PMI RESEARCH & DEVELOPMENT

Integrating multi-omics and high-content screening data to link biological network perturbations with cellular phenotypes to elucidate pathways of toxicity

Ignacio Gonzalez Suarez, Ph.D.

Senior scientist,
Philip Morris International R&D

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How to apply omics and cellular imaging to toxicological assessment

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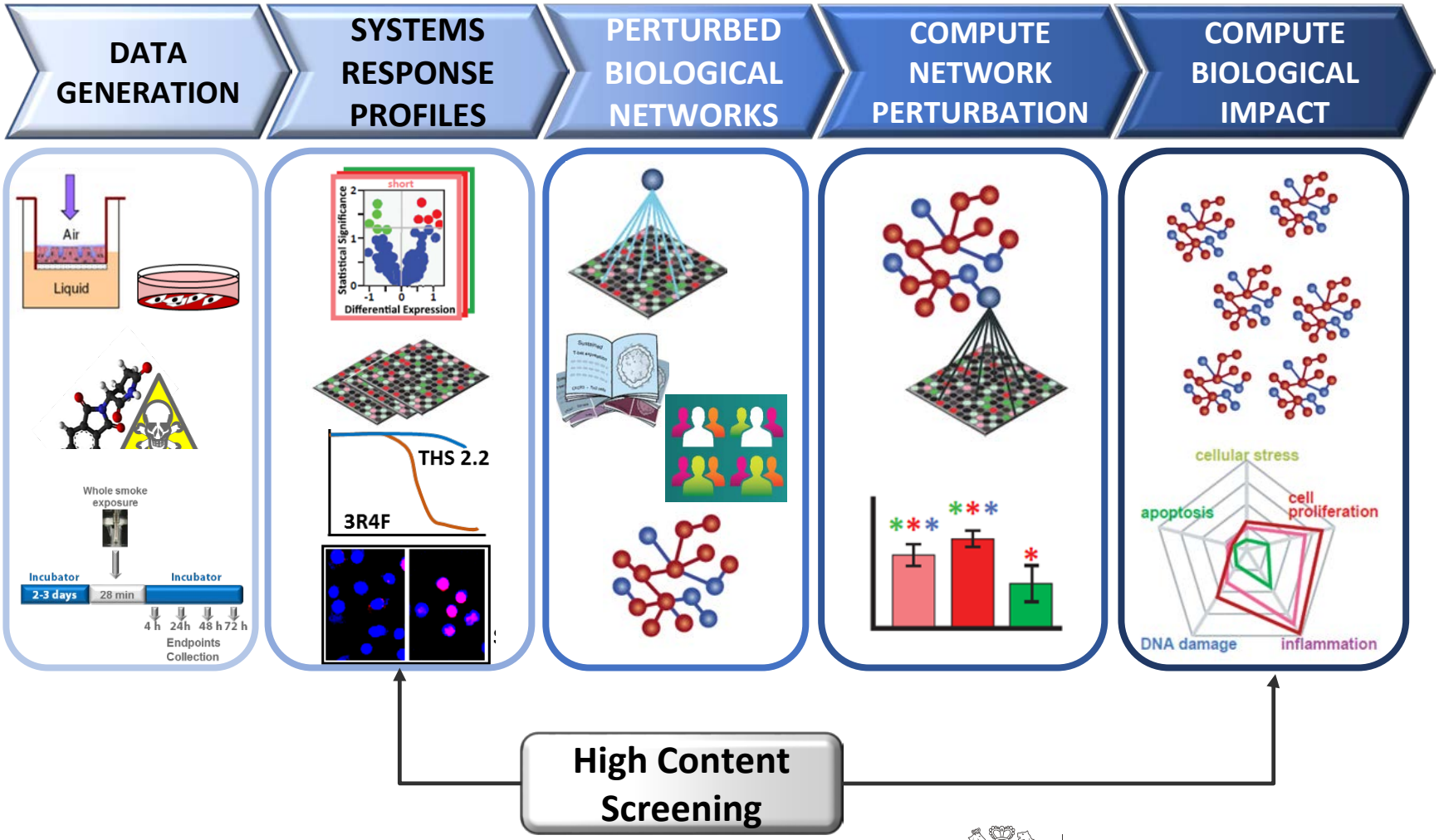
Systems Toxicology Assessment workflow at PMI

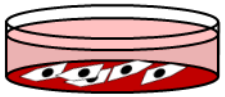


Toxicological Assessment of Tobacco Products

Toxicological Assessment of Tobacco Products

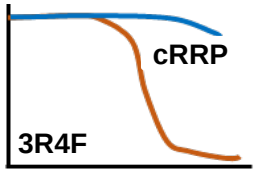
Study Design

Primary Human Bronchial epithelial cells (NHBE)

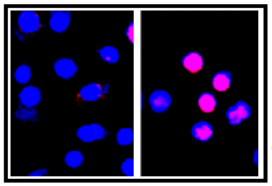


Exposure

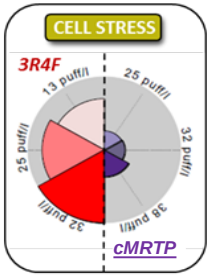
- **Conventional cigarette (3R4F)**
- **Candidate Reduced Risk Product (cRRP)**



