Diversity Outbred: In Vivo and In Vitro Applications for Toxicology

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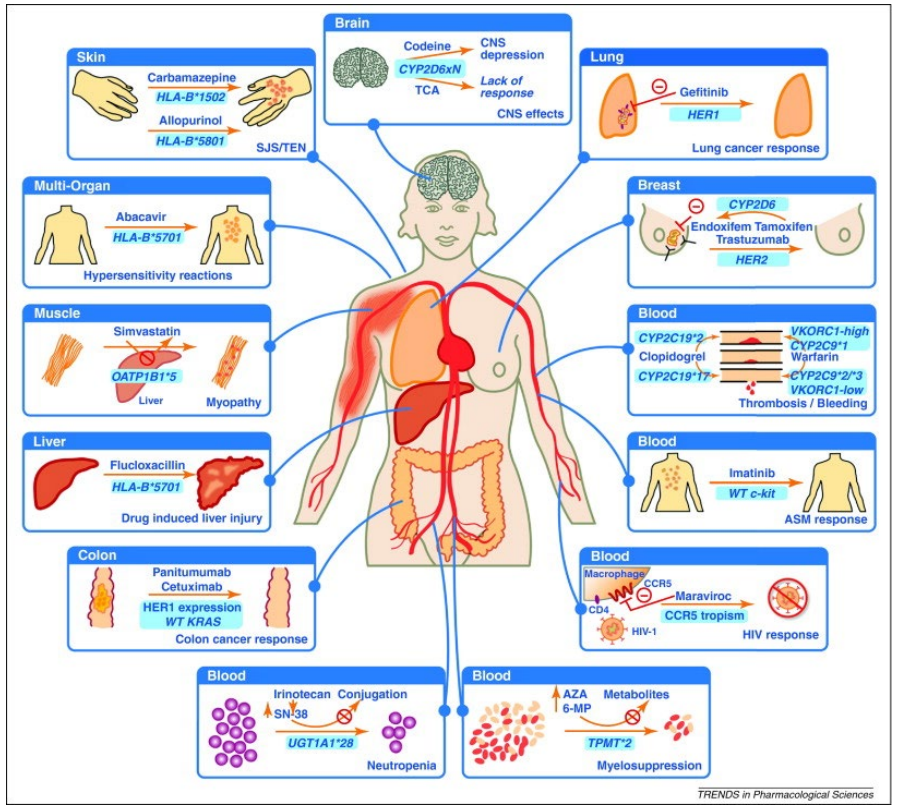
The views expressed in this presentation are those of the presenter and do not necessarily reflect the views or policies of the U.S. EPA

Precision Medicine

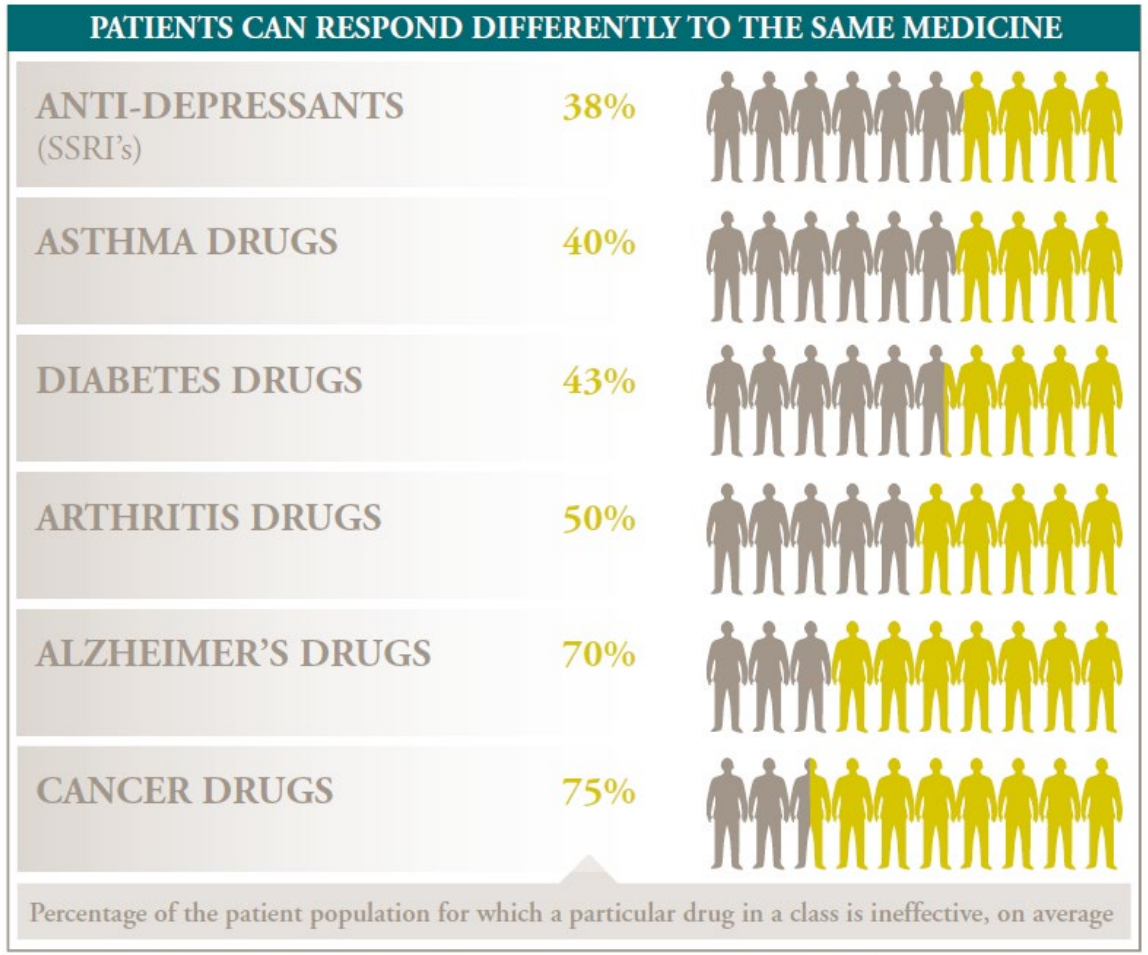
- Genetic differences can contribute to **toxicity** (adverse events) and to **efficacy** (pharmacotherapy)

Image: ranchcreekrecovery.com, June 2017



Sim and Ingelman-Sunberg. *Trends in Pharm Sci*. 2011

Who exactly are we predicting with our models??
Even humans aren't a good model for humans.....



