTP) halt or delay the progression of vascular and respiratory pathologies?
  If so, what are the cellular and molecular mechanisms affected by switching to cMRTP exposure and how similar are these mechanisms to smoking cessation?

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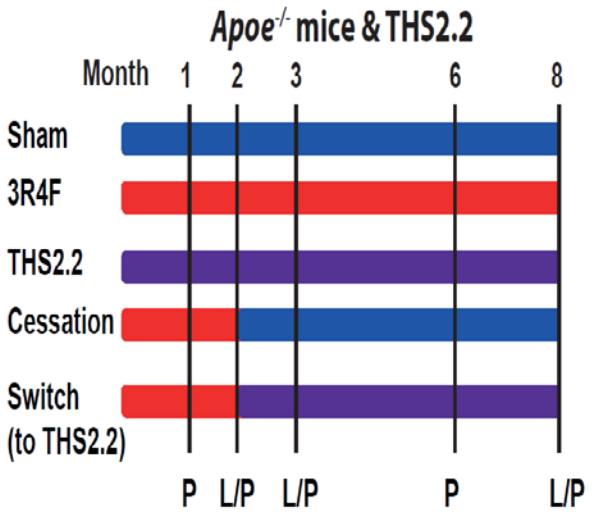
ApoE-/- Study Design



- **ApoE-/- mice model** is an accepted model for the study of cardiovascular diseases. These mice are prone to develop premature atherosclerosis and emphysema.
- **8-month study** on the effects of Sham (control), cigarette smoke (3R4F), cMRTP (THS2.2) aerosol, cessation, and switching to THS2.2 in Apolipoprotein E-deficient (ApoE-/-) mice. All groups n=8 biological replicates analyzed. Months analyzed are: 1, 2, 3, 6 and 8.
- **Nicotine concentration matched between 3R4F and THS2.2** exposure groups (29.9 mg/m^3 nicotine)
- **Conducted comprehensive system toxicology study** with special emphasis on respiratory and cardiovascular effects including:
  - In-life observations and biomarkers of exposure
  - Hematology and clinical chemistry
  - Histopathology
  - Aortic arch plaque formation
  - Lung function and BALF analysis
  - Transcriptomics, **proteomics**, and lipidomics

Phillips, B. et al. **An 8-Month Systems Toxicology Inhalation/Cessation Study in Apoe-/- Mice to Investigate Cardiovascular and Respiratory Exposure Effects of a Candidate Modified Risk Tobacco Product, THS 2.2, Compared with Conventional Cigarettes**. Toxicological sciences : doi:10.1093/toxsci/kfv243 (2015).

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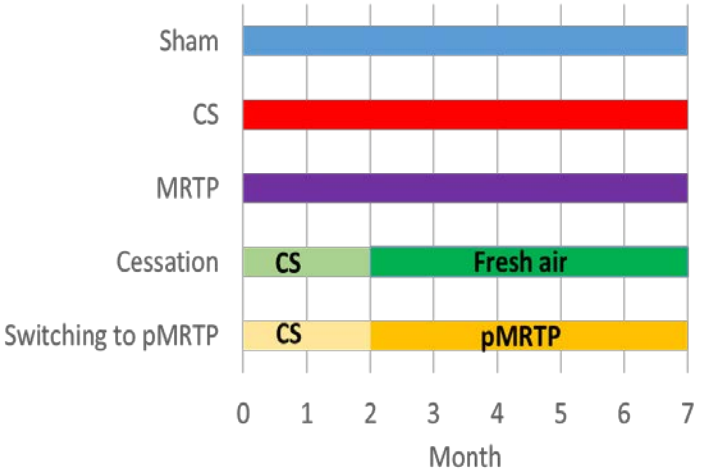
COPD II Study Design



- **C57BL/6 mice** are the accepted model for the study of COPD disease. The mice are prone to developing emphysema, a form of chronic obstructive pulmonary disease (COPD).

- **7-month study** on the effects of cigarette smoke (3R4F), pMRTP aerosol, cessation, and switching to pMRTP. All groups n=6 biological replicates analyzed. Months analyzed are: 1, 3, 5, and 7.

- **Nicotine concentration matched between 3R4F and pMRTP** exposure groups (34.4 mg/m^3 nicotine).

- **Conducted comprehensive system toxicology study** with special emphasis on respiratory and cardiovascular effects including:
  - In-life observations and biomarkers of exposure
  - Hematology and clinical chemistry
  - Histopathology
  - Lung function and BALF analysis
  - **proteomics**, transcriptomics and lipidomics

Phillips, B. et al. **A 7-month cigarette smoke inhalation study in C57BL/6 mice demonstrates reduced lung inflammation and emphysema following smoking cessation or aerosol exposure from a prototypic modified risk tobacco product.** Food and chemical toxicology : 80, 328-345, doi:10.1016/j.fct.2015.03.009 (2015).