Cell viability



High Content Screening

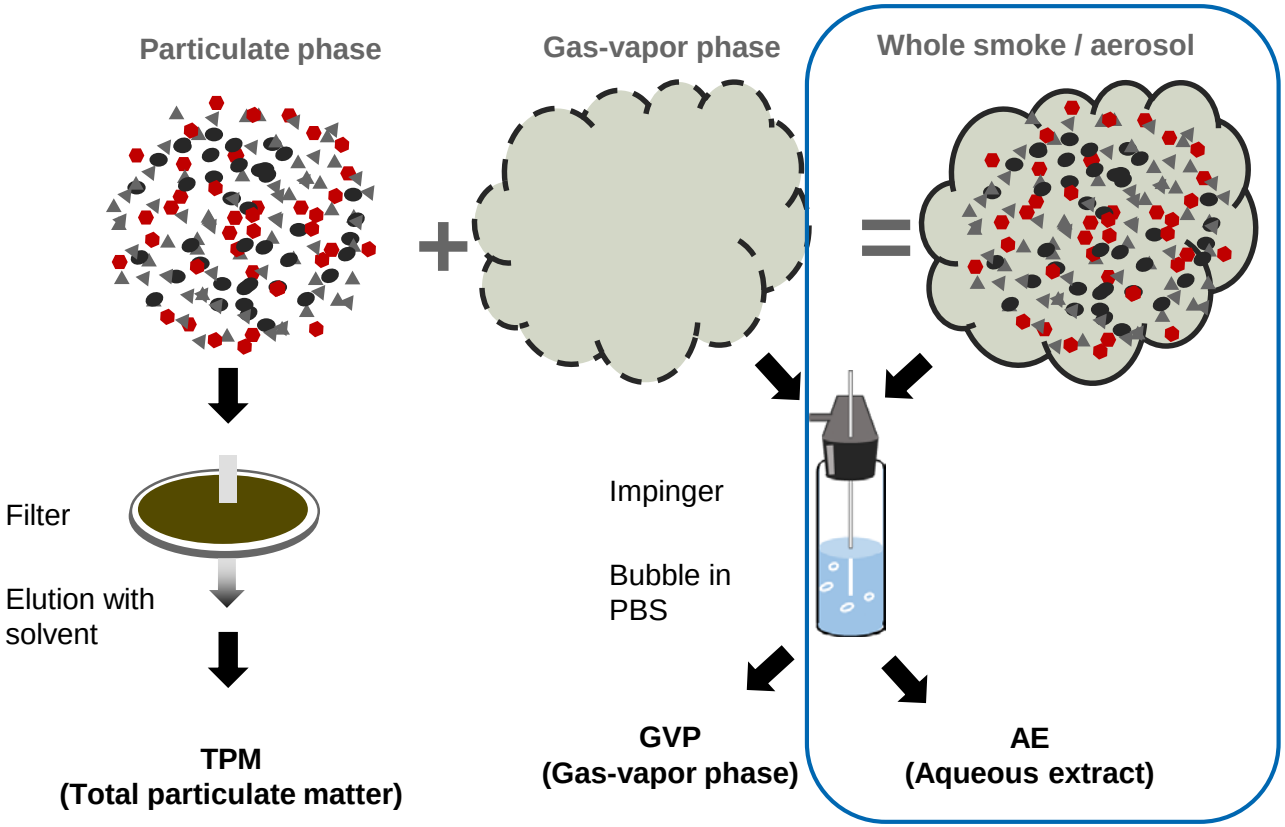


Gene Expression

What is the Biological impact of a cRRP compared to 3R4F?

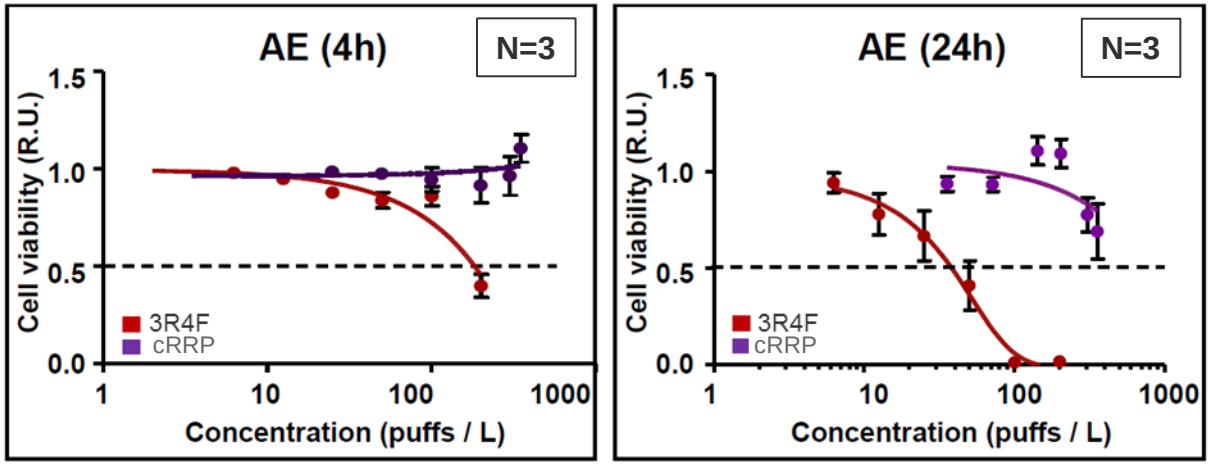
Toxicological Assessment of Tobacco Products

Cigarette smoke / cRRP aerosol



Toxicological Assessment of Tobacco Products

Step I: Cell Viability

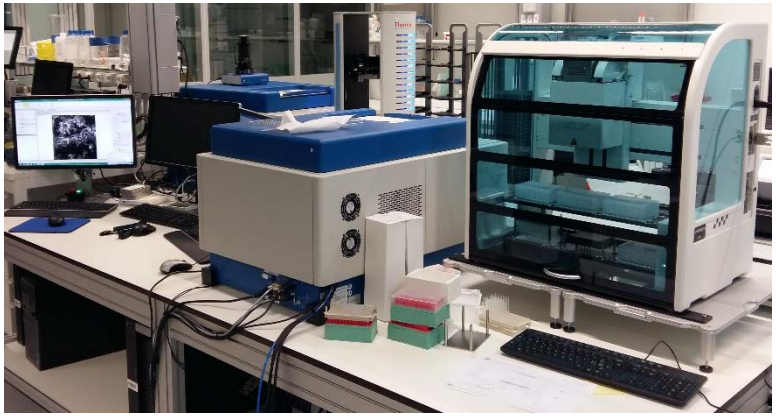


Gonzalez-Suarez et al. Chem. Res. Tox. 2015

- ✓ **Dose-dependent decrease in cell viability upon exposure to 3R4F AE.**
- ✓ **Selected appropriate doses for HCS analysis**

Toxicological Assessment of Tobacco Products

Step II: High-Content Screening

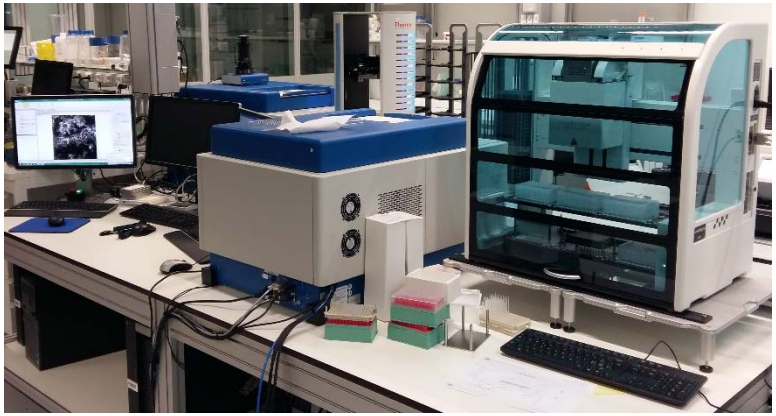


- **7** Different Assays
- **14** Toxicological endpoints
- **6** Doses + vehicle
- **3** replicate wells per dose
- **2** time points (4h & 24h)
- ≥ **3** independent experiments

• Cell Count • Cell cycle	**Proliferation**
• Phospho-H2AX	**DNA Damage**
• Phospho cJun • ROS formation • GSH content	**Cell / Oxidative stress**
• Caspase 3/7 • Cytochrome C • Membrane permeability • Nuclear size	**Apoptosis / necrosis**
• Mitochondrial mass • Mitochondrial potential	**Mitochondrial health**
• NF-κβ translocation	**Inflammation**
• Gap-Junction	**Cell communication**