Source of data: Brian B. Spear, Margo Heath-Chiozzi, Jeffrey Huff, "Clinical Trends in Molecular Medicine, Volume 7, Issue 5, 1 May 2001, Pages 201-204.

In vitro toxicology collapses the population space even further, because we typically use **one or a few** human donors.

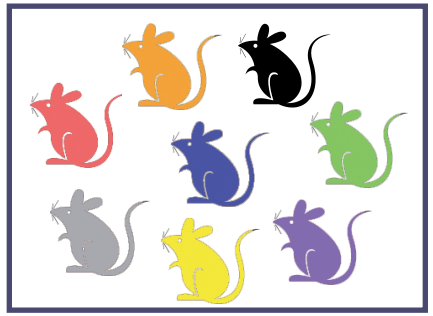
To study toxicity mechanisms, you must first find a good model

For *toxicity* studies, when a single genetic background is used, the results may be misleading:

You didn't choose the "right" strain or donor → Most strains or donor lines react "like" average humans, but the one you chose is idiosyncratically more/less susceptible

In principle, the likelihood of this scenario is reduced when using multiple genetic backgrounds

*This observation can be specific to the chemical or MOA.

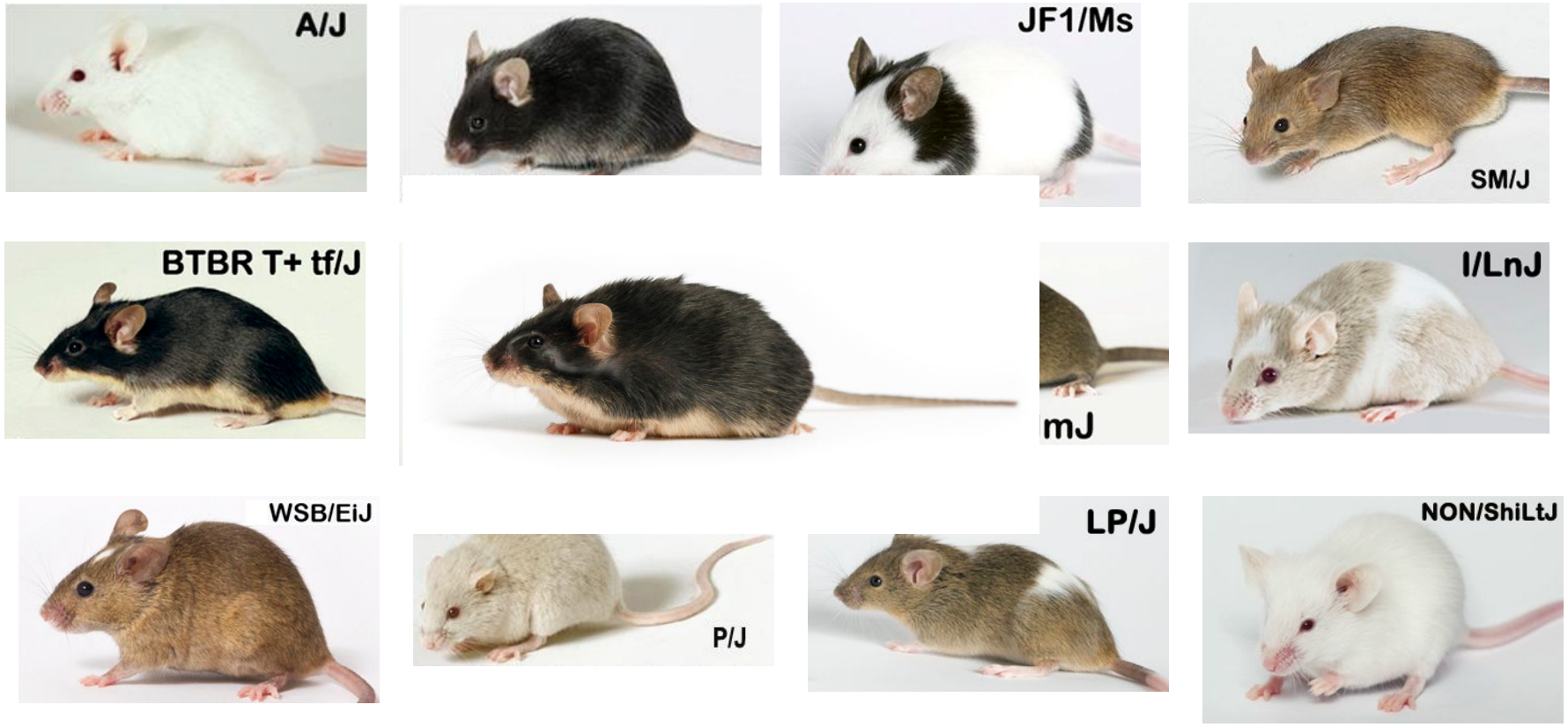


Genetically Diverse Mouse Population

Model population space

Genetically Diverse Human Population