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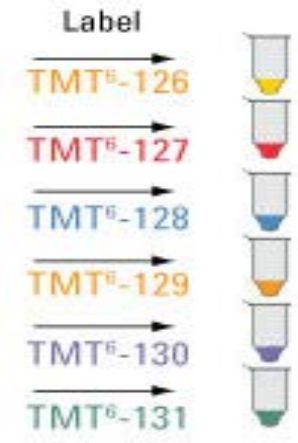
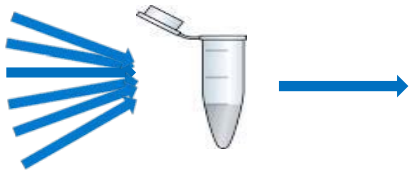
Proteomics Analysis Methodology

Quantitative Proteomics iTRAQ/TMT LC MS/MS Approach

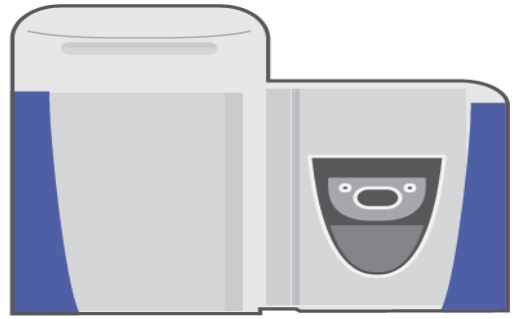
randomized processing order

Biological sample → Proteins → Peptides





Peptide pool
6-plex =
6 samples in 1



nanoLC connected to a
Q-Exactive (Thermo Scientific)

Quality Control:

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

TMT-Labeling:

-Sham
-3R4F
-cMRTP/pMRTP
- Cessation
- Switch
- refmix
→ Pool treatments
→ Months separated

randomized channel/pool assignments

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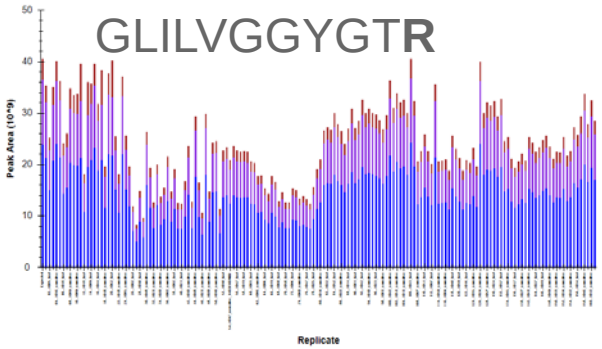
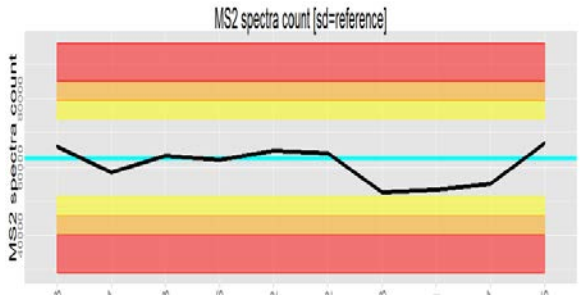
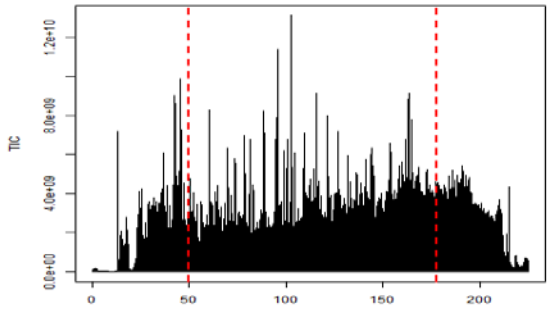
In-House Developed QC Pipeline







LC-MS/MS performance over batch/study analysis

Freeware/open source tools:

- Skyline, OpenMS, R

Checked samples:

- Reference peptide standard
- Reference complex proteome sample
- Study Samples

QC parameters include:

- LC-parameters:
  - Peak-shape, RT-stability,…
- MS-parameters:
  - Total ion-current, acquired spectra, assigned spectra, number of PSMs, peptides, protein IDs, …
- iTRAQ/TMT
  - labeling efficiency

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Isobaric-Tag based Approach 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)

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

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).