Toxicological Assessment of Tobacco Products

Step II: High-Content Screening



- **7** Different Assays
- **14** Toxicological endpoints
- **6** Doses + vehicle
- **3** replicate wells per dose
- **2** time points (4h & 24h)
- ≥ **3** independent experiments

	3R4F		cRRP	
	4h	24h	4h	24h
Cell Count	-	100	-	200*
DNA Damage (p-H2AX)	200*	200*	-	-
Cell Stress (p-cJun)	-	100	-	200*
ROS Formation	-	100*	-	-
GSH Content	50	100	-	200*
Cell cycle	NA	13	NA	140
Apoptosis (Cytochrome C)	-	100	-	280*
Necrosis (membrane permeability)	100	100	350*	-
Mitochondrial Membrane Potential	-	100	-	280
Mitochondrial Mass	50*	200*	-	-

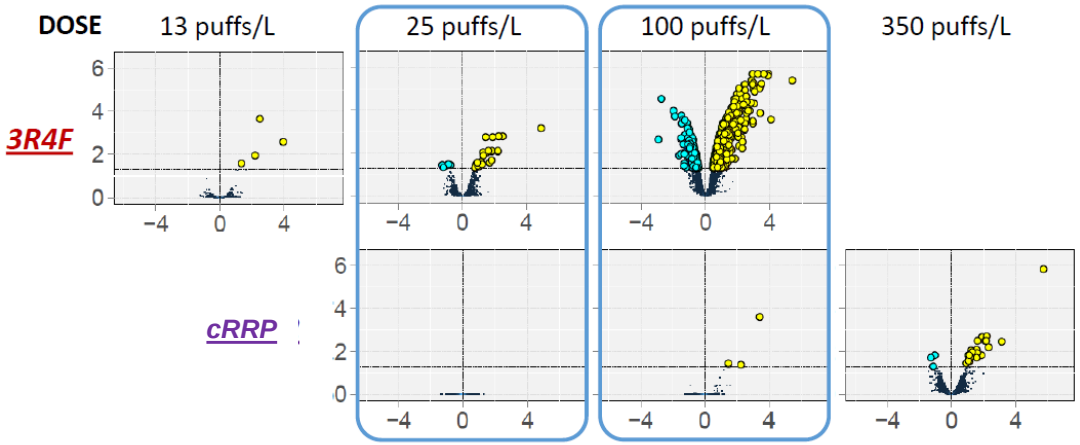
Gonzalez-Suarez et al. Chem. Res. Tox. 2015

- ✓ **Dose-dependent responses in multiple endpoints upon exposure to 3R4F.**
- ✓ **Selected appropriate doses for Transcriptomics**

Toxicological Assessment of Tobacco Products

Gene expression (DEG)

- **2** items: 3R4F & cRRP
- **3** Doses + vehicle
- **1** time points (4h)
- ≥ **3** independent experiments

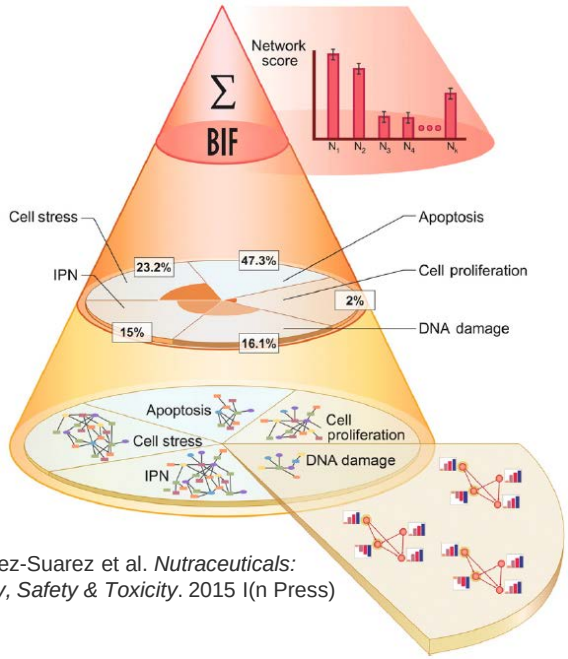


Gonzalez-Suarez et al. *Chem. Res. Tox*. 2015

✓ **Increased number of DEG in response to 3R4F compared to cRRP.**

Toxicological Assessment of Tobacco Products

Gene expression (Network Biology)



Gonzalez-Suarez et al. *Nutraceuticals: Efficacy, Safety & Toxicity*. 2015 I(n Press)

1. Biological Impact
2. Network Level Perturbation
3. Subnetwork Level Perturbation
4. Leading nodes

Step-wise approach

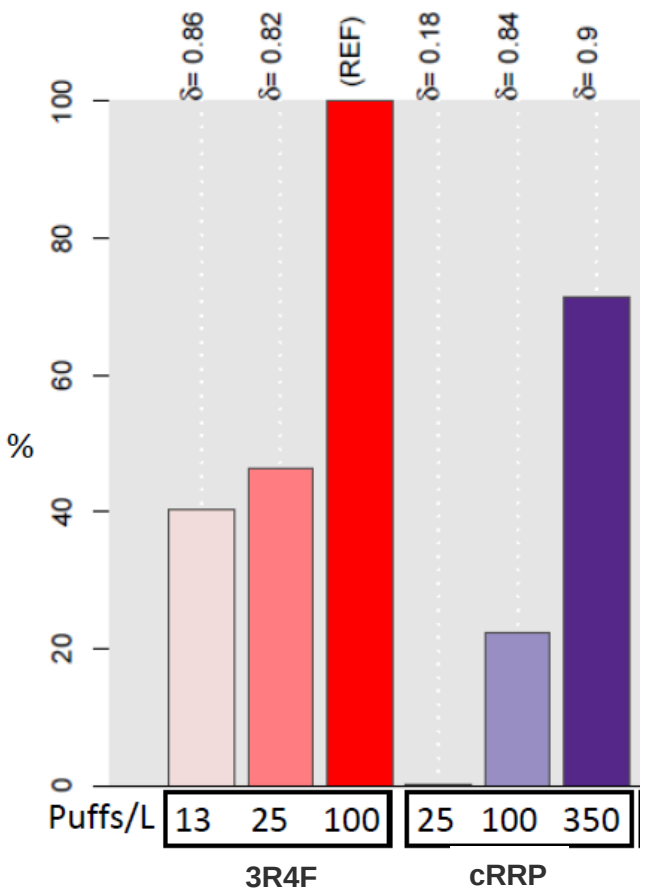
Increased Granularity

Increased mechanistic insight

Toxicological Assessment of Tobacco Products

Gene expression: Biological Impact Factor (BIF)

