Hepatic Transcriptome Comparative Analysis Reveals Similar Pathways and Targets Altered by Legacy and Alternative Per- and Polyfluoroalkyl Substances

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Office of Research and Development
Environmental Protection Agency

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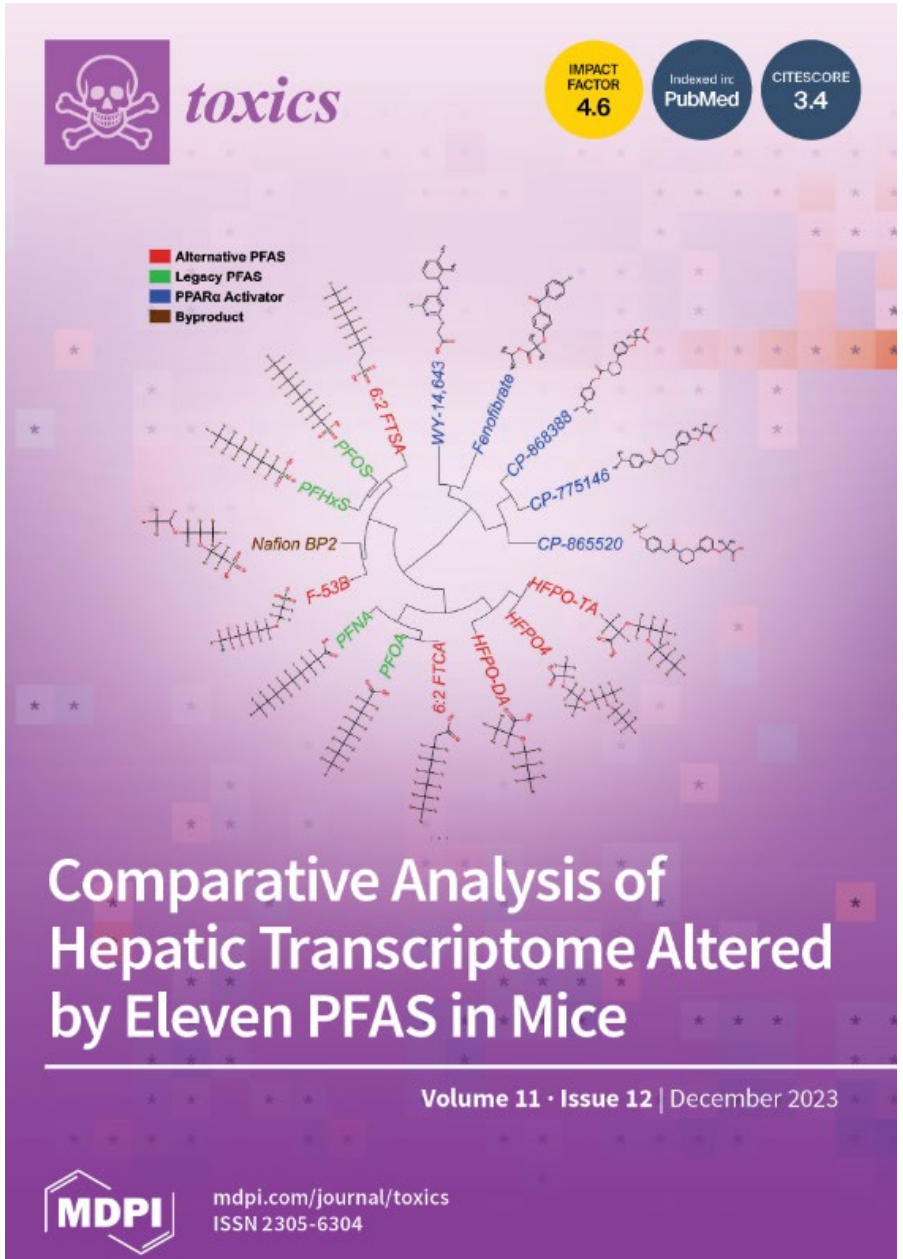
Agency



toxics

MDPI

Article

Hepatic Transcriptome Comparative In Silico Analysis Reveals Similar Pathways and Targets Altered by Legacy and Alternative Per- and Polyfluoroalkyl Substances in Mice

Dakota R. Robarts [1,2], Jiayin Dai [3], Christopher Lau [4], Udayan Apte [1] and J. Christopher Corton [2,*]

Dakota Robarts, PhD

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Chris Lau – EPA

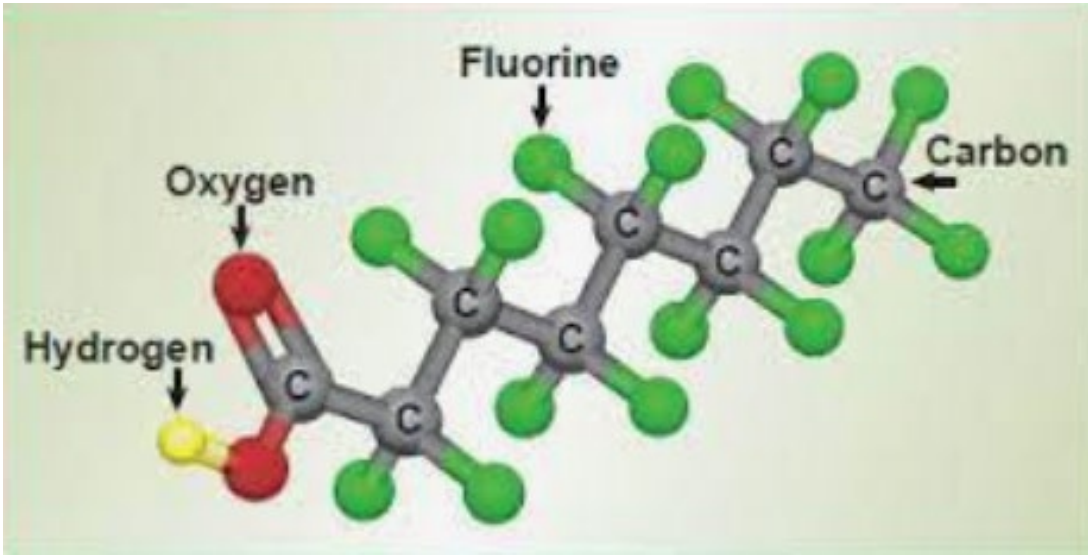
Jiayin Dai – Shanghai Jiao Tong University, China

Udayan Apte – University of Kansas Medical Center

What will be covered

- Background on PFAS
- Summary of work in our Center related to PFAS
- The Robarts et al. study
  - Why we did it
  - What we found

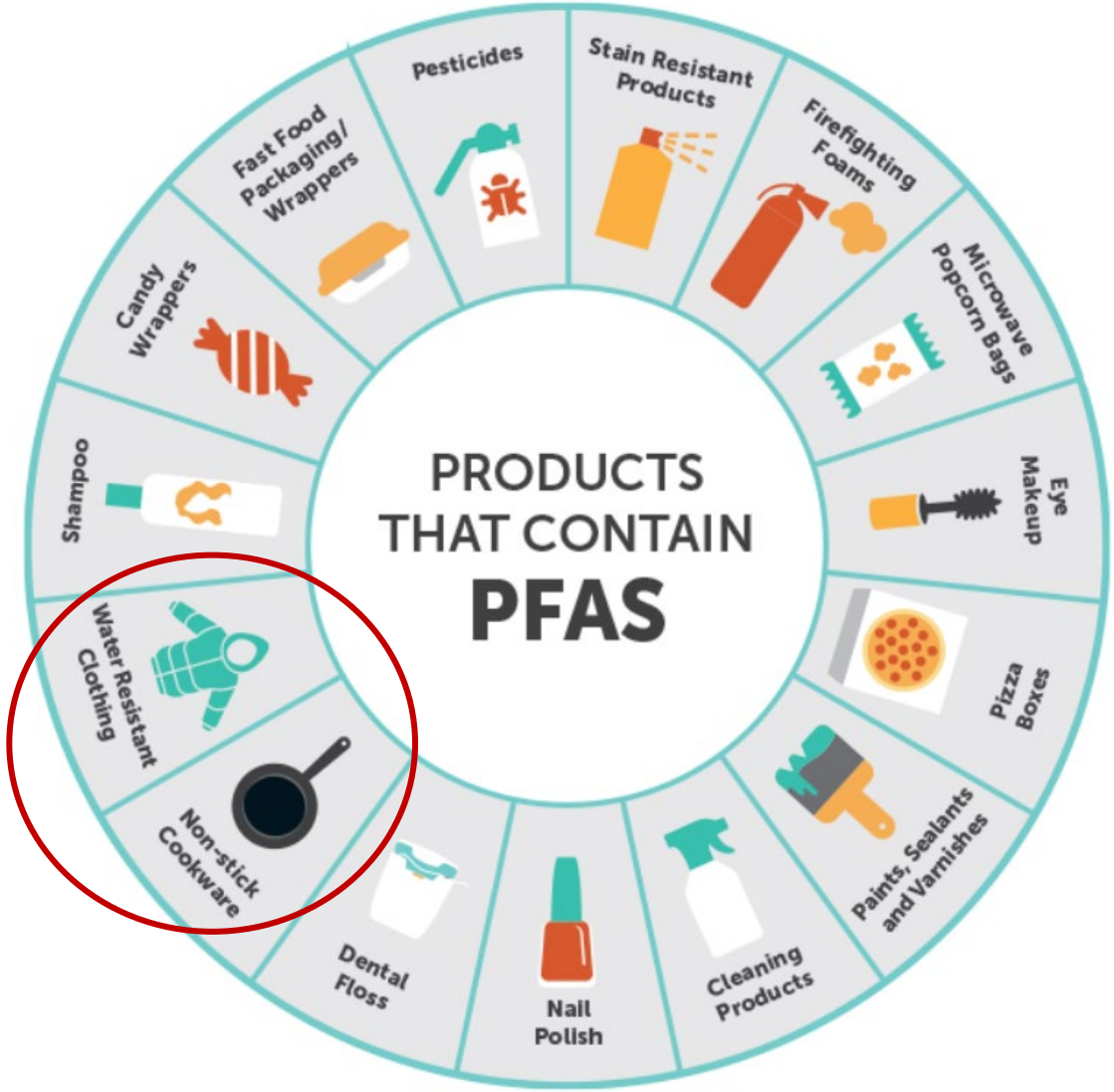
PFAS = Per- and poly-fluoroalkyl substances