.raw files ***PSMS file*** ***result files***

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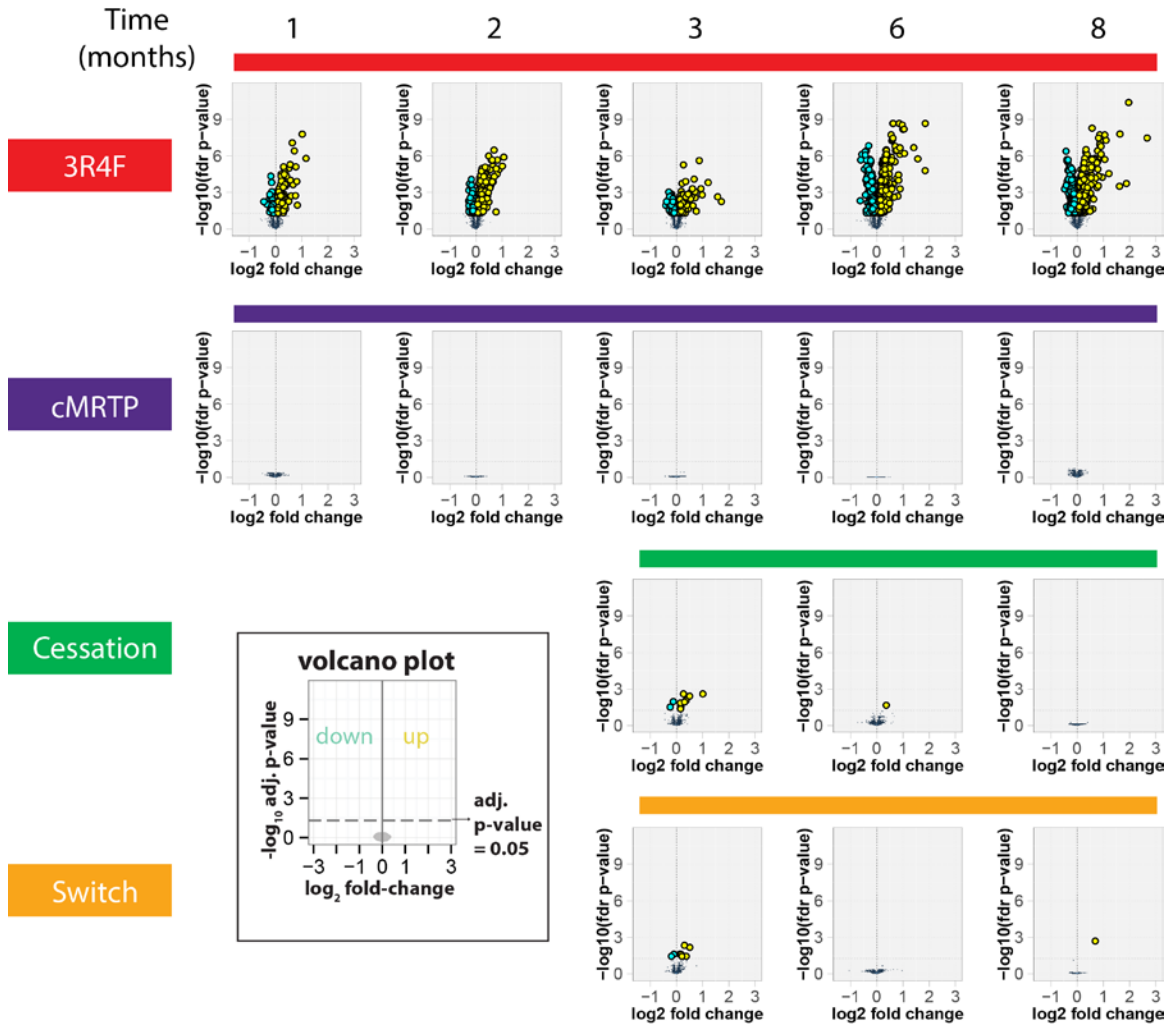
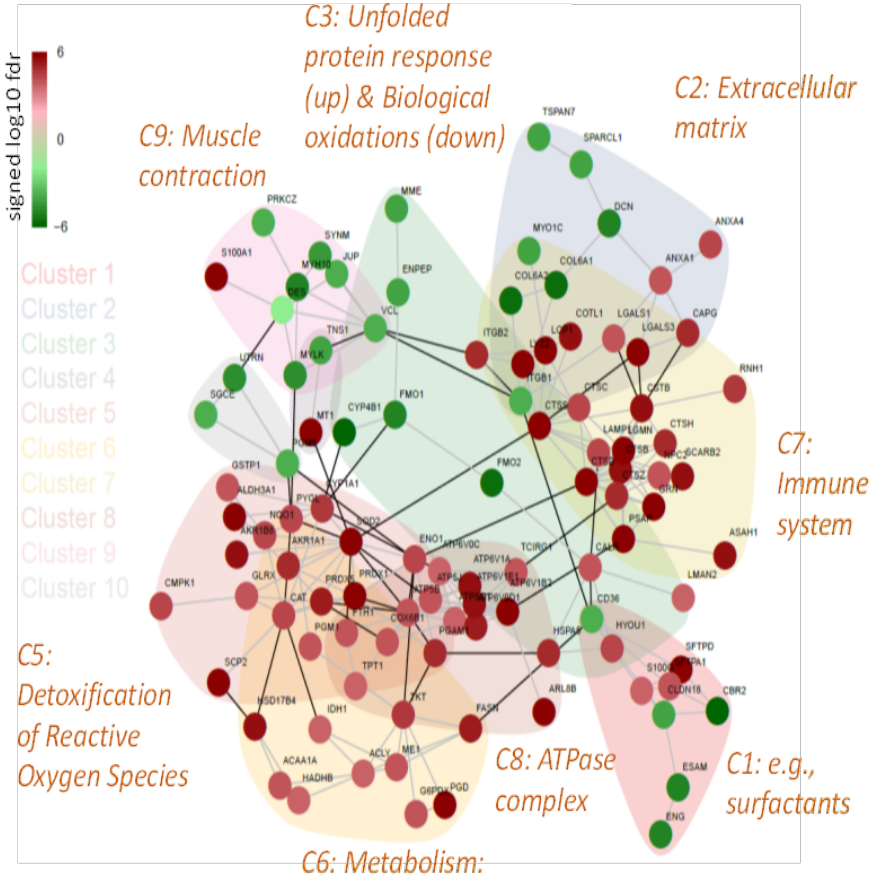
Results