- **Sum of all perturbations** across all biological networks
- **Vehicle control (0%)**
- **Reference value (100%)**
- **Reference:** highest level of overall perturbation
- **δ value (-1 to 1):** compares underlying biology to reference

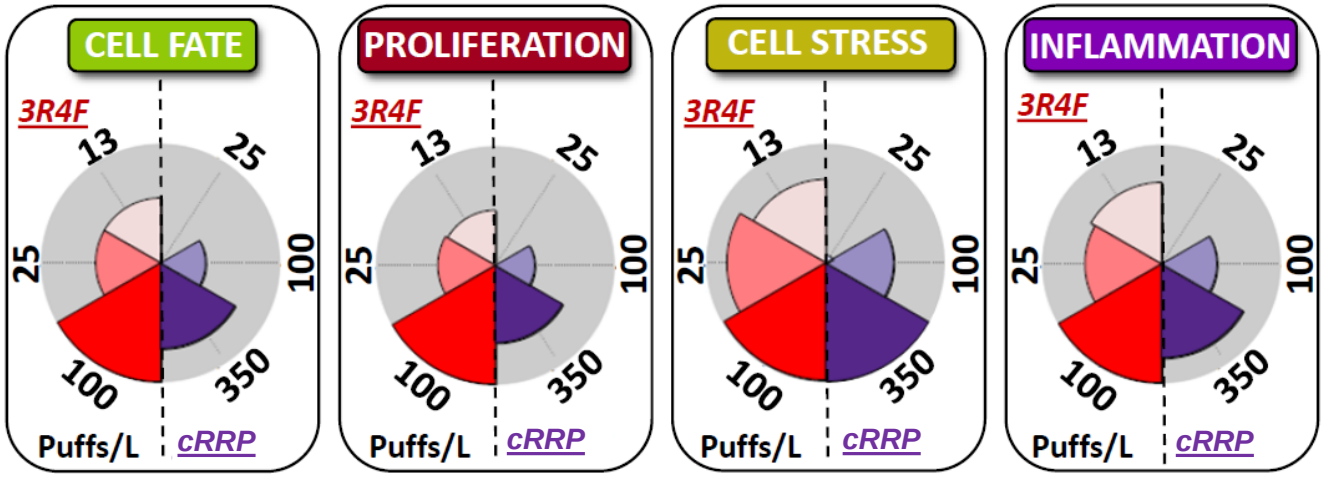


Gonzalez-Suarez et al. *Chem. Res. Tox*. 2015

- ✓ **Dose-dependent responses in 3R4F and cRRP.**
- ✓ **At comparable doses, lower biological impact of cRRP**
- ✓ **δ suggest similar underlying biology between 3R4F and cRRP**

Toxicological Assessment of Tobacco Products

Gene expression: BIF Mechanistic Components



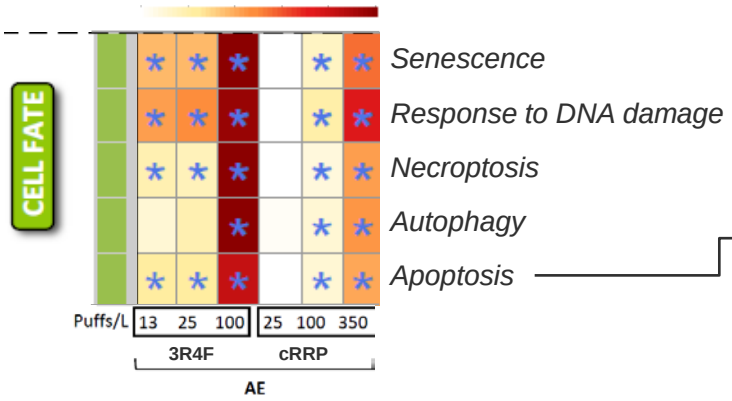
Gonzalez-Suarez et al. *Chem. Res. Tox*. 2015

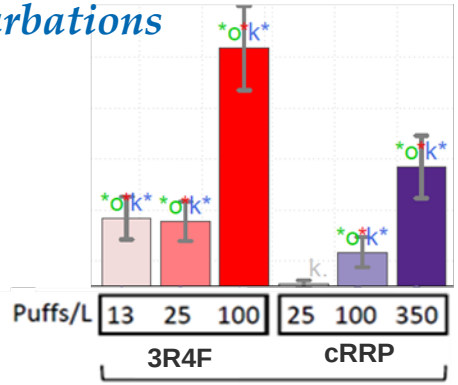
- **BIF values can be separated into its mechanistic components**
- **Surface area proportional to level of network perturbation**
- **Vehicle control (0%)**
- **Values normalized to reference**

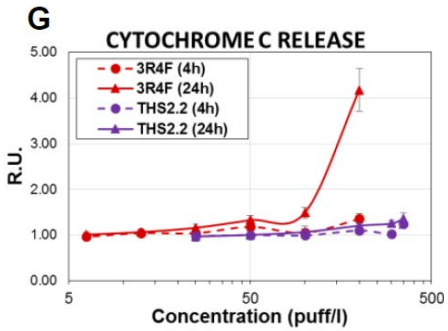
- ✓ **Dose-dependent responses in 3R4F and cRRP.**
- ✓ **At comparable doses, lower biological impact of cMRTP**

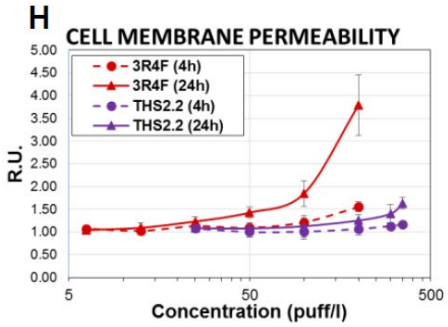
Toxicological Assessment of Tobacco Products