Taking advantage of decades of mouse genetics research to create a diverse model

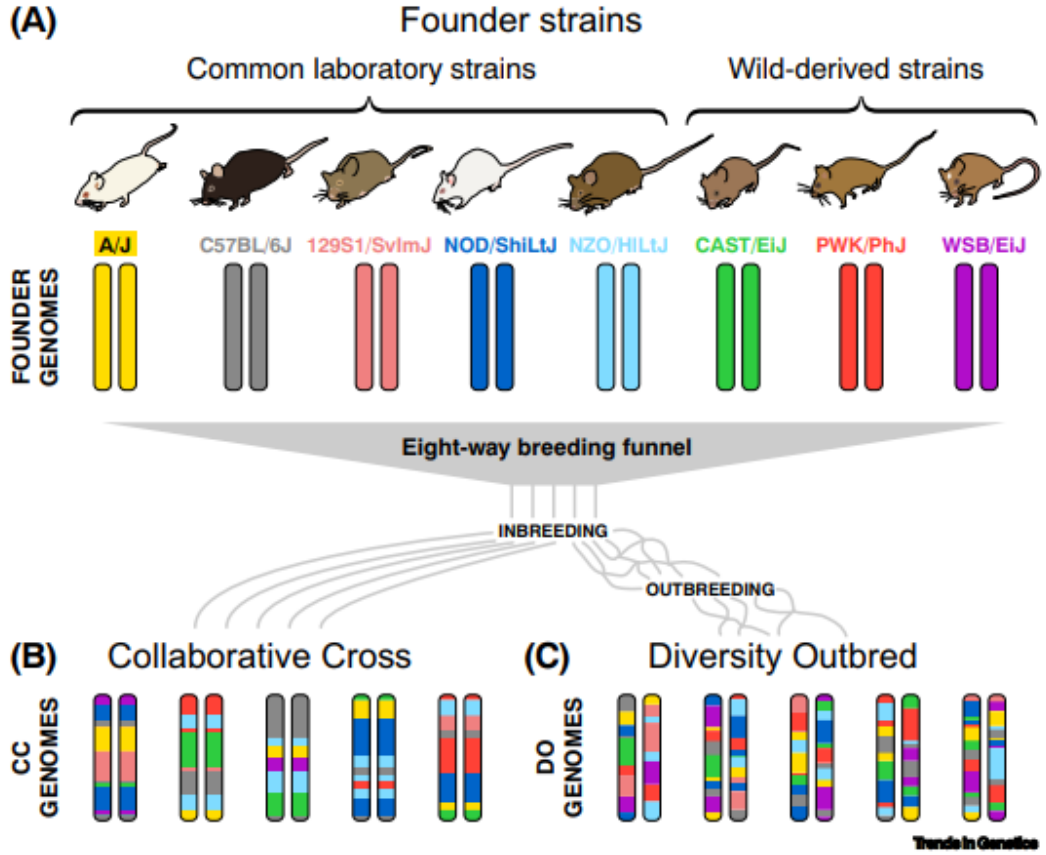


Photos by Stanton Short, Jackson Laboratory

Wide variation in toxicity response, behavior, exercise patterns, glucose tolerance, cancer susceptibility, coat color, weight, etc…

Diversity Outbred mice provide genetic diversity

Rationally interbred population that mimics human genetic diversity, but polymorphisms are highly randomized



- **Diversity Outbred mice** are highly genetically diverse, with a randomization of polymorphisms that is superior to human populations. Each mouse is ***genetically unique***.

Saul et al. *Trends in Genetics* 2019

Complex traits

Mouse populations have primarily been used for basic and translational research questions, not for Toxicology.

DO mice offer advantage over human studies due to randomization of genetic variants (need far fewer DO mice vs comparable human study)