ApoE-/- Study Results





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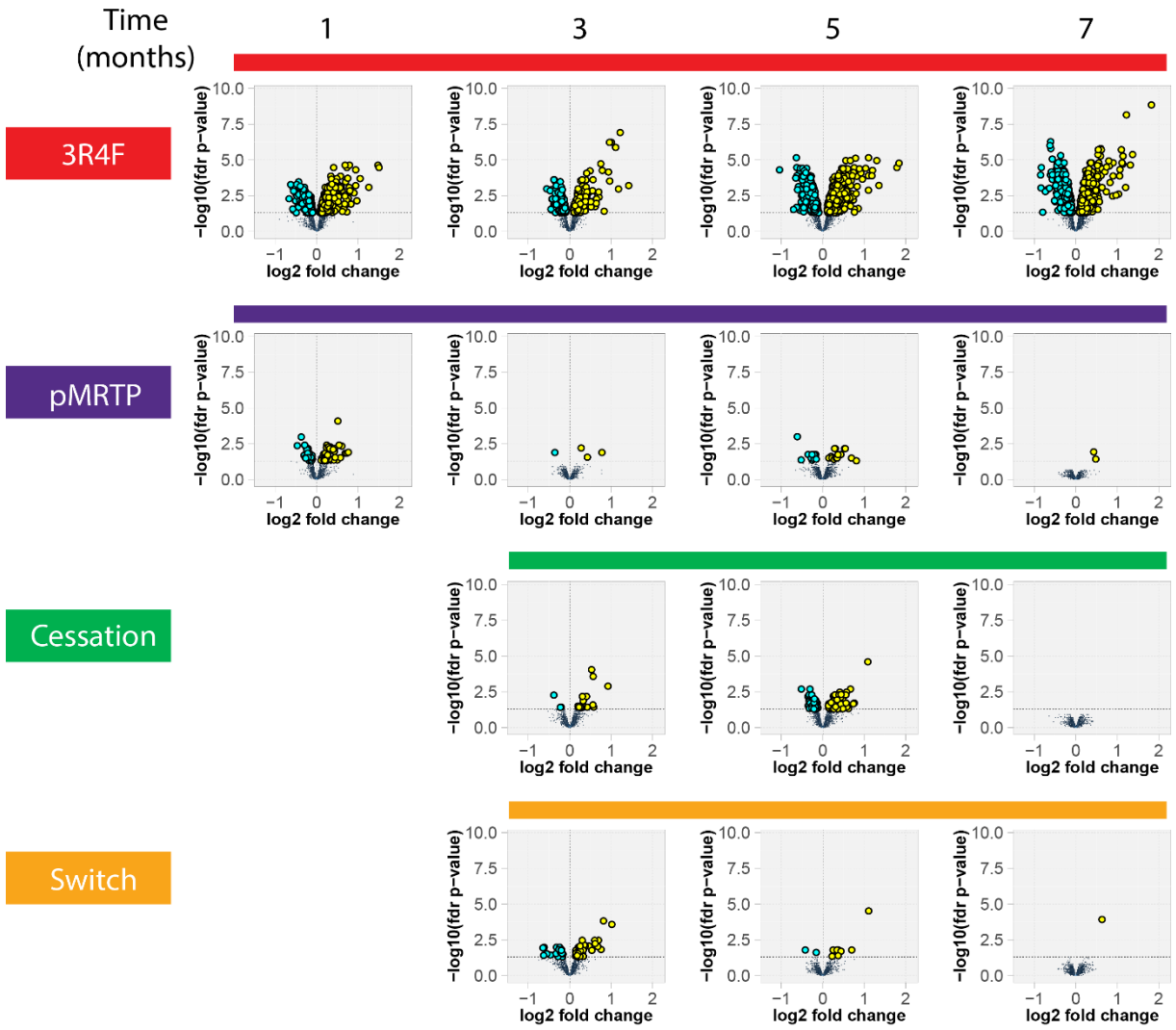
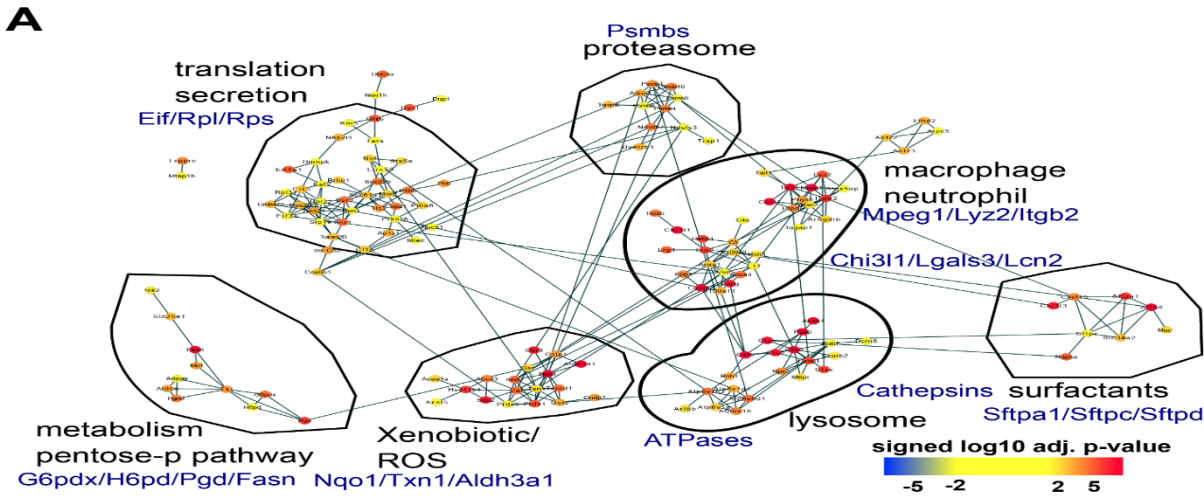
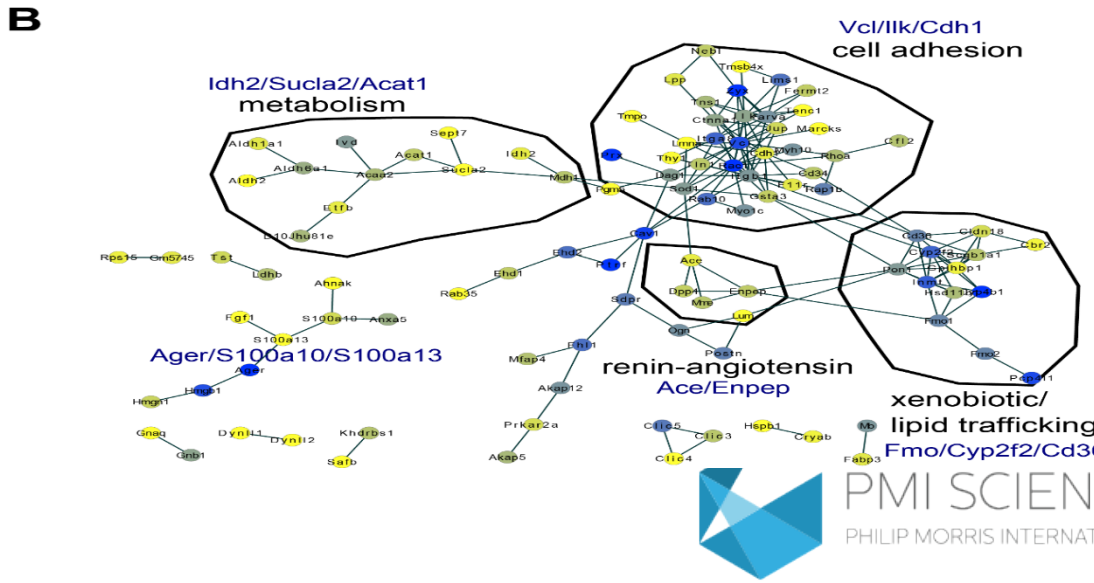
COPD II Study Results