Gene expression: Cell Fate Subnetwork Perturbations





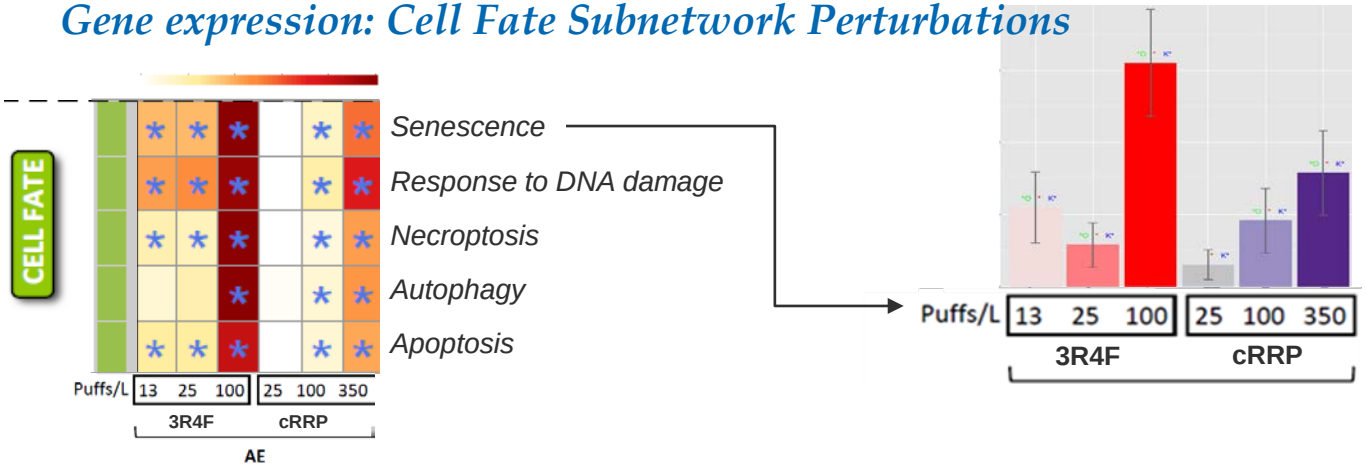


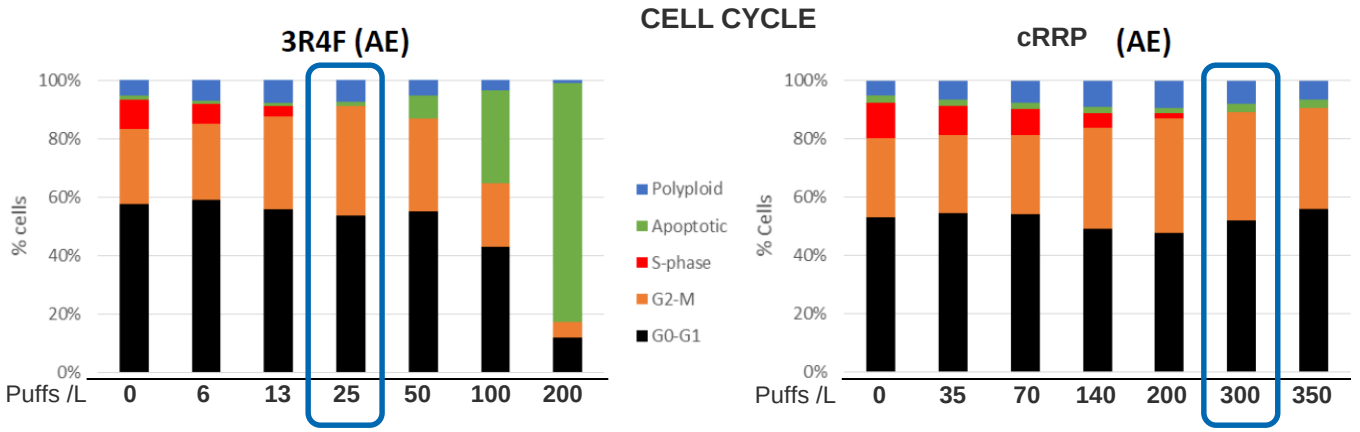


Gonzalez-Suarez et al. *Chem. Res. Tox*. 2015

Toxicological Assessment of Tobacco Products

Gene expression: Cell Fate Subnetwork Perturbations





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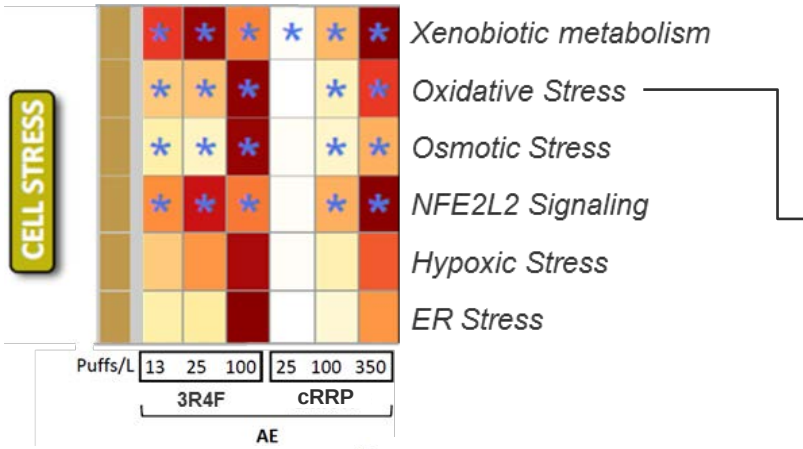
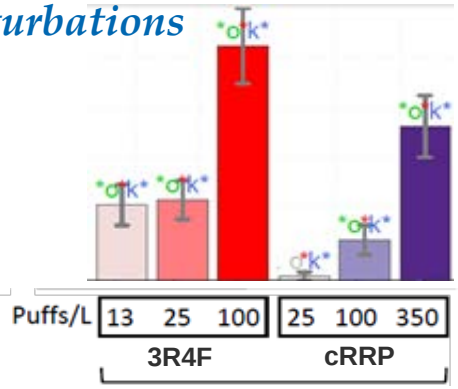
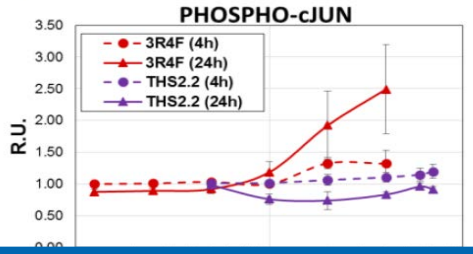
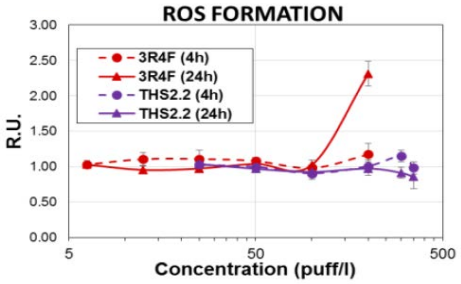
Toxicological Assessment of Tobacco Products

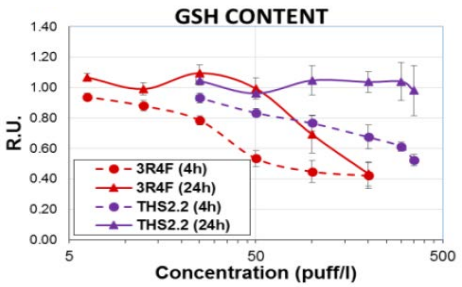
Gene expression: Cell stress Subnetwork perturbations











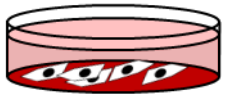
Gonzalez-Suarez et al. *Chem. Res. Tox*. 2015