https://www.xdd-llc.com/remediation/pfas-introduction-and-chemical-properties/

Why do we care about PFAS?

- They are everywhere and environmentally persistent
  - Globally distributed, detected in water, air, house dust, soil, sediment, sludge from wastewater treatment plants, biosolids
  - Non-biodegradable in environment
  - Found in consumer products



https://pinellas.gov/per-and-polyfluoroalkyl-substances-pfas/

Slide modified from Dr. Chris Lau

Why do we care about PFAS?

- **They are present in the blood of humans and wildlife**

Serum Levels (ppb)	PFOS (C8)	PFOA (C8)	PFHxS (C6)	PFNA (C9)	PFDA (C10)
Production workers	1,500-2,000	500-1,000	~500	N/A	N/A
NHANES '99-'00	30.4	5.2	2.1	0.6	N/A
NHANES '01-'02	20.8	3.7	2.8	0.6	N/A
NHANES '03-'04	20.7	3.9	1.9	1.0	N/A
NHANES '05-'06	17.1	3.9	1.7	1.1	0.36
NHANES '07-'08	13.2	4.1	2.0	1.2	0.29
NHANES '09-'10	9.3	3.1	1.7	1.3	0.28
NHANES '11-'12	6.3	2.1	1.3	0.9	0.20
NHANES '13-'14	5.0	1.9	1.4	0.7	0.19
NHANES '15-'16	4.7	1.6	1.2	0.6	0.15
NHANES '17-'18	4.3	1.4	1.1	0.4	0.19
Arnsburg, Germany '06	5.8-10.5	23.4-25.3	1.1-2.5	N/A	N/A
Little Hocking, WV '07	19.2	32.9	3.3	1.4	0.4
Lake trout	121	4.4	0.6	2.9	N/A
Polar bear	~1,200	~10	--	~100	N/A

https://thehill.com/

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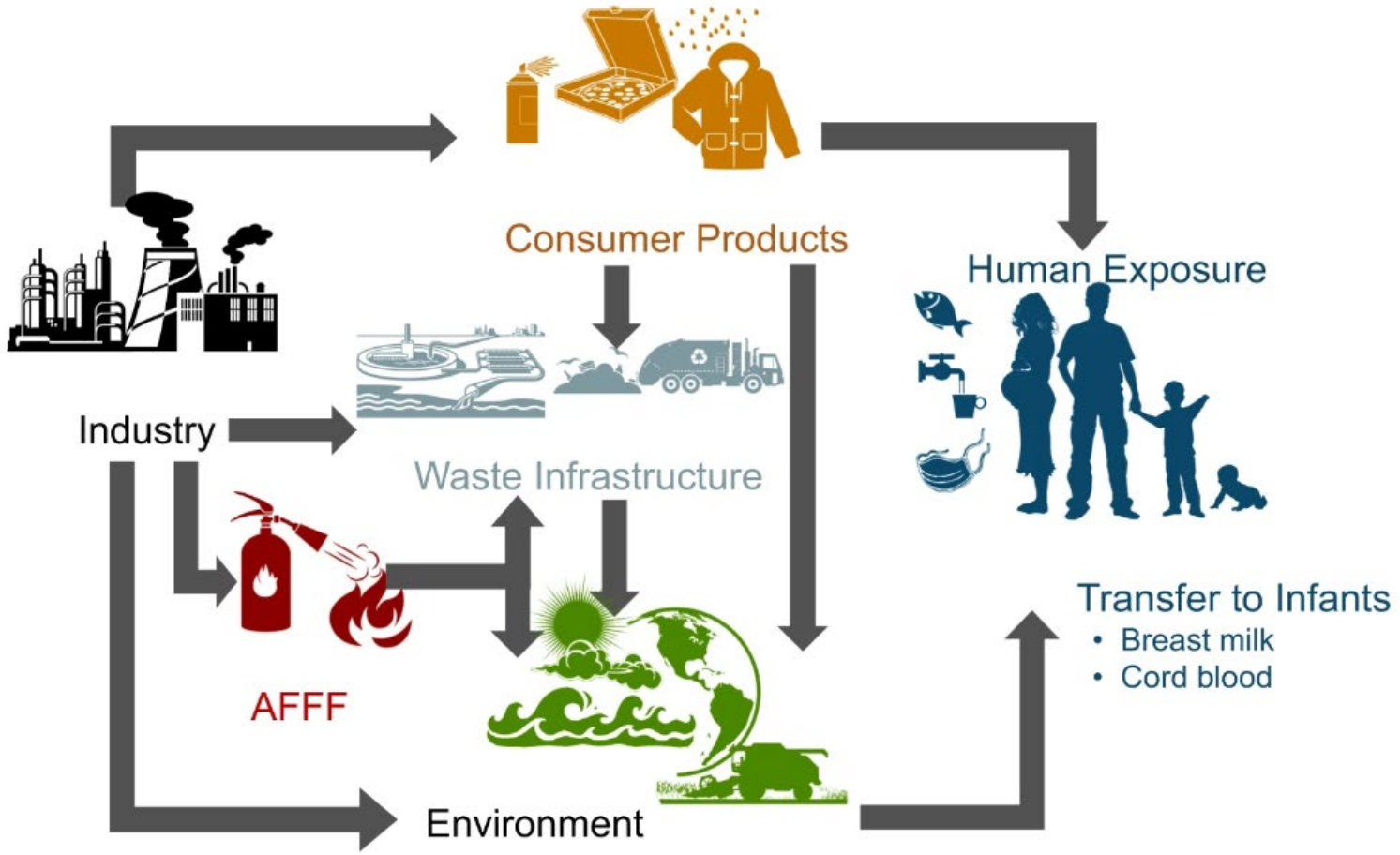
Why do we care about PFAS?

- **People can be exposed to PFAS through multiple routes**
  - **food (fish, some edible plants), migration from food packaging**
  - **drinking water (contamination sites)**
  - **house dust, air**
  - **Consumer products**



https://www.nature.com/articles/s41370-018-0094-1/figures/1

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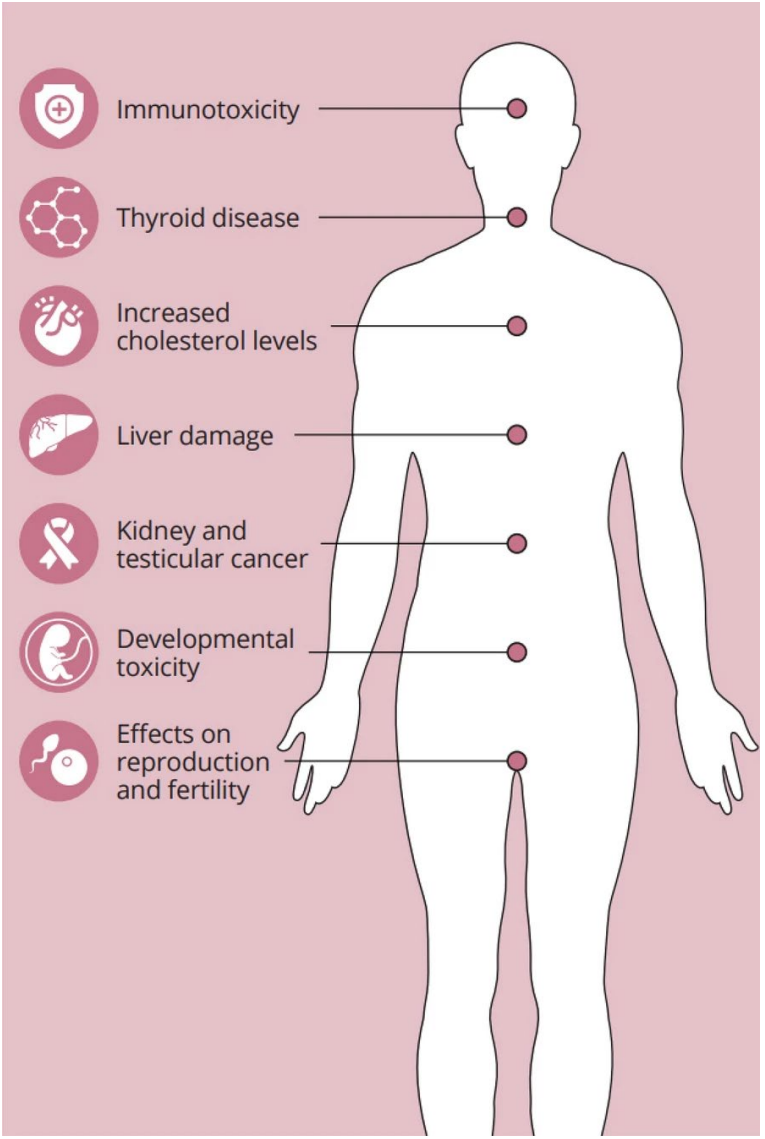
Why do we care about PFAS?

- Toxic effects have been identified in animal studies
  - Hepatotoxicity, reproductive and developmental toxicity, immunotoxicity, tumor induction, endocrine disruption, neurotoxicity
- Epidemiology studies indicate adverse effects in humans
- Human health risks assessed by regulatory bodies internationally
  - Legally enforceable Maximum Contaminant Levels (MCLs) for 5 PFAS in drinking water were issued by the US EPA on 4/10/2024:
    - PFOA (4 ppt),
    - PFOS (4 ppt)
    - PFHxS (10 ppt),
    - PFNA (10 ppt),
    - HFPO-DA (GenX, 10 ppt)



https://enveurope.springeropen.com/articles/10.1186/s12302-023-00721-8/figures/2

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Why do we care about PFAS?

- **Some PFAS are very persistent in the blood of humans**

F-53B

Serum half-life estimates of some per- and polyfluoroalkyl substances

	PFBS (C4)		PFHxS (C6)		PFOS (C8)		PFBA (C4)		PFHxA (C6)		PFHpA (C7)		PFOA (C8)		PFNA (C9)		PFDA (C10)		F-53B		GenX	
	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
Rat	4.0 hours	4.5 hours	1.4 days	26-27 days	28-43 days	34-36 days	1.8 hours	9.2 hours	0.5-7.3 hours	1.3-11 hours	1.2-2.1 hours	1.5-24 hours	1.7-4.8 hours	8.1-8.5 days	6.4 days	31-55 days	45-59 days	55-83 days			0.9-2.8 days	3.0-3.7 days
Mouse	4.5 hours	5.8 hours	25-27 days	28-30 days	31-38 days	36-43 days	6.2 hours	12 hours	~1.2 hours	~1.6 hours			16 days	22 days	26-68 days	34-69 days					1.0 day	1.5 days
Monkey	1.1 days	1.6 days	87 days	140 days	110 days	130 days	1.7 days		2.4 hours	5.3 hours			30 days	21 days							3.3 days	2.7 days
Humans	35 days	36 days	13 years	14 years	3.4 years	3.7 years	3 days		32 days		140 days	130 days	2.1-3.8 years		1.7 years	3.2 years			15.3 years		3.4 days	

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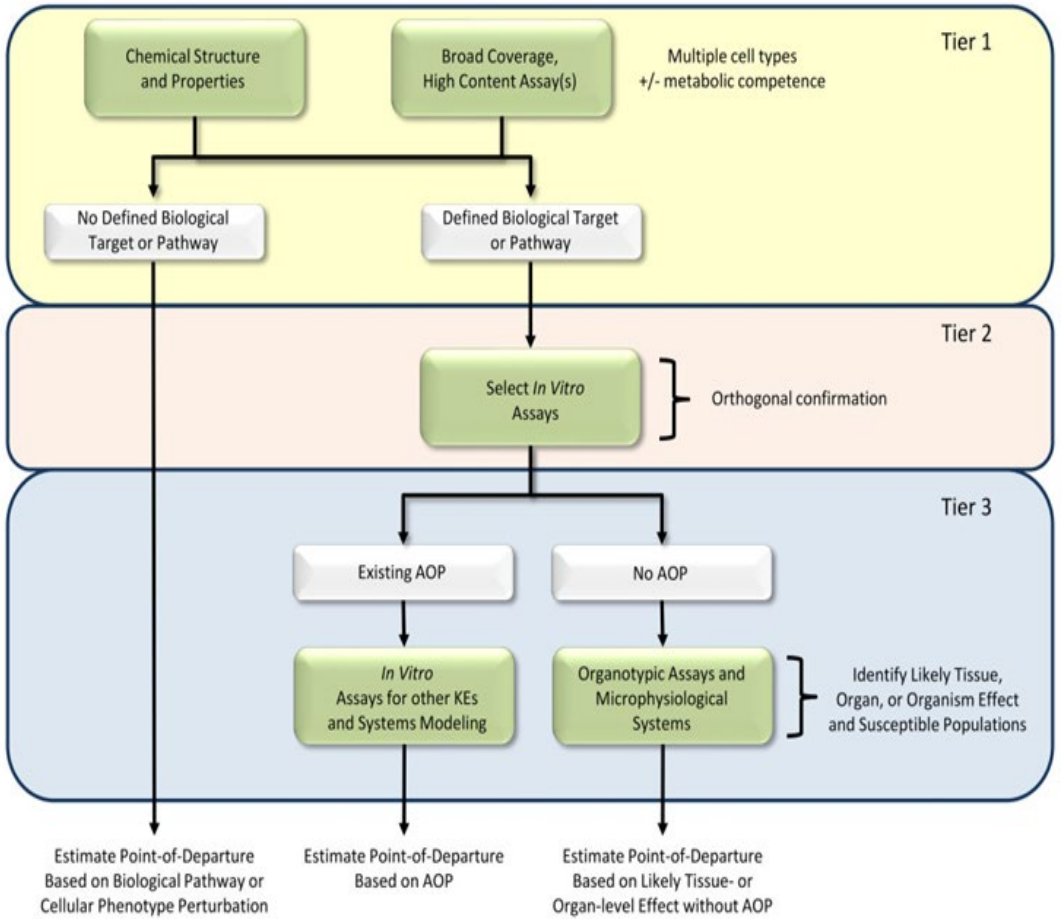
Center for Computational Toxicology and Exposure – Understanding the potential risks to PFAS exposure

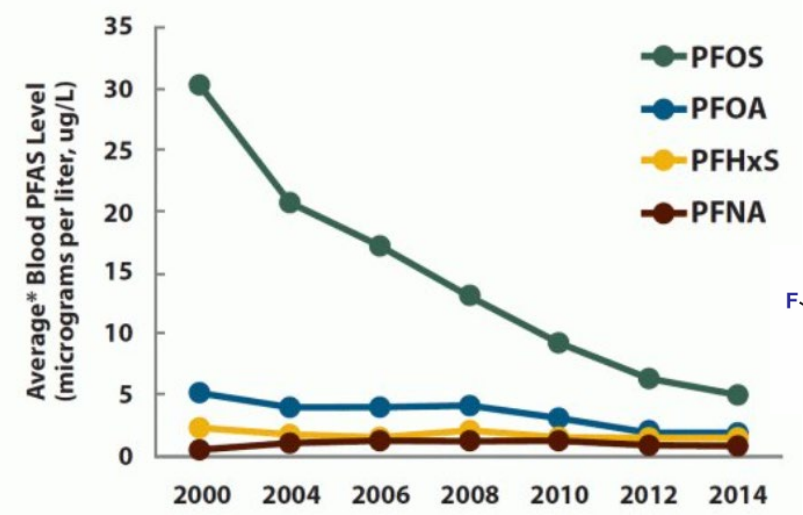
- Considering the universe of PFAS (thousands)
- Tiered testing strategies
  - Tier 1 – high throughput testing examining cellular transcript or phenotypic changes in different cell lines; computational approaches for extrapolating test concentrations to exposure in humans; grouping by structure and biology allowing read across
  - Tier 2 – in vitro assays that are part of our new approach methodologies (NAMs)
  - Tier 3 – expose rats to different doses of individual PFAS to generate benchmark doses (EPA transcriptomic assessment product; ETAP)



Thomas et al. (2019) ToxSci 169(2):317-332

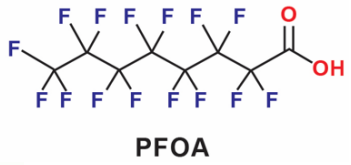
Background and Goal of Study

The levels of legacy PFAS in human blood are decreasing



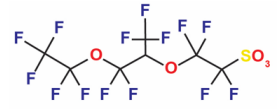
Brennan et al. (2021) Intl J Environ Res Pub Health 18:10900

Straight chain PFAS



The levels of alternative PFAS are increasingly detectable

- Increased blood levels of per- and polyfluoroalkyl ether acids (PFEAs)
  - Kotlarz et al. (2024) EHP 132(2):27701; 132(2):27702
  - Rosen et al. (2022) EHP 130(9):97002

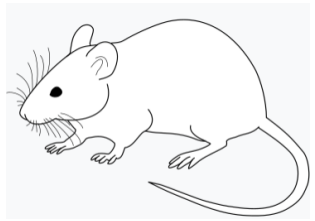


Nafion BP2

- **Goal of study: Use available archived transcript profile data to identify molecular targets of legacy and alternative PFAS**
  - **Are there differences?**
  - **How do alternative PFAS toxicities compare to toxicities of legacy PFAS?**

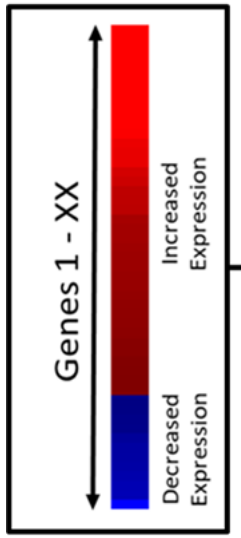
Methods

Treat mice with 11 legacy or alternative PFAS

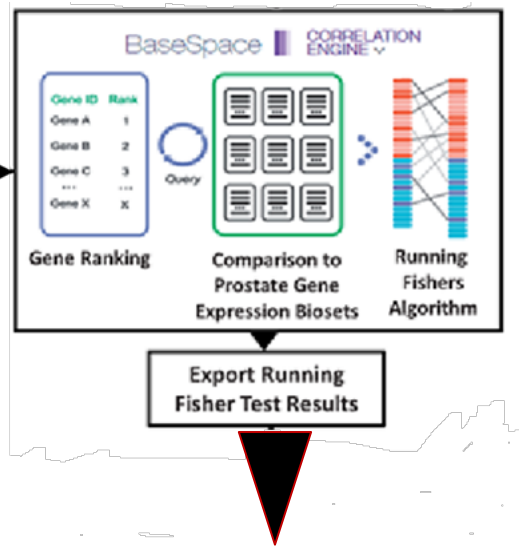


Collect liver; Isolate RNA; Measure transcript profile changes using microarrays or RNA-Seq

Archived transcript profiles



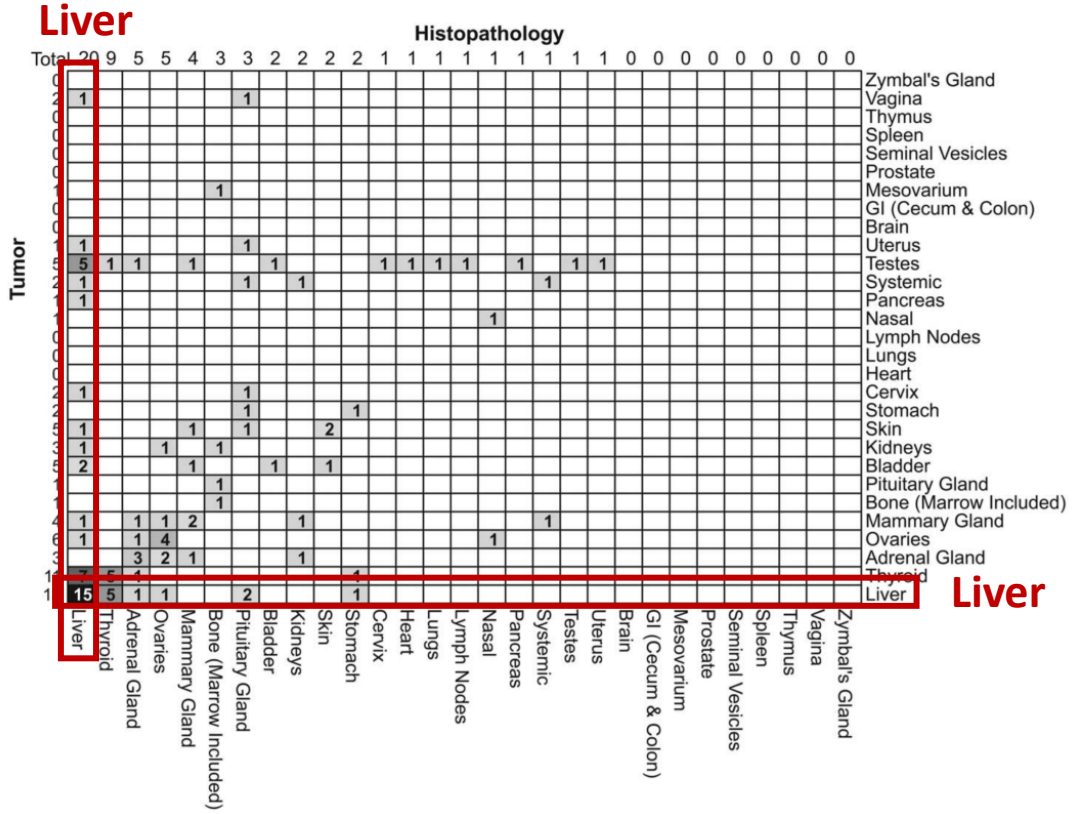
Illumina's BaseSpace Correlation Engine



Compare to gene lists and biomarkers to understand biological effects

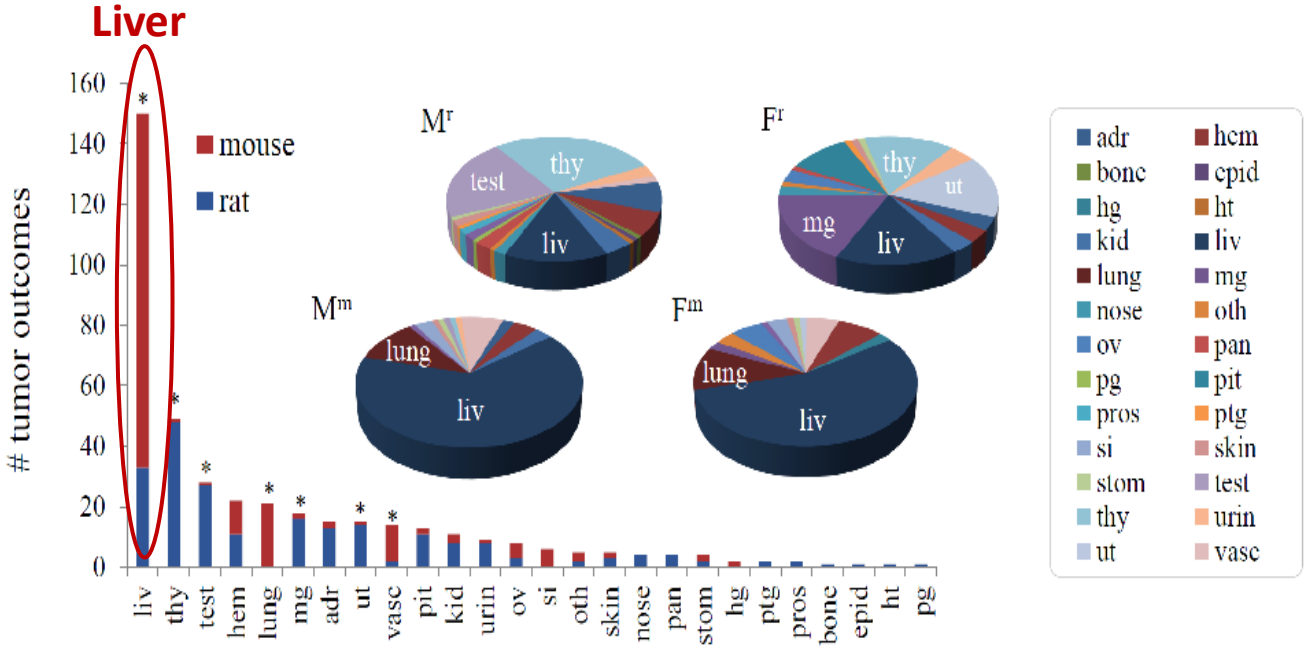
Liver is a major site for chemical-induced carcinogenesis in rodents

Marketed Pharmaceuticals in Rats



From Sistare et al. Toxicol Pathol. 2011 Jun; 39(4):716-44.

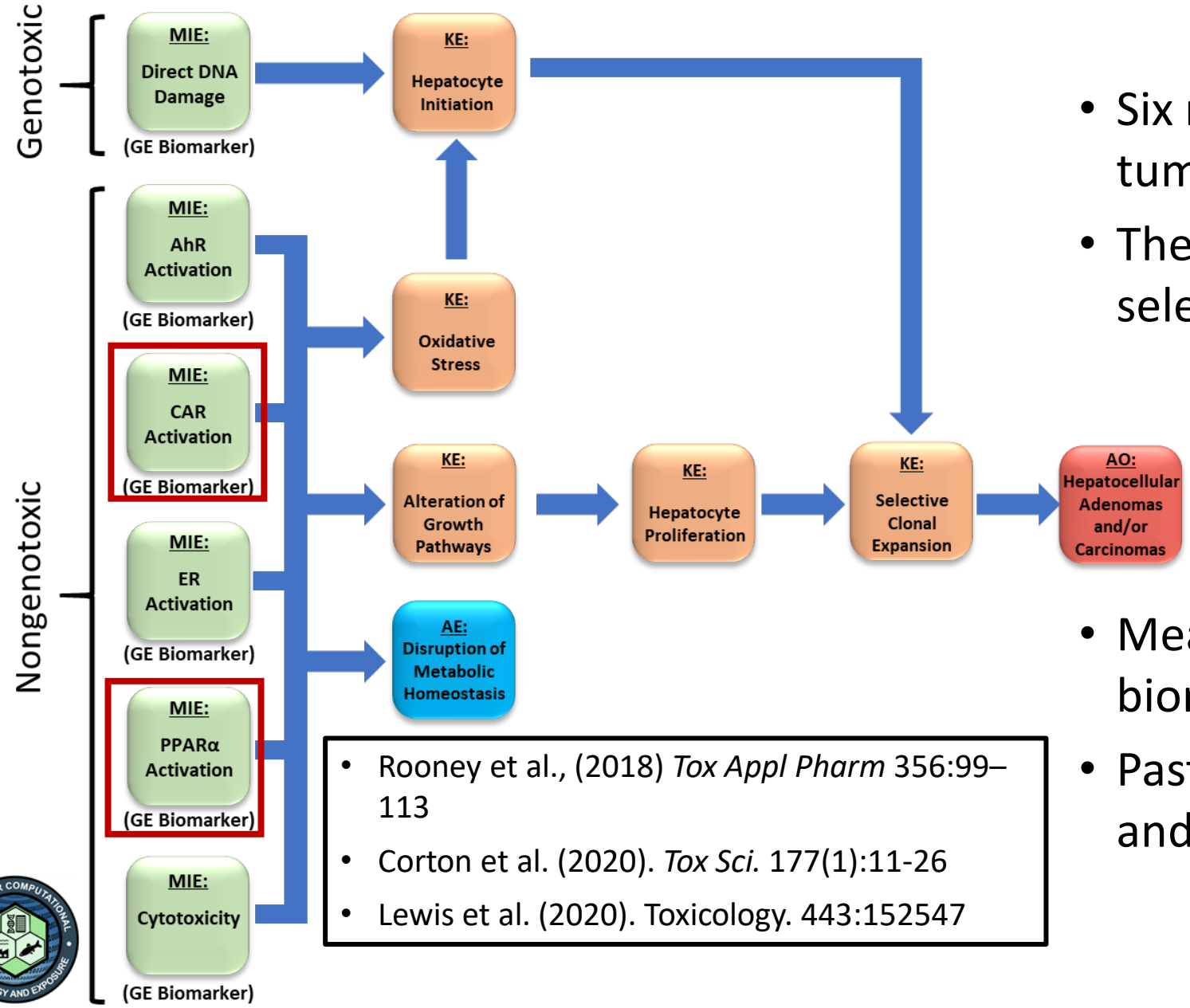
Environmental Chemicals in Mice and Rats



- **Results of 628 two-sex carcinogenicity studies (n = 324 rat, n = 304 mouse) available in ToxRefDB**
- **Studies covered 336 unique compounds (n = 307 rat, n = 288 mouse), 259 of which were tested in both species**

From Hill et al. Toxicol Sci. 2017 Jan; 155(1):157-169

Major Adverse Outcome Pathways That Lead to Rodent Liver Tumors



- Rooney et al., (2018) *Tox Appl Pharm* 356:99–113
- Corton et al. (2020). *Tox Sci.* 177(1):11-26
- Lewis et al. (2020). Toxicology. 443:152547

- Six major AOPs lead to rodent liver tumors
- The AOPs converge on the key event of selective clonal expansion

- Measure MIEs with gene expression biomarkers
- Past studies have revealed that PPARα and CAR are targets of the legacy PFAS

EPA United States Environmental Protection Agency

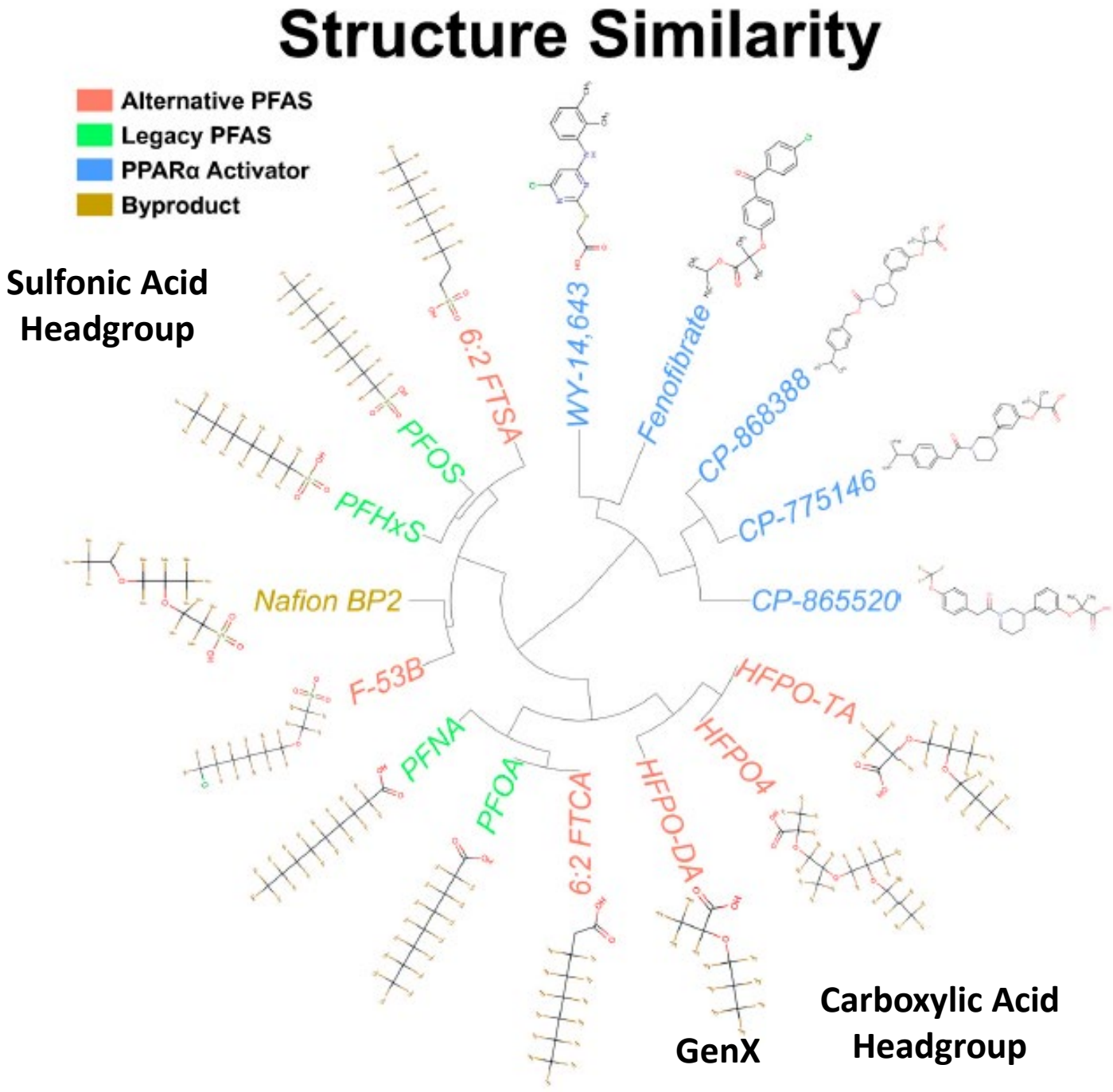
Compiled Studies Examined

- Studies in the public domain
- Two main profiling platforms (Affymetrix microarrays, RNA-Seq)
- All profiles from male mouse liver
- Exposures were 7-28d
- One to four doses
- Examined 11 PFAS and 5 PPARα activators

Chemical Name	Abbreviation	DTXSID	PMID	Dose	Timepoint
Perfluorooctane sulfonate	PFOS	DTXSID3031864	20936131	Daily 3 mg/kg or 10 mg/kg	7 days
Perfluorooctanoic acid	PFOA	DTXSID8031865	18281256	Daily 3 mg/kg	7 days
Perfluorononanoic acid	PFNA	DTXSID8031863	28558994	Daily 1 mg/kg or 3 mg/kg	7 days
perfluorohexane sulfonate	PFHxS	DTXSID7040150	28558994	Daily 3 mg/kg or 10 mg/kg	7 days
Perfluoro-2-{[perfluoro-3-(perfluoroethoxy)-2-propanyl]oxy}ethanesulfonic acid	Nafion BP2	DTXSID10892352	#	Daily 0.03, 0.3, 3, or 6 mg/kg	7 days
Ammonium perfluoro-2-methyl-3-oxahexanoate	HFPO-DA (HFPO2) (GenX)	DTXSID40108559	27553808 32138627	Daily 1 mg/kg Daily 0.1, 0.5, or 5 mg/kg	28 days 90 days
Perfluoro-(2,5,8-trimethyl-3,6,9-trioxadodecanoic)acid	HFPO4	DTXSID70276659	27553808	Daily 1 mg/kg	28 days
Perfluoro-2,5-dimethyl-3,6-dioxanonanoic acid	HFPO-TA	DTXSID00892442	29927593	Daily 0.02, 0.1, 0.5 mg/kg	28 days
Potassium 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate	F-53B	DTXSID60881236	#	Daily 5 mg/kg	28 days
6:2 Fluorotelomer sulfonic acid	6:2 FTSA	DTXSID6067331	28032147	Daily 5 mg/kg	28 days
2-Perfluorohexyl ethanoic acid	6:2 FTCA	DTXSID50472556	28032147	Daily 5 mg/kg	28 days
(S)-2-methyl-2-(3-(1-(2-(4-(trifluoromethoxy)phenyl)acetyl)piperidin-3-yl)phenoxy)propanoic acid sodium salt	CP-865520	DTXSID4044032	18971326	Daily 1 mg/kg	5 days
(S)-2-(3-(1-(2-(4-isopropylphenyl)acetyl)piperidin-3-yl)phenoxy)-2-methylpropanoic acid sodium salt	CP-775146	DTXSID9044033	18971326	Daily 1 mg/kg	5 days
(S)-2-(3-(1-((4-isopropylbenzyloxy)carbonyl)piperidin-3-yl)phenoxy)-2-methylpropanoic acid sodium salt	CP-868388	DTXSID4044034	18971326	Daily 1 mg/kg	5 days
Propan-2-yl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate	Fenofibrate	DTXSID2029874	18301758	Single 4 mg/mL	6 h
([4-Chloro-6-(2,3-dimethylanilino)pyrimidin-2-yl]sulfanyl)acetic acid	WY-14,643	DTXSID4020290	26215100	Single 250 mg/kg	8 h

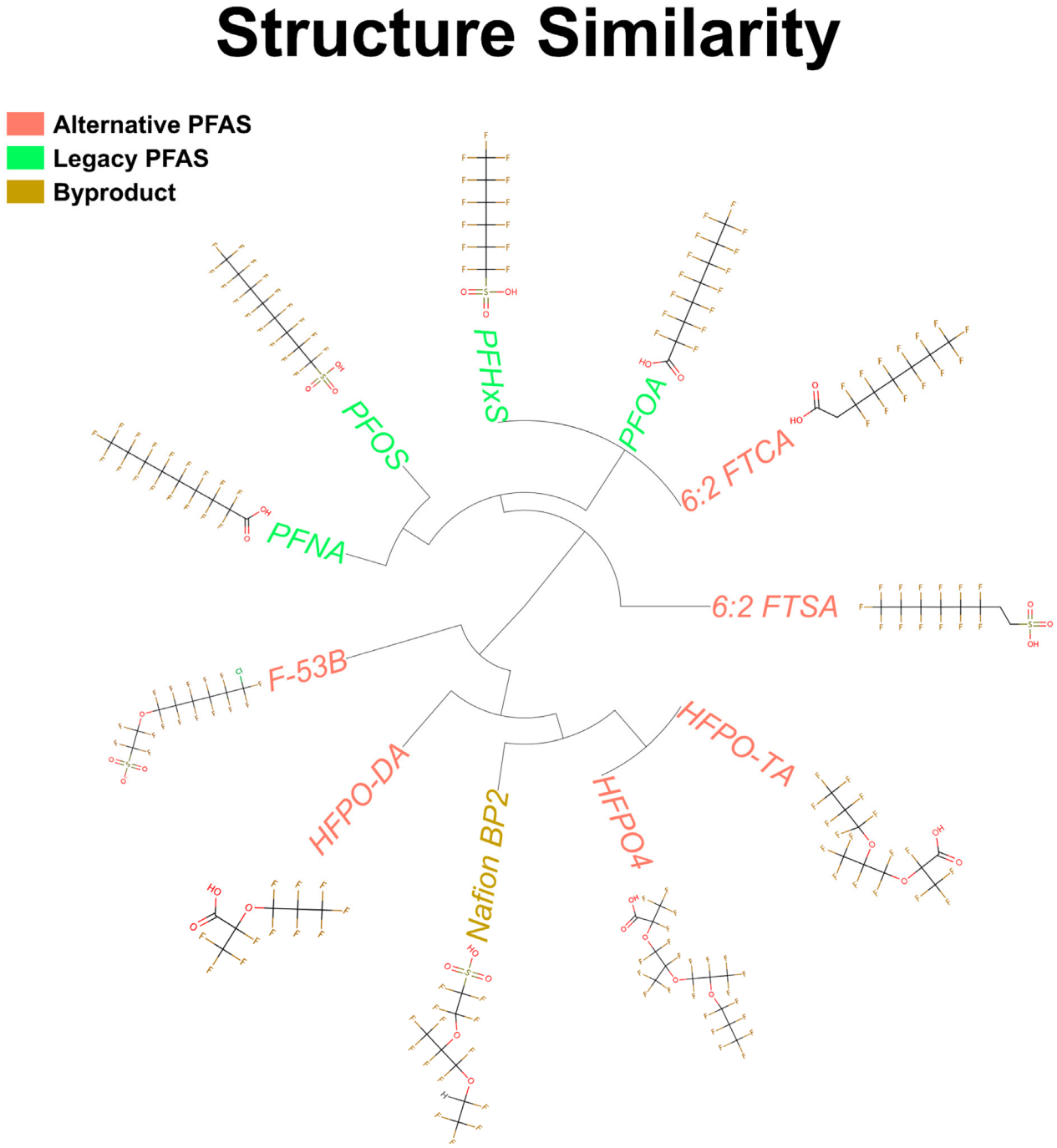
Structural similarity of the compounds examined in the study

- Used ToxPrint to determine structural relatedness
- PPARα agonists clustered together
- PFAS cluster based on the head groups



Structural similarity of the compounds examined in the study

- Used ToxPrint to determine structural relatedness
- Removed the head groups
- Clustered based on whether straight chain or contains ether linkages

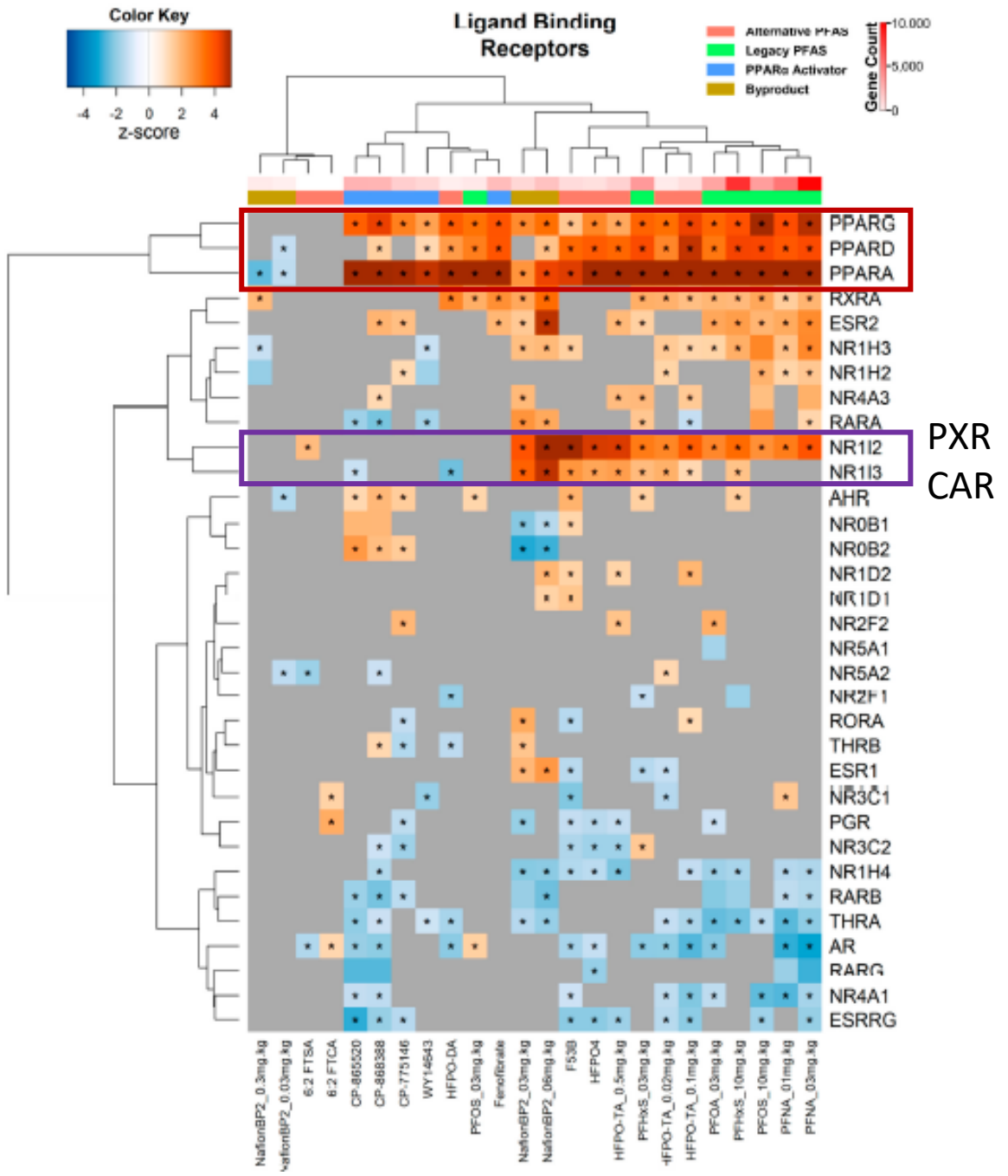


(B)

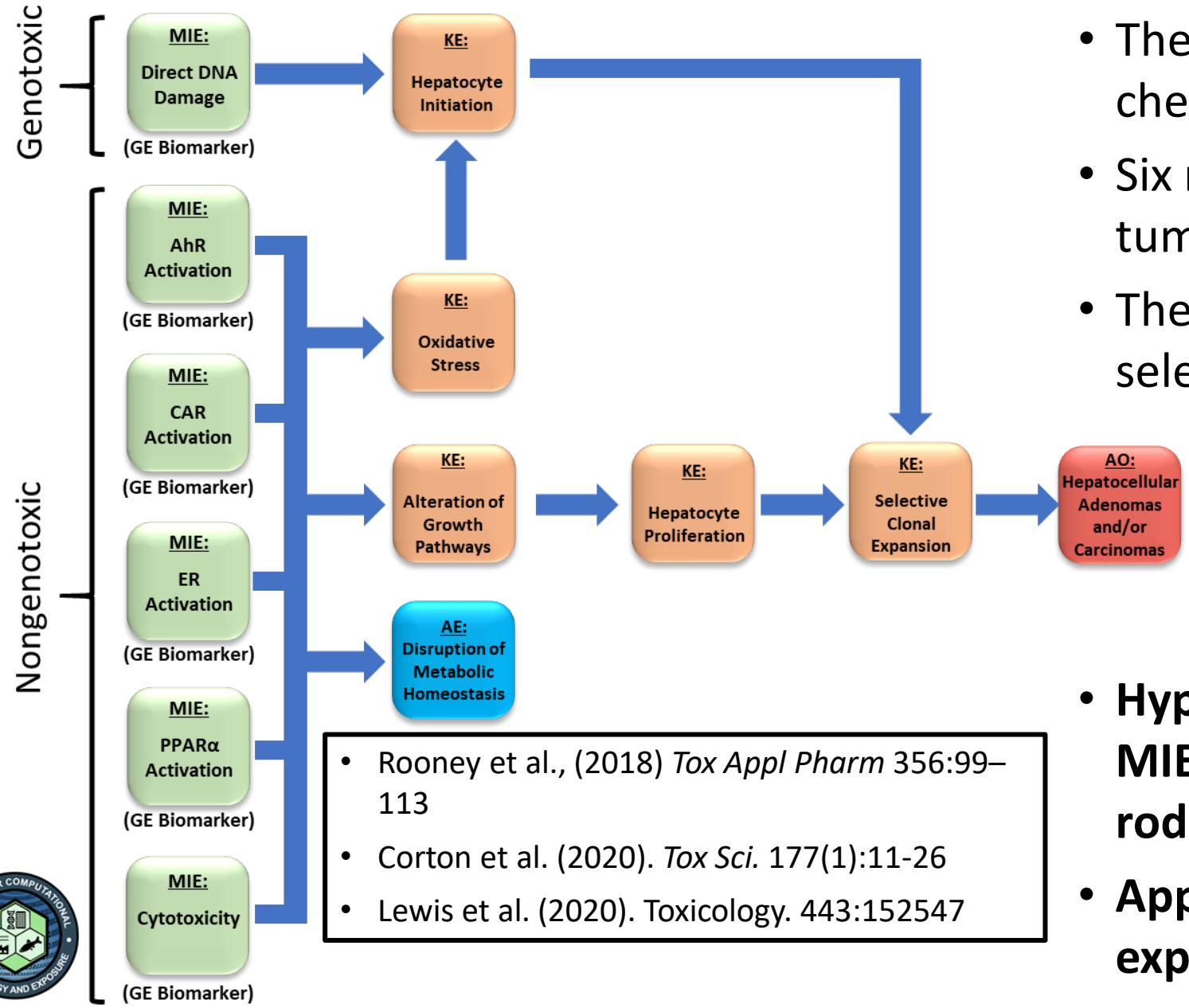
Examination of the role of nuclear receptors

- Use the upstream activator analysis function of Ingenuity Pathways Analysis (IPA)
- Most PFAS activate PPAR family members
- A subset of PFAS activate CAR and/or PXR
- No major distinctions between legacy and alternative PFAS

- Qualifier: the gene lists in IPA have not been characterized for prediction – they are hypothesis generating tools



Major Adverse Outcome Pathways That Lead to Rodent Liver Tumors

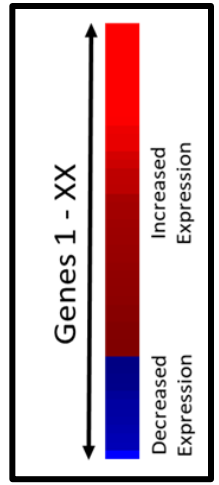


- Rooney et al., (2018) *Tox Appl Pharm* 356:99–113
- Corton et al. (2020). *Tox Sci.* 177(1):11-26
- Lewis et al. (2020). Toxicology. 443:152547

- The liver is the most frequent target of chemical tumorigens
- Six major AOPs lead to rodent liver tumors
- The AOPs converge on the key event of selective clonal expansion

- **Hypothesis: measurement of the six MIEs will be sufficient to predict rodent liver tumors**
- **Approach: measure MIEs with gene expression biomarkers**

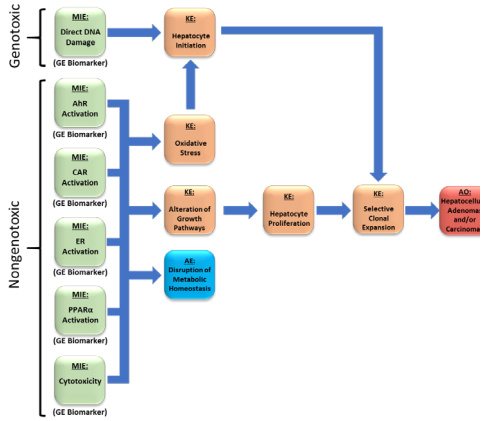
Gene expression biomarkers

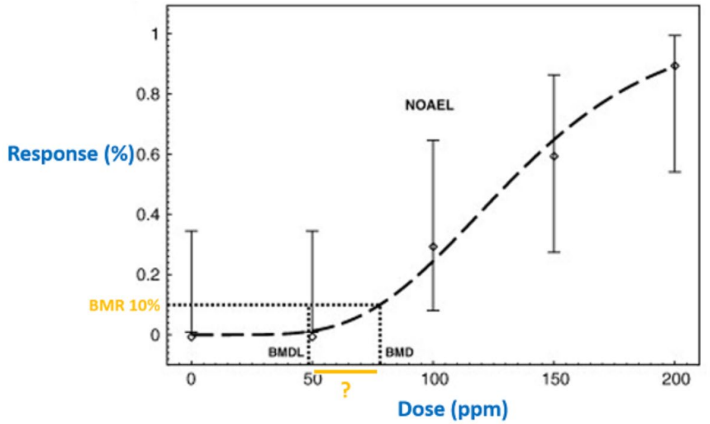


- Biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” (1998, the National Institutes of Health Biomarkers Definitions Working Group)

- A gene expression biomarker is a short list of genes and associated fold-change values or ranks used to predict the activity of a factor important in mediating effects of chemicals or toxicity

- Can be used to
  - Identify mode of action
  - Predict tumorigenic potential
  - Determine a benchmark dose

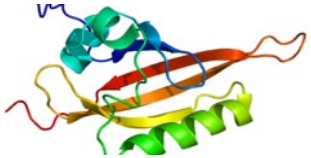




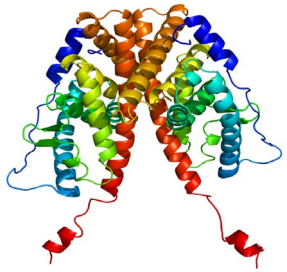
- Very few examples of well characterized gene expression biomarkers with known accuracies
  - Gene lists as signature/pathway analysis often used as hypothesis generators

Biomarkers that predict key events in the livers of mice and rats

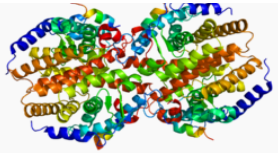
AhR



Estrogen Receptor α

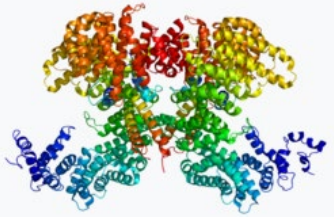


CAR

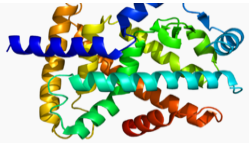


NRF2

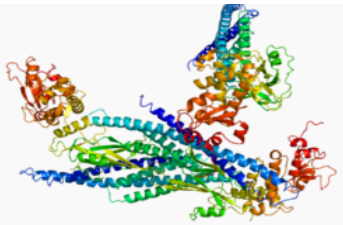
SREBP



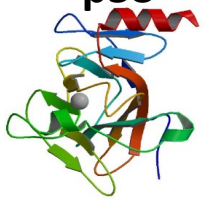
PPARα



STAT5b



p53



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The mouse biomarkers have excellent predictive accuracy

Mouse Biomarker	Number of Genes	Mutant mice used	Predictive Accuracy for Activation	Publication
PPARα	131	*Ppara*	98%	PLoS One. 2015 10(2):e0112655
CAR	83	*Nr1i3*	97%	Nucl Recept Signal. 2015 13:e002
AhR	63	*Ahr*	95%	Toxicology. 2015 336:99-112
Nrf2		*e2l2, Keap1*	96%	PLoS One 2018 13(8):e0200004
Stat5b			97%	PLoS One 2016 11(3):e0150284
Srebp				10 (2019) 77

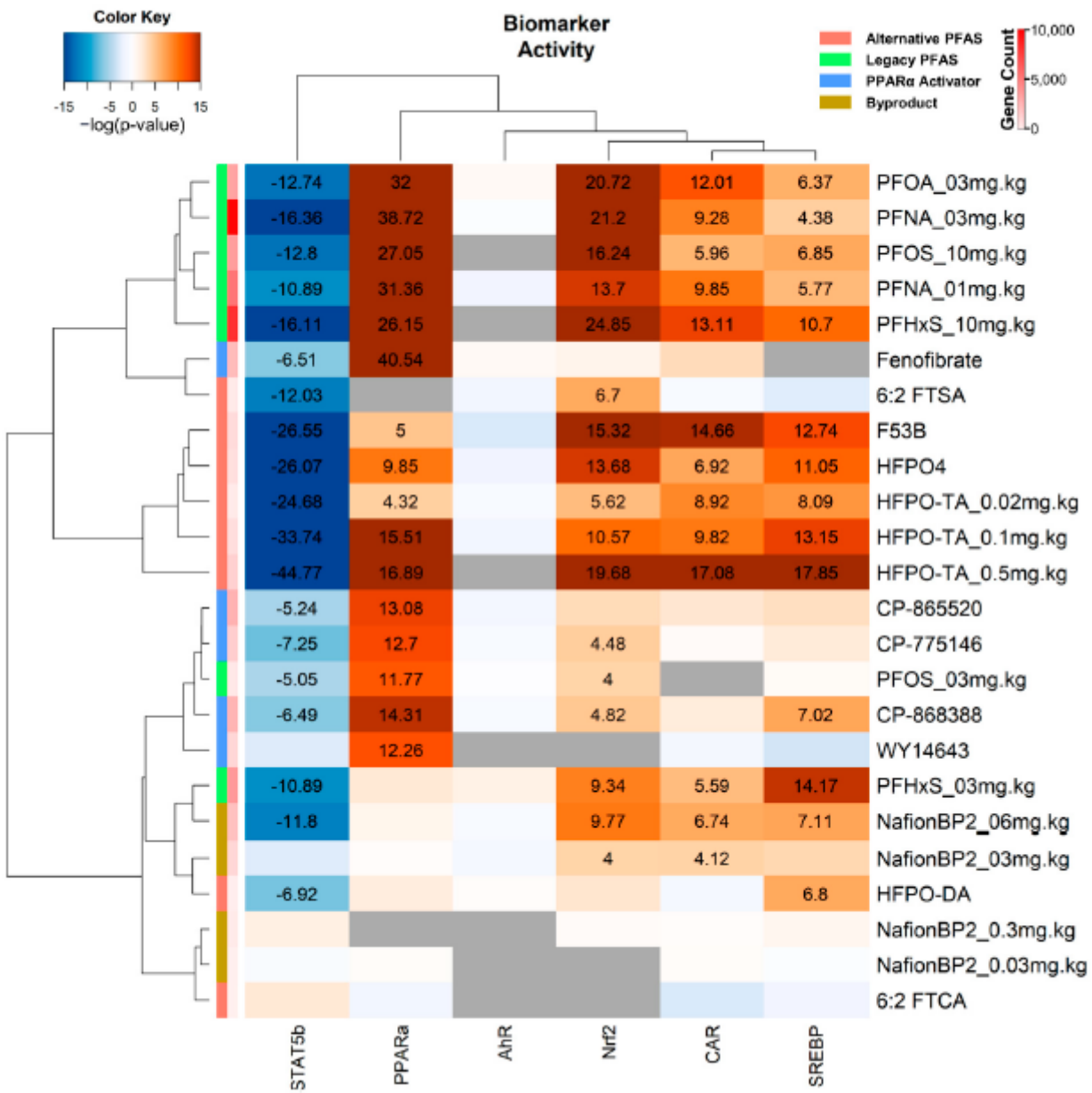
Activated by oxidative stress

Regulates growth hormone responsive genes

Regulates genes involved in the synthesis of cholesterol and triglycerides

Biomarker analysis

- Most PFAS suppress Stat5b – indicative of suppression of growth hormone signalling
- Most chemicals activate PPARα (not Nafion BP2, 6:2 FTSA and 6:2 FTCA) that regulates fatty acid and glucose metabolism
- Many activate CAR and Nrf2 – indicative of increases in xenobiotic metabolism and associated oxidative stress
- Many activate SREBP – linked to steatosis commonly observed in the livers of treated rodents
- No clear distinctions between the legacy and alternative PFAS
- 6:2 FTCA and 6:2 FTSA activate fewer factors - 6:2 FTCA is more quickly metabolized



Conclusions

- To understand the diversity of molecular targets of the PFAS in the mouse liver, we performed a comparative toxicogenomics analysis of the gene expression changes after exposure to 11 PFAS
- Using hierarchical clustering, pathway analysis, and predictive biomarkers, we found that most of the alternative PFAS modulate molecular targets that overlap with legacy PFAS
- Only three of the 11 PFAS tested did not appreciably activate PPARα (Nafion BP2, 6:2 FTSA, and 6:2 FTCA)
- Predictive biomarkers showed that most PFAS (PFHxS, PFOA, PFOS, PFNA, HFPO-TA, F-53B, HFPO4, Nafion BP2) activated CAR
- PFNA, PFHxS, PFOA, PFOS, HFPO4, HFPO-TA, F-53B, Nafion BP2, and 6:2 FTSA activated NRF2
- A subset of PFAS activated SREBP that may underlie the steatosis observed

- The work highlights the similarities in molecular targets between the legacy and alternative PFAS
- We predict that the alternative PFAS (except 6:2 FTCA) would be no less toxic than the legacy PFAS

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Thanks for listening!

Questions?