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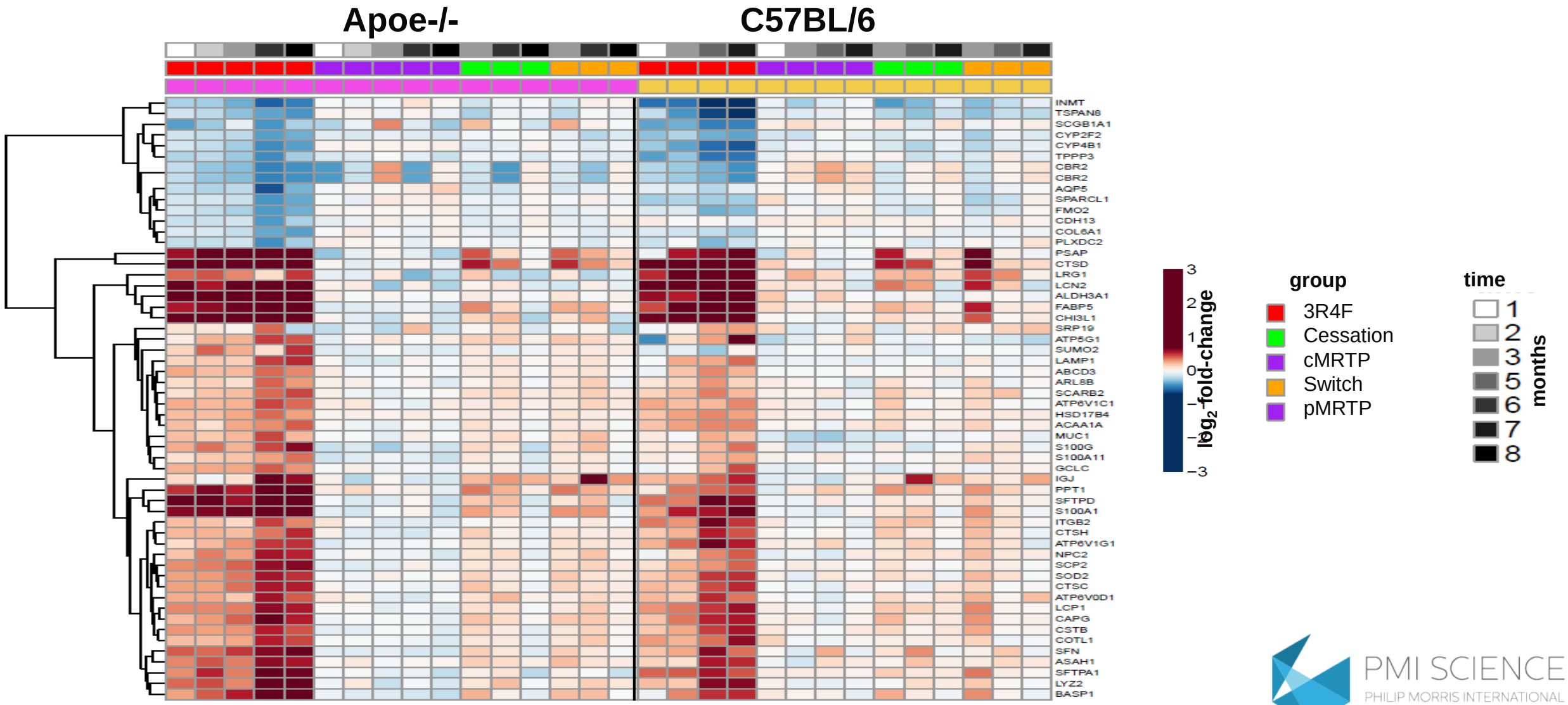
Integration of ApoE-/- and COPD II Studies Datasets