Exposure to cRRP has a lower biological impact on NHBE cells compared to 3R4F

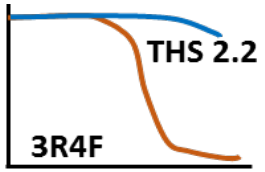
Toxicological Assessment of Environmental toxicants

Study Design

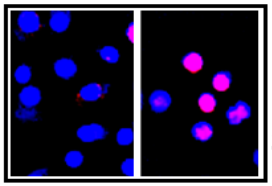
Primary Human Bronchial epithelial cells (NHBE)



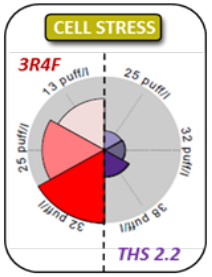
Exposure



Cell viability



High Content Screening



Gene Expression

- **Phase I (2012): 15 compounds***
- **Phase I (2014): 32 compounds***

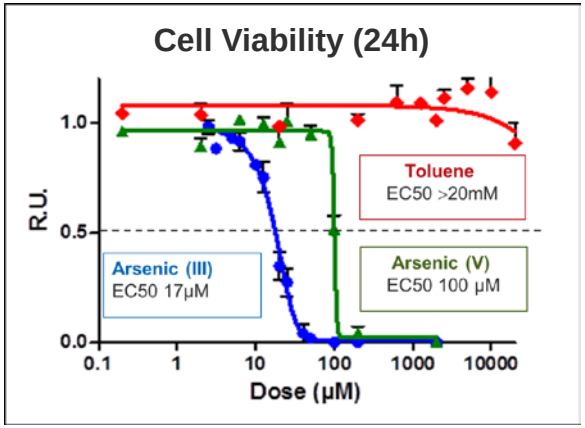
**HPHC (harmful & potentially harmful smoke constituents)*

What is the Biological impact of these chemicals in NHBE cells?

Toxicological Assessment of Environmental toxicants

Step I: Cell Viability

- **32** toxicants
- **6** Doses + vehicle
- **24h** exposure
- **≥ 3** independent experiments



	HPHC	EC50 Value	R^2
1	Chromium (VI)	4 µM	0.995
2	Arsenic (III)	17 µM	0.968
3	5-Methylchrysene	28 µM	0.961
4	Arsenic (V)	100 µM	0.990
5	Mercury (II)	110 µM	0.999
6	Selenium (IV)	338 µM	0.982
7	Crotonaldehyde	501 µM	0.994
8	Nickel (II)	520 µM	0.999
9	Lead (II)	528 µM	0.918
10	1-Aminonaphthalene	1000 µM	0.964
11	Naphthalene	1176 µM	0.902
12	m-Cresol	2028 µM	0.936
13	o-Cresol	2170 µM	0.912
14	p-Cresol	5060 µM	0.900
15	Acrilamide	5880 µM	0.981
16	Phenol	6680 µM	0.982

	HPHC	EC50 Value	R^2
17	o-Anisidine	11970 µM	0.968
18	2-nitropropane	> 20 mM	-
19	Acetamide	> 20 mM	-
20	Acetone	> 20 mM	-
21	Benzene	> 20 mM	-
22	MEK	> 20 mM	-
23	Nitrobenzene	> 20 mM	-
24	Quinoline	> 20 mM	-
25	Toluene	> 20 mM	-
26	Benz [a] anthracene	> 100 µM	-
27	Benzo [a] pyrene	> 100 µM	-
28	Benzo [b] fluoranthene	> 100 µM	-
29	Benzo [k]fluoranthene	> 100 µM	-
30	Dibenz [a,h] anthracene	> 100 µM	-
31	Dibenzo [a,l] pyrene	> 100 µM	-
32	Indeno [1,2,3-cd] Pyrene	> 100 µM	-

✓ **Dose-dependent decrease in cell viability observed in 17 toxicants.**

✓ **Selected appropriate doses for HCS analysis**

Toxicological Assessment of Environmental toxicants

High-Content Screening

- **6** Different Assays
- **13** Toxicological endpoints
- **6** Doses + vehicle
- **3** replicate wells per dose
- **2** time points (4h & 24h)
- ≥ **3** independent experiments

✓ **10 toxicants selected for Transcriptomics**

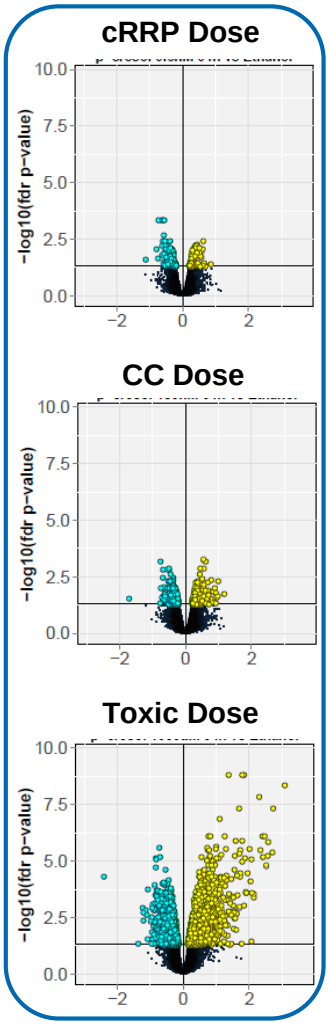
✓ **3 Doses selected for Transcriptomics:**

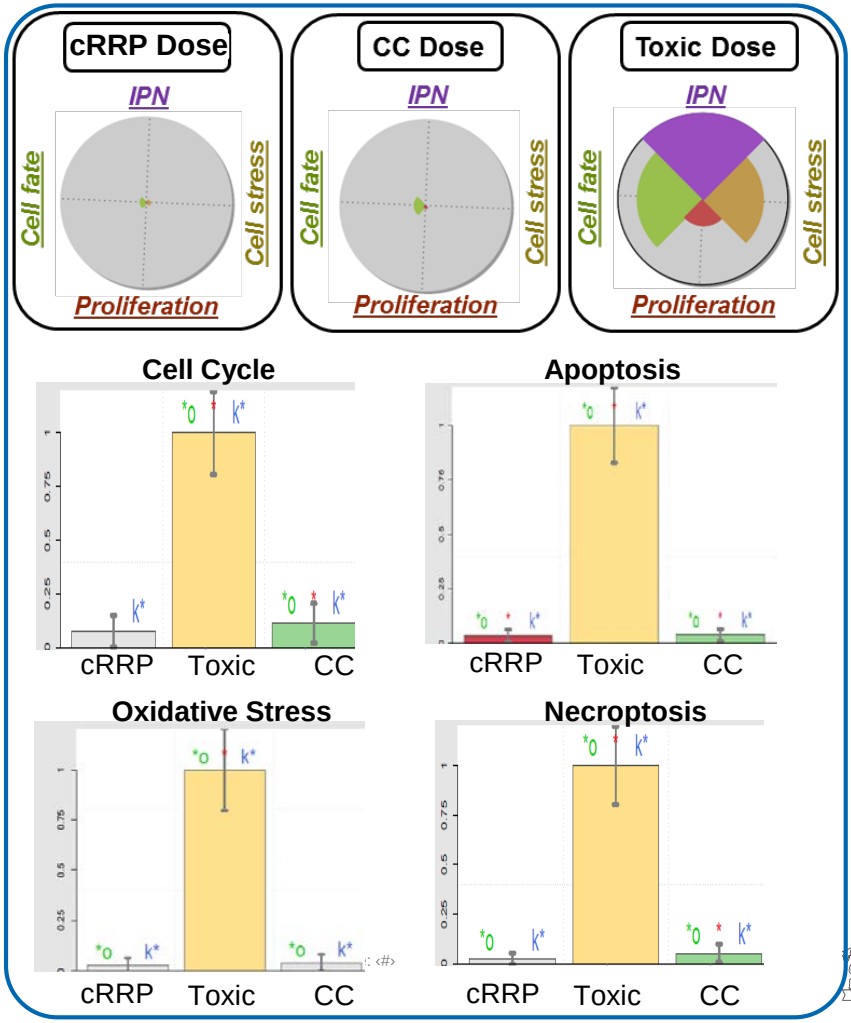
- cRRP dose
- 3R4F (CC) dose
- HCS-toxic dose