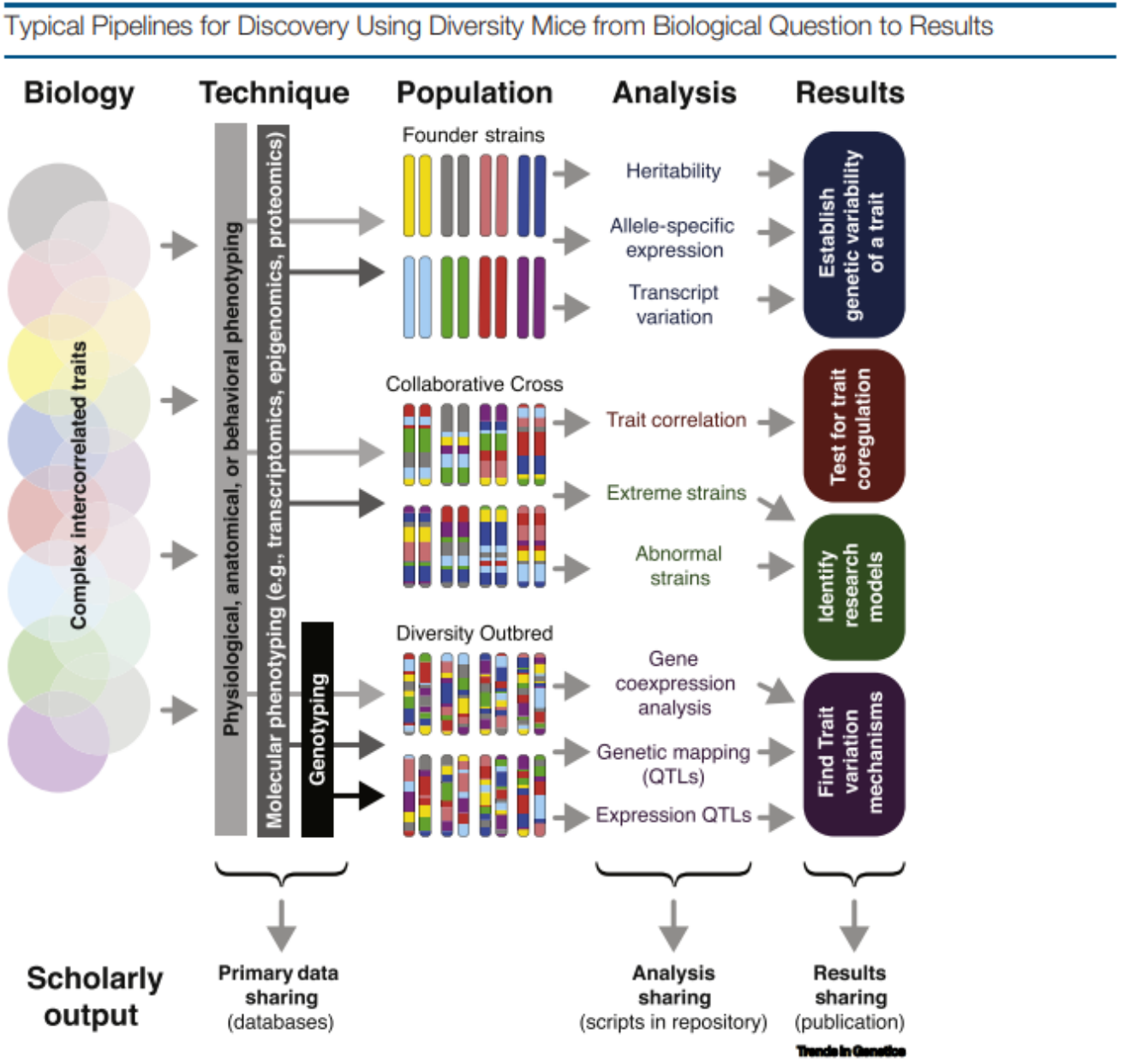
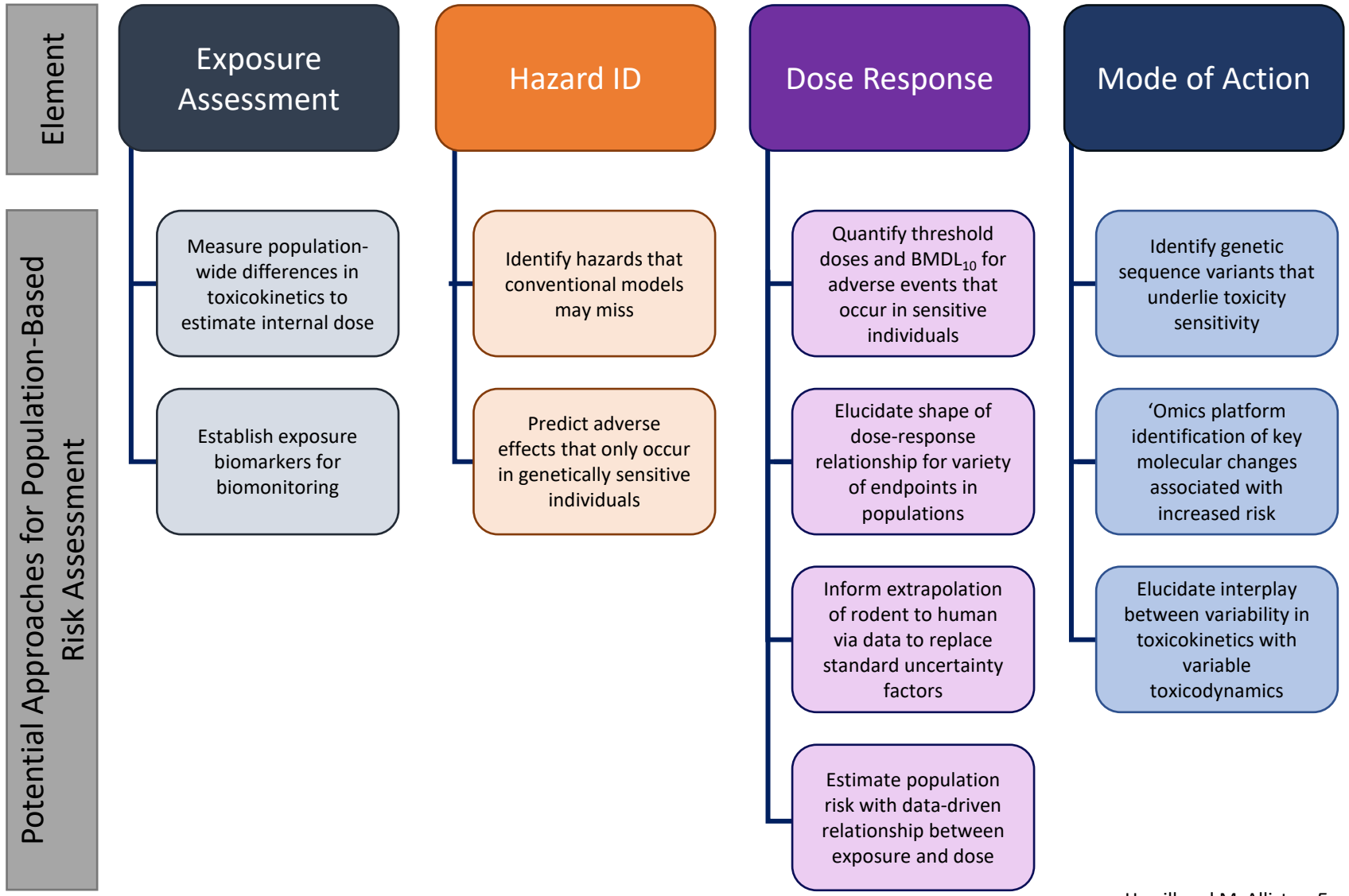


Figure 2. Diversity mice can contribute to research on complex traits through multiple integrated applications. The selection of the ideal mouse population is dependent on the research question being asked. Complex traits can be established as heritable, then dissected into multiple phenotypic and genotypic outputs. Furthermore, extreme and multivariate outlier strains allow the establishment of research models that can correlate and dissociate important aspects of biology.