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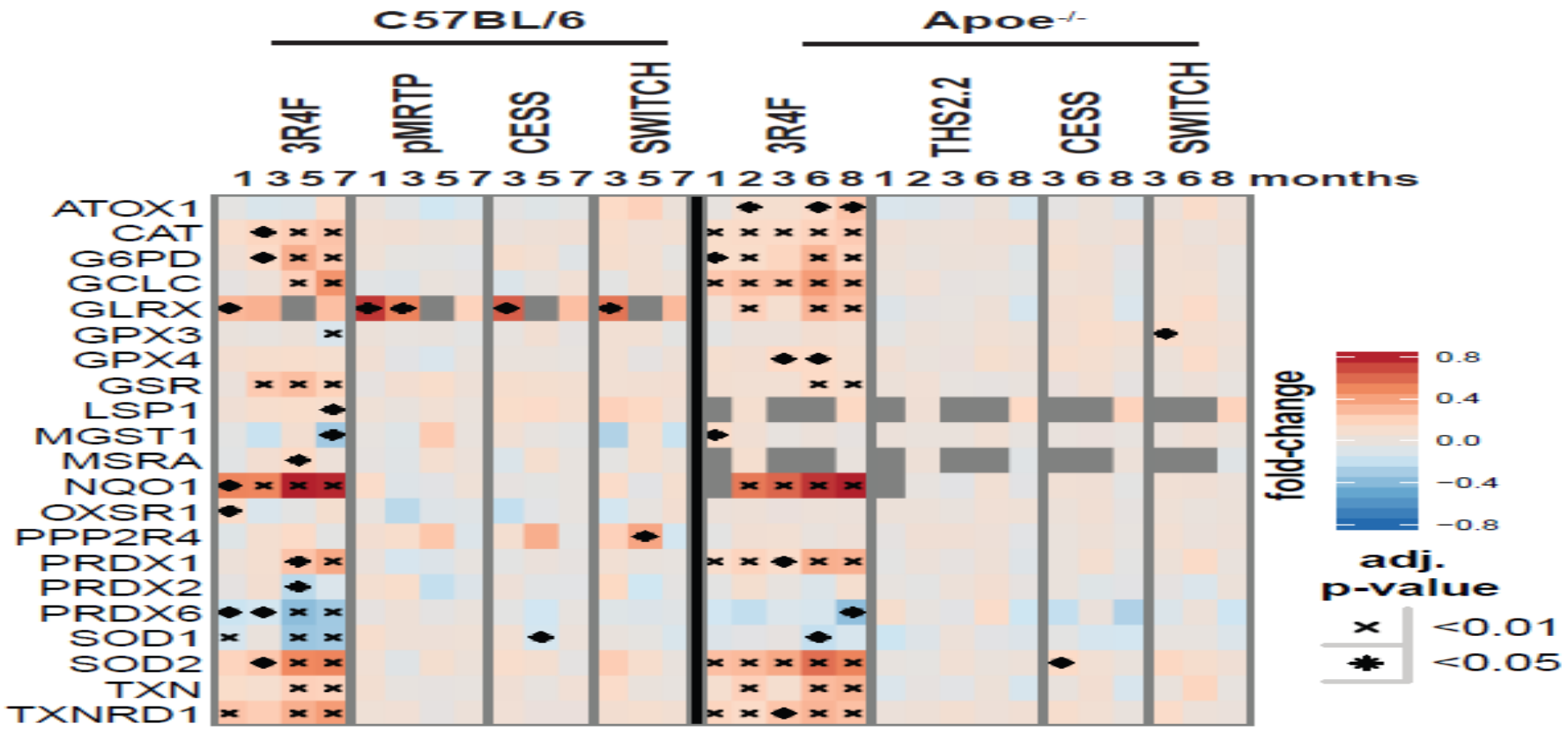
Response of Proteins in Oxidative Stress Pathway