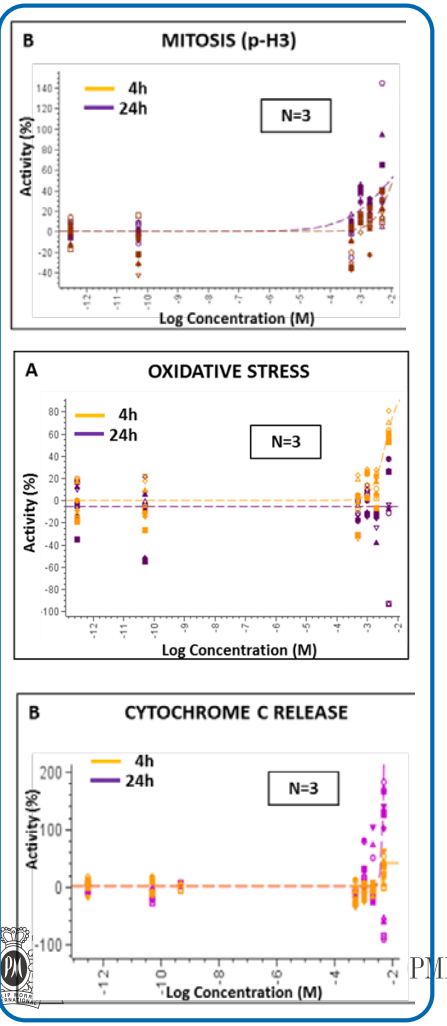
HPHC		Cell Loss	DNA Damage	Stress Kinase	GSH Content	Oxidative Stress	Caspase 3/7	Cytochrome C Release	Cell Membrane Permeability	Mitochondrial Membrane Potential	Mitochondrial Mass
5-Methylchrysene	4h	-	-	✓	✓	-	-	-	-	-	-
	24h	✓	✓	✓	✓	-	-	-	-	-	-
Arsenic (III)	4h	-	-	✓✓	✓	-	-	-	-	-	-
	24h	✓	✓	✓✓	✓✓	-	✓	✓✓	✓	✓	-
Lead (II)	4h	-	-	-	✓	-	-	-	-	✓	✓✓
	24h	✓	-	-	✓	-	-	✓	-	✓	✓✓
m-Cresol	4h	-	✓✓	✓	✓	✓	-	-	-	-	-
	24h	✓	✓✓	-	✓✓	-	✓	-	✓	✓	-
Mercury (II)	4h	-	✓✓	-	✓✓	✓	✓	✓✓	✓✓	✓✓	✓✓
	24h	✓	✓✓	-	✓✓	✓	✓✓	✓✓	✓✓	✓✓	✓✓
Naphthalene	4h	-	-	-	✓✓	✓	-	-	-	-	-
	24h	✓	✓	-	✓✓	-	-	-	-	-	-
o-Anisidine	4h	-	✓	-	✓✓	-	-	-	-	-	-
	24h	✓	✓✓	-	✓✓	-	✓✓	-	✓✓	-	✓
o-Cresol	4h	-	✓✓	-	✓✓	✓	-	-	-	-	-
	24h	✓	✓	-	✓✓	-	✓✓	✓	✓✓	-	-
p-Cresol	4h	-	✓✓	✓	✓	-	-	-	-	-	✓
	24h	✓	✓✓	✓	✓✓	-	✓	-	-	-	✓
Selenium (IV)	4h	-	✓✓	-	✓	✓	-	✓✓	-	-	✓✓
	24h	✓	✓✓	✓	✓✓	-	✓	✓✓	✓✓	-	-
1-aminonaphthalene	4h	-	✓✓	-	✓	✓✓	-	-	✓✓	-	-
	24h	✓	✓✓	✓	✓	✓✓	✓	✓	✓✓	-	-
Chromium (VI)	4h	-	✓	-	✓	-	-	-	-	-	-
	24h	-	✓✓	-	✓	-	✓✓	-	✓✓	-	-
Crotonaldehyde	4h	-	✓✓	-	-	✓✓	✓	-	✓✓	-	-
	24h	✓	✓✓	✓	-	✓	✓	✓✓	✓	✓	-
Acrylamide	4h	-	✓✓	-	✓	-	-	-	-	-	-
	24h	✓	✓✓	✓	✓	-	-	-	✓	-	-
Phenol	4h	-	✓	-	✓	-	-	-	-	-	-
	24h	✓	✓✓	-	✓	-	✓	-	✓	-	✓
Nickel (II)	4h	-	-	-	✓✓	-	-	-	-	-	-
	24h	✓	-	-	✓✓	-	✓	-	-	-	-
Arsenic (V)	4h	-	-	-	✓	-	-	-	-	-	-
	24h	✓	-	-	✓	-	-	-	-	-	-