Saul et al. *Trends in Genetics* 2019

Population tools can be leveraged for Toxicology



Harrill and McAllister, *Environmental Health Perspectives* 2017

Are DO mice TOO variable? No!

Analyte	DO Reference Range		B6C3F1 Reference Range	"Mouse" Reference Range
Albumin (g/dl)	2.4 - 3.2		2.5 - 4.2	2.5 - 3.0
ALP (U/L)	35 - 97		20 - 85	35 - 96
ALT (U/L)	11 - 46		20 - 50	17 - 77
Anion Gap	8.8 - 30.8			
BUN (mg/dl)	16 - 39		12 - 34	8 - 33
Ca (mg/dl)	8.6 - 9.8			7.1 - 10.1
Cholesterol (mg/dl)	72 - 96		80 - 130	50 - 250
CK (U/L)	24 - 270			
Cl (mEq/dl)	108 - 118			88 - 110
CO2 (mEq/L)	13 - 33			
Creatinine (mg/dl)	0.1 - 0.2		0.2 - 0.8	0.2 - 0.9
Fasting glucose (mg/dl)	69 - 157		81 - 165	62 - 175
Glob (g/dl)	1.6 - 2.7			
HDL (mg/dl)	47 - 113			
Iphos (mg/dl)	4.8 - 9.8			5.7 - 9.2
K (mEq/dl)	4.2 - 7.4		3.6 - 7.3	5.0 - 7.5
LDL (mg/dl)	6-22			
Na (mEq/dl)	145 - 155		147 - 163	140 - 160
NEFA (mEq/dl)	0.8 - 2.1			
SDH (U/L)	9.9 - 32.9		18 - 57	
Total bile acids (uMol/L)	0.4 - 4.2			
Total protein (mg/dl)	4.2 - 5.3		4.0 - 6.0	3.5 - 7.2
Triglycerides (mg/dl)	69 - 388			

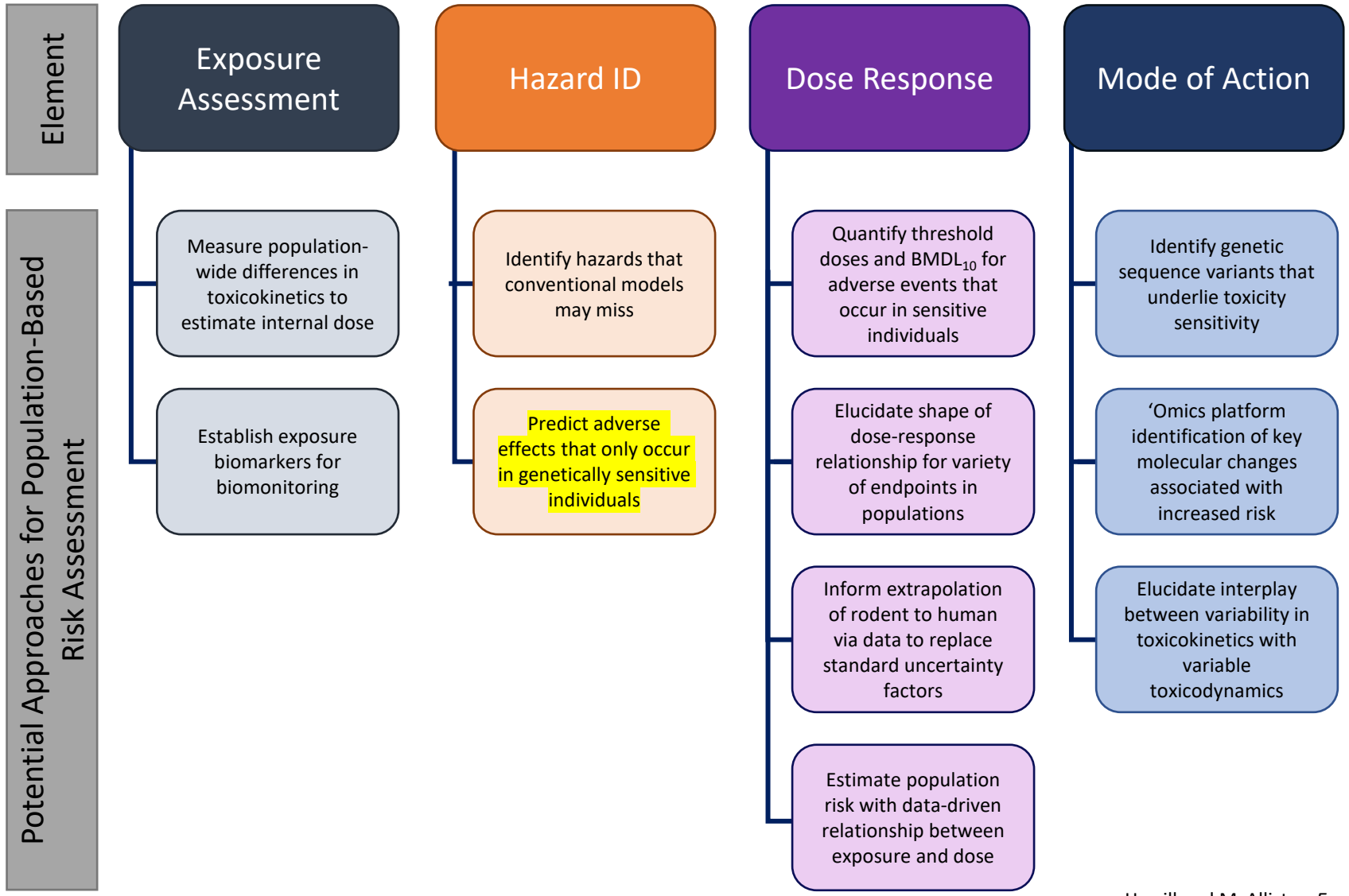
Analyte	DO Reference Range
Sperm concentration	0 - 27.7
Sperm motility	19.4 - 79.8
Path velocity	80.2 - 178.6
Progressive velocity	50.3 - 169.5
Track speed	157.6 - 300.4
Lateral Amplitude	8.7 - 14.0
Beat frequency	28.3 - 42.7
Straightness	62.8 - 88.9
Linearity	29.5 - 63.9
% Hyperactivity	0 - 8.08
Glucose AUC	31283 - 64065
Glucose AUC/mg	461.1 - 1076.1
Fasting T0 glucose	69 - 157
Glucose T0/T180	0.33 - 1.23
Wk 1 insulin (ng/ml)	0.112 - 3.19
Wk 14 insulin (ng/ml)	0.0727 - 3.49
Wk 1 leptin (ng/ml)	0.350 - 5.20
Wk 14 leptin (ng/ml)	0.415 - 17.20