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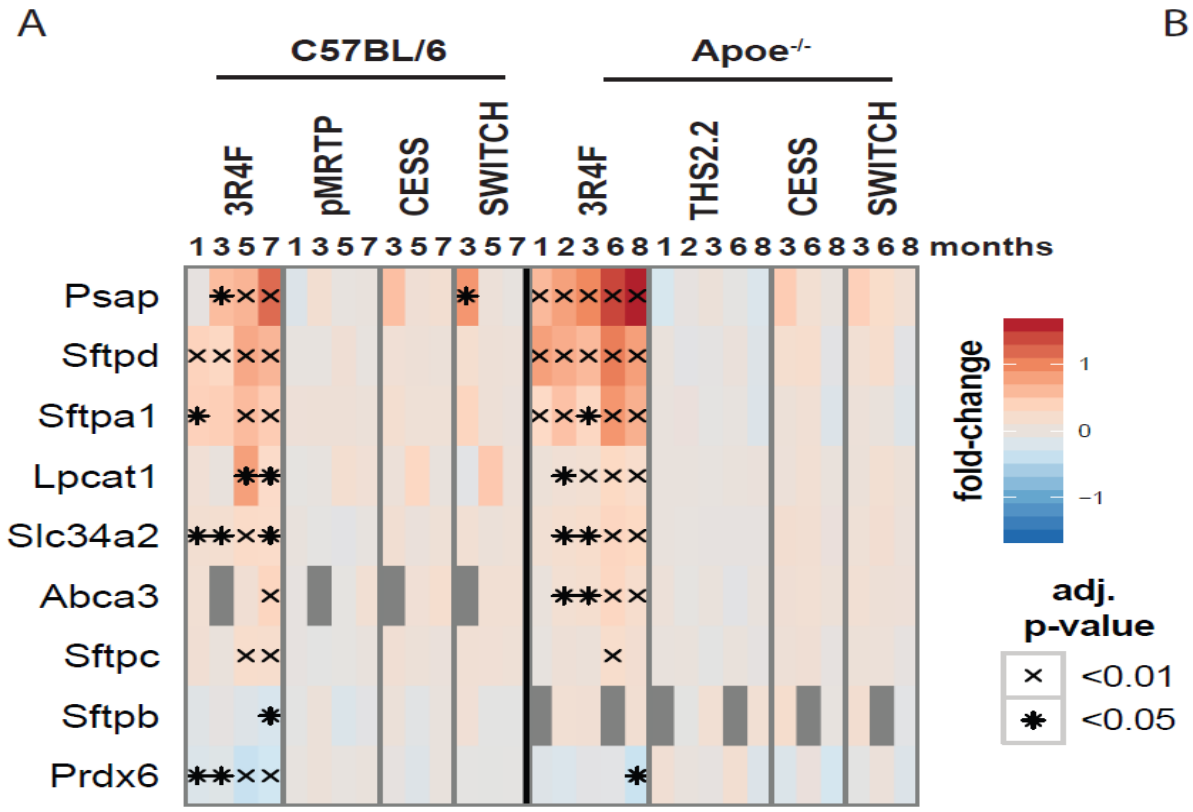
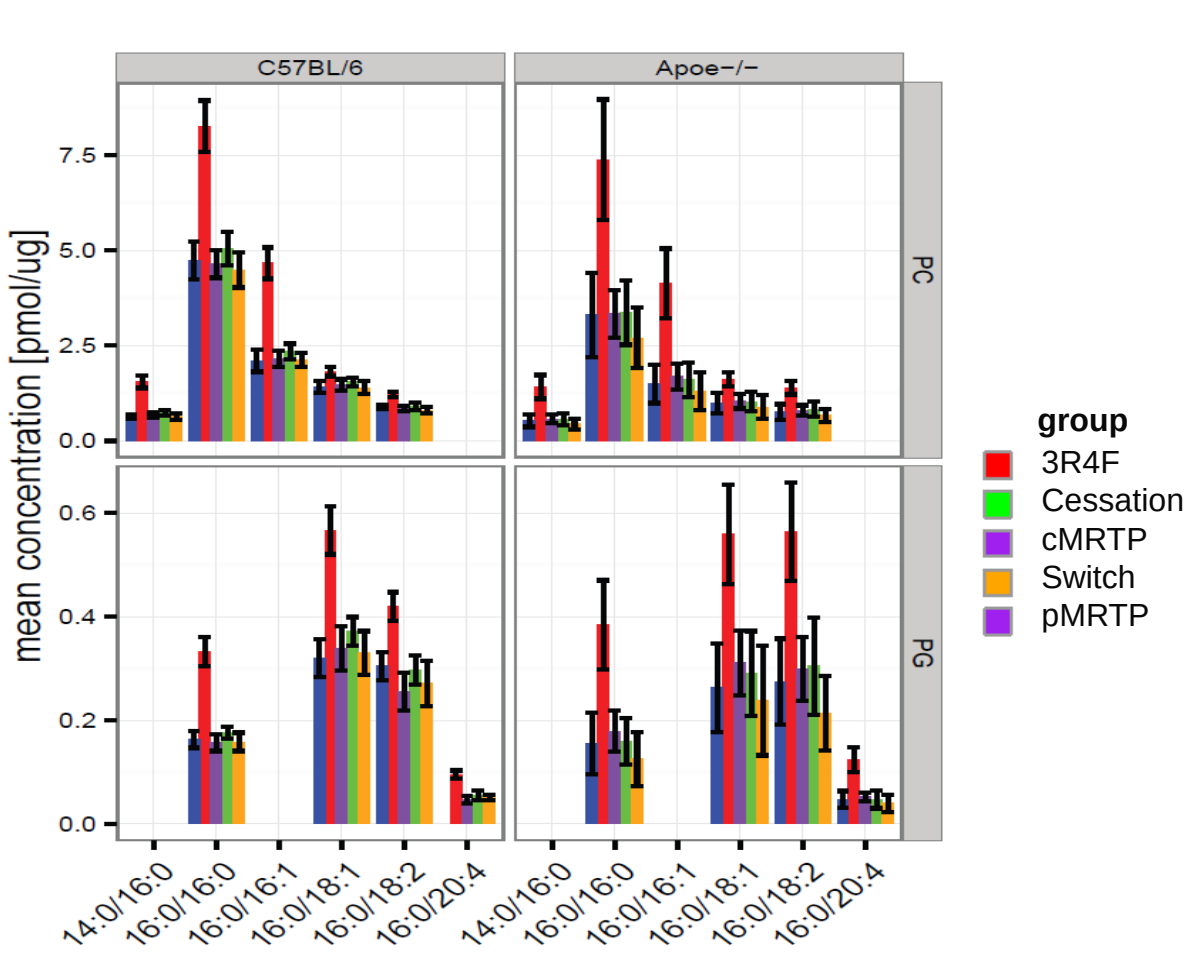
Surfactant Proteins and Lipids Responses