In vitro Toxicological assessment of p-Cresol

Gene Expression *Network Biology* *High Content Screening*

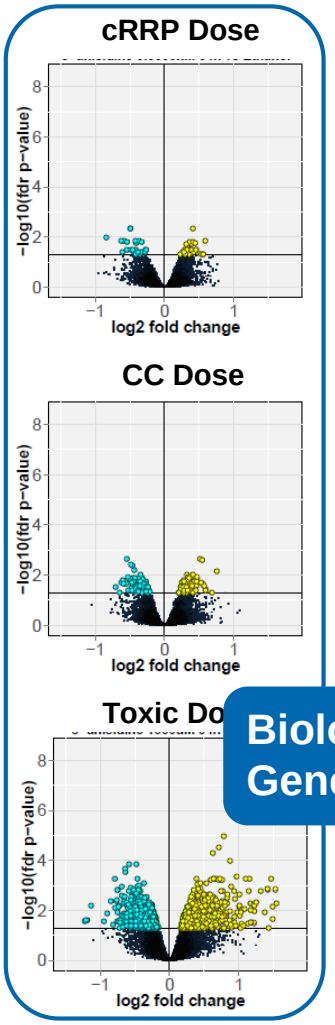


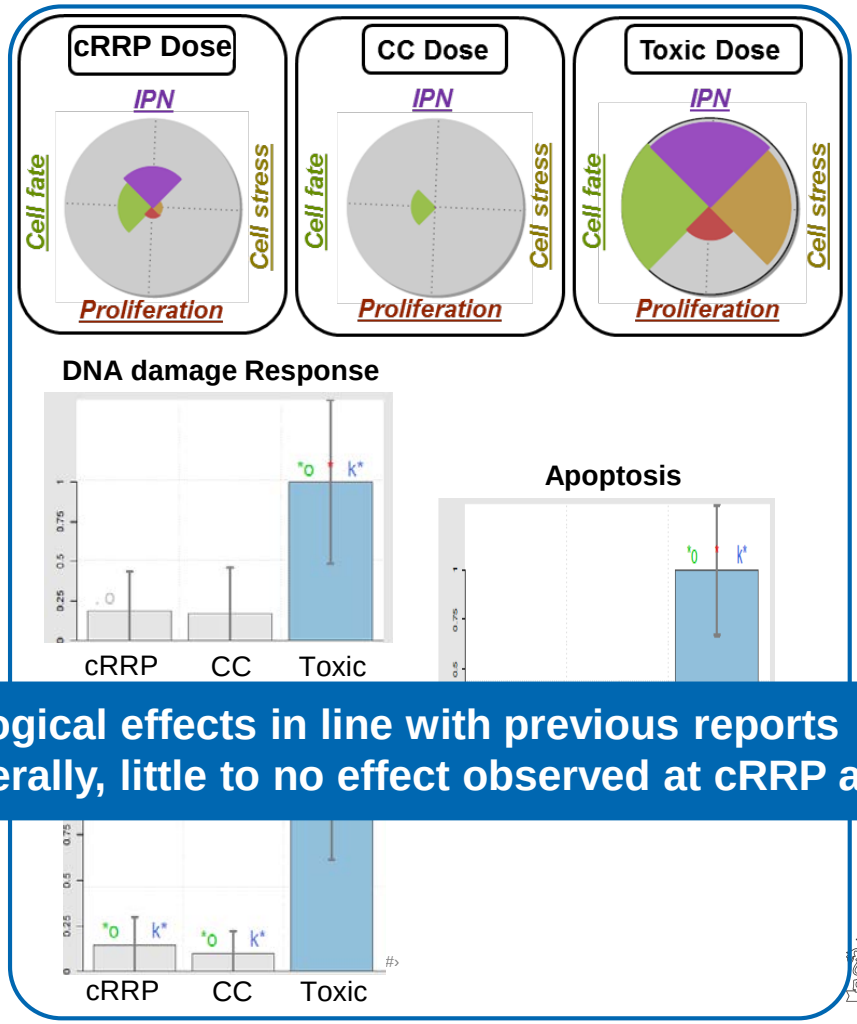


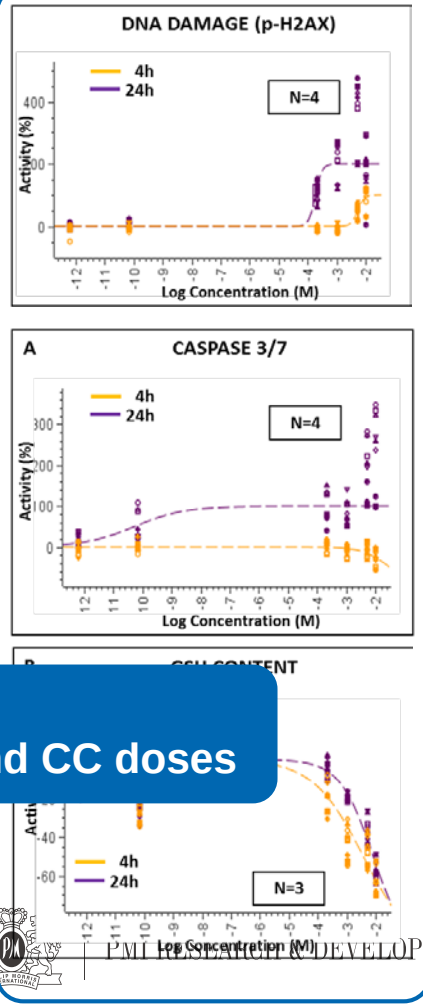


In vitro Toxicological assessment of o-Anisidine

Gene Expression *Network Biology* *High Content Screening*







Biological effects in line with previous reports
Generally, little to no effect observed at cRRP and CC doses

PMI RESEARCH & DEVELOPMENT

Summary

- The combination of systems biology and high-throughput imaging tools is a valuable approach to investigate molecular mechanisms of toxicity:
  - *Mechanistic insight into toxicity pathways activated upon exposure*
  - *Investigate biological perturbations at sub-cytotoxic exposures*
  - *Systematic and robust assessment*
- Challenges and future directions:
  - *Continuous improvement of Biological Networks*
  - *Incorporation of additional "omics" endpoints*
  - *Increase the number of HCS-based endpoints*
  - *Expand the number of cellular models*
- Ensure transparency and data traceability
- Foster collaboration and data sharing

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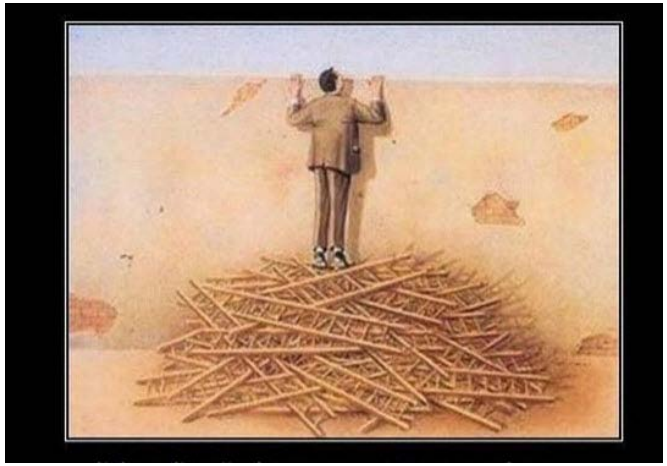
…and many more…

Thank you for your attention and Q&A



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“It doesn’t matter how many resources you have,

if you don’t know how to use them, they will never be enough”