N=~20-35 would work for most biomarker and histopathology observations.

Data based on 75 adult male DO mice maintained on D12450J diet.

B6C3F1: Handbook of Toxicology, 3rd Ed. From NIEHS Data
Mouse: From vet school pages of UMN and WikiVet, and *UPenn

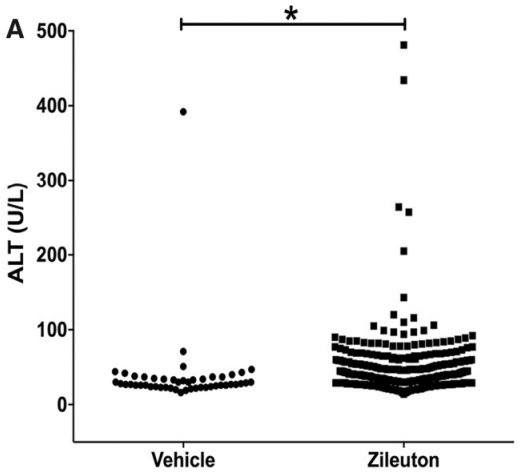
Adverse FX in genetically sensitive individuals



Harrill and McAllister, *Environmental Health Perspectives* 2017

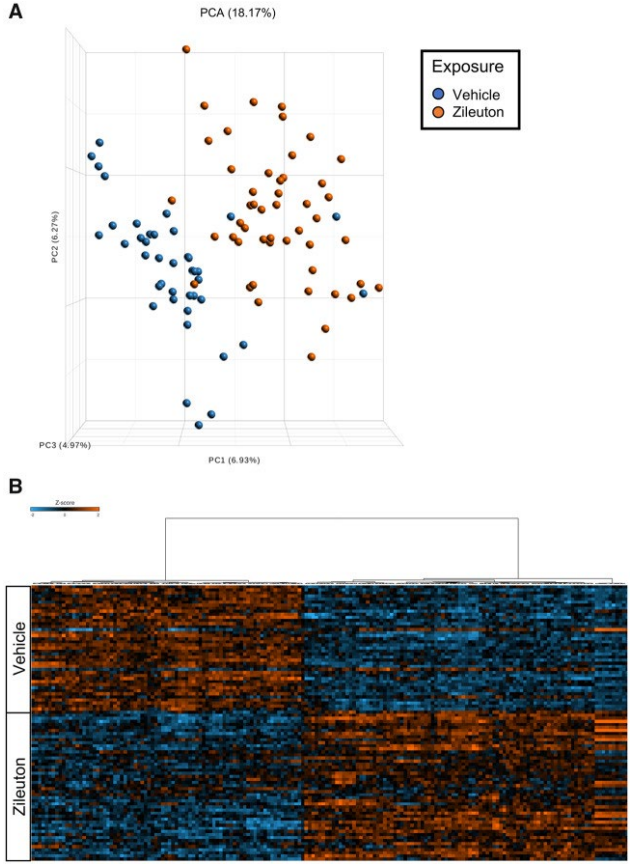
DO studies can detect human-relevant hazards (Liver)

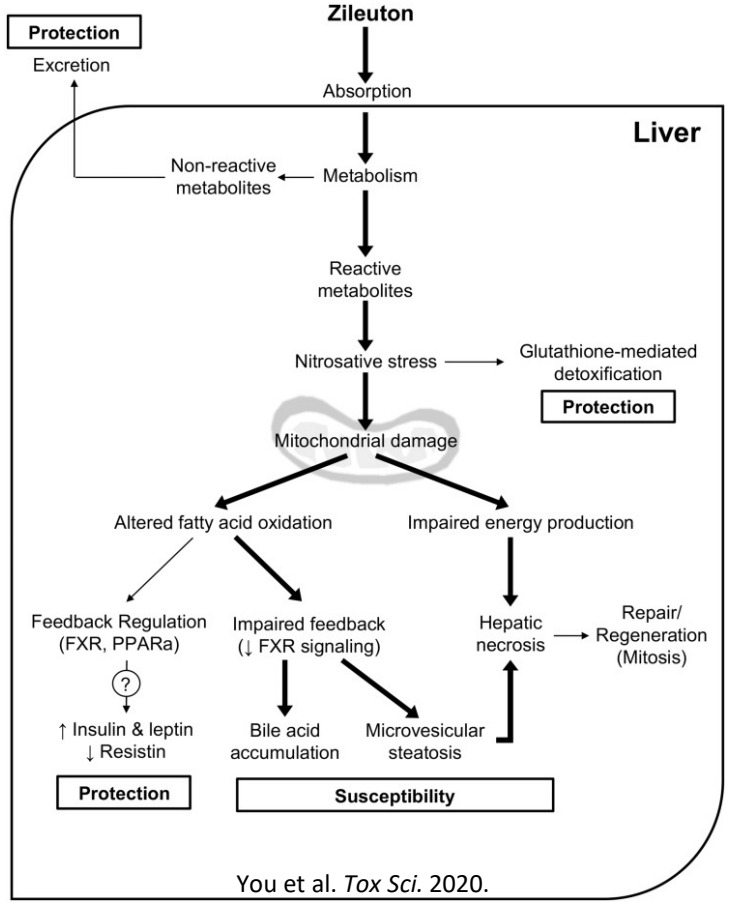
Liver injury occurred in the clinic, but was missed by conventional nonclinical testing (idiosyncratic).



Zileuton
Asthma medication
assoc. with
idiosyncratic DILI
7 days exposure (ig)
300 mg/kg

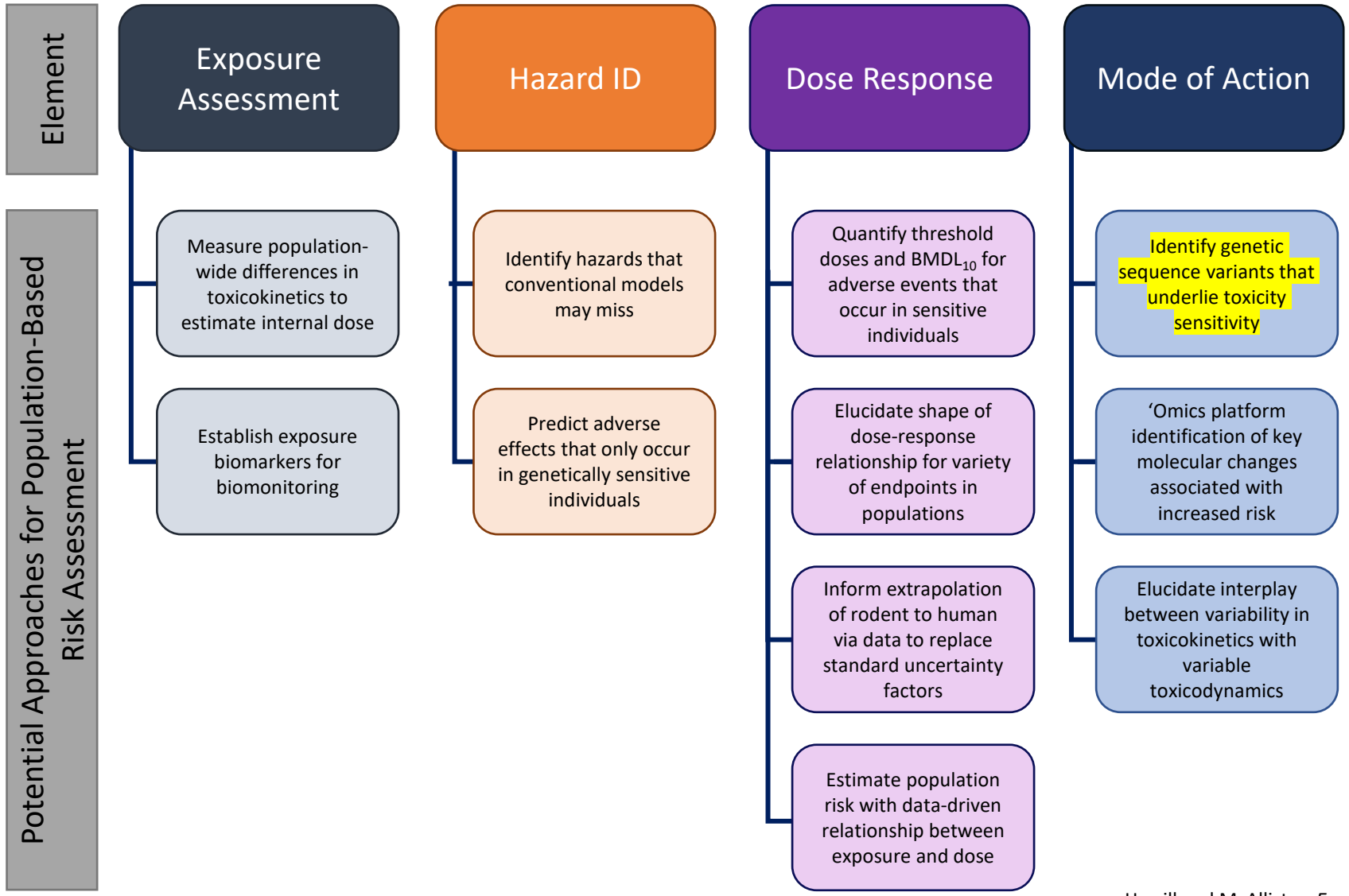
Susceptibility Mode of Action





You et al. *Tox Sci.* 2020.

Identify genetic susceptibility genes

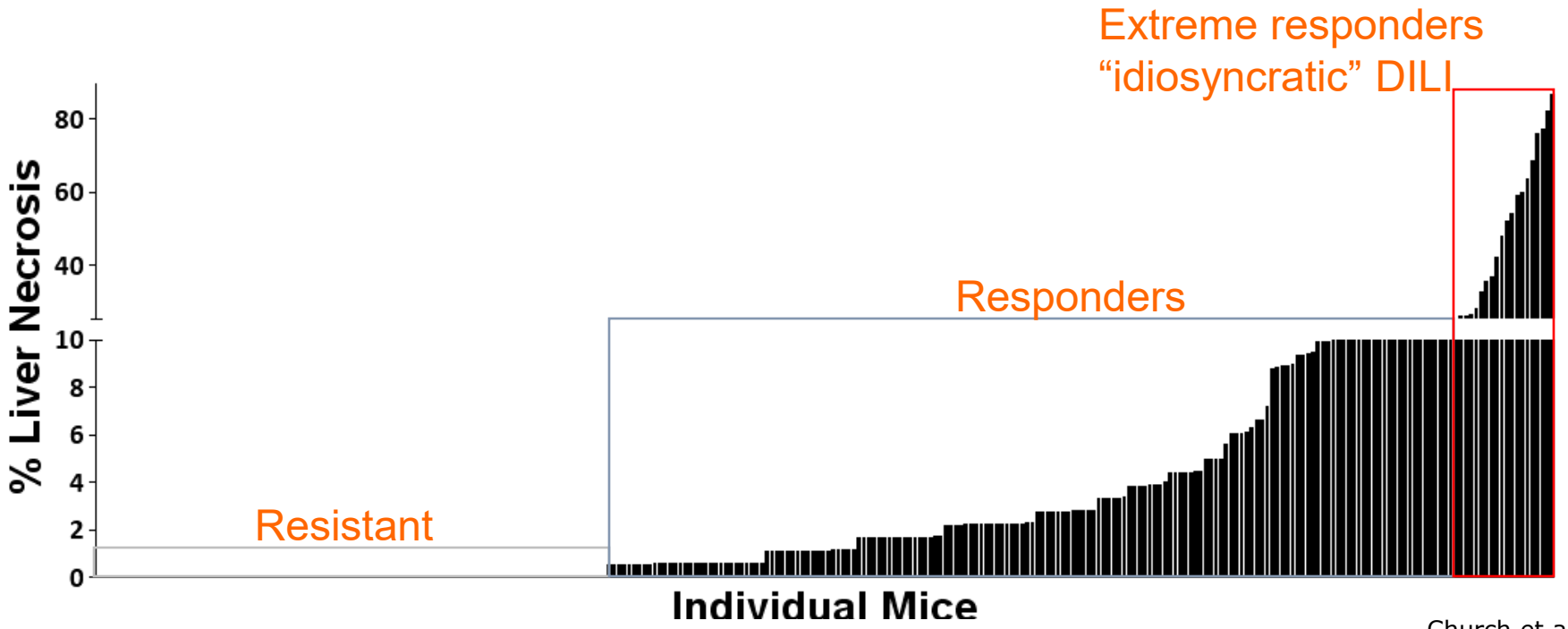


Harrill and McAllister, *Environmental Health Perspectives* 2017

Human-relevant pharmacogenetic risk factors

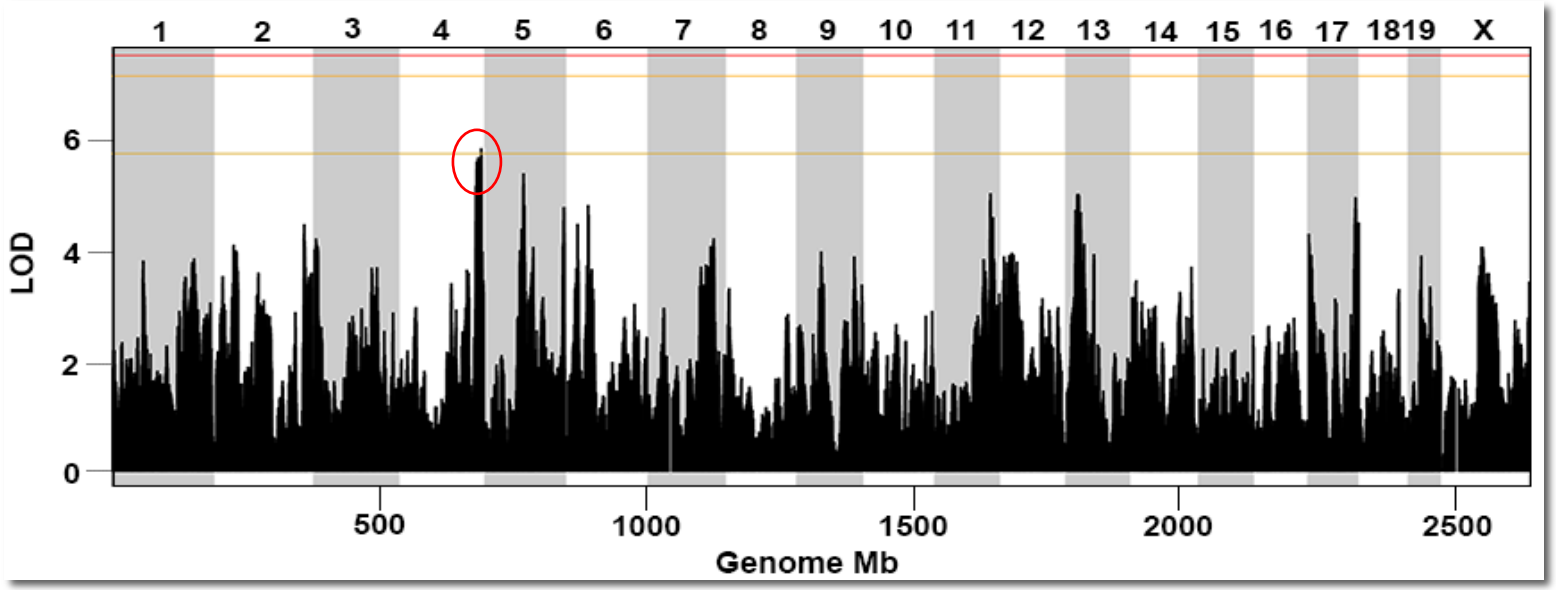
Green tea extract containing supplements cause rare and non-dose dependent liver injury in susceptible people