Titz, B. et al. **Effects of cigarette smoke, cessation and switching to two heat-not-burn tobacco products on lung lipid metabolism in C57BL/6 and Apoe-/- mice - an integrative systems toxicology analysis.** Toxicological sciences: doi:10.1093/toxsci/kfv244 (2015).

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Conclusions

- **Isobaric-tag based quantitative proteomics approach** was successfully implemented to assess the effects of cigarette smoke (CS), cMRTP and pMRTP aerosols on lung proteome of 2 strains of mice.
- Exposure to mainstream **cigarette smoke (CS) induced a strong effect on the lung proteome** with more than 700 differentially regulated proteins were detected after 7/8 months exposure to 3R4F. The **other experimental groups showed only a limited number differentially expressed proteins** as compared to the CS group. Effects detected were comparable on both strains of mice.
- **Proteomics analysis captured** the effect of CS exposure on a **broad range of biological functions** including xenobiotic response, ROS response, immune-response, metabolism, surfactants, ECM, and unfolded-protein response. These effects were not detected in the cMRTP and pMRTP exposure groups and reversed back to levels close to sham levels upon switching and cessation.
- **The integration of proteomics datasets with transcriptomics (data not shown), lipidomics and histopathological datasets proved to be valuable mechanistic insight in our systems toxicology approach in the assessment of MRTPs.**

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The Singapore research laboratories
Blaine Phillips

Declaration of Interest
All mentioned names in this presentation were employees of Philip Morris Products S.A. (or NAME OF ENTITY IN SINGAPORE) (both part of Philip Morris International group of companies) when they made their contributions to the study. Philip Morris Products S.A. is the sponsor of this project.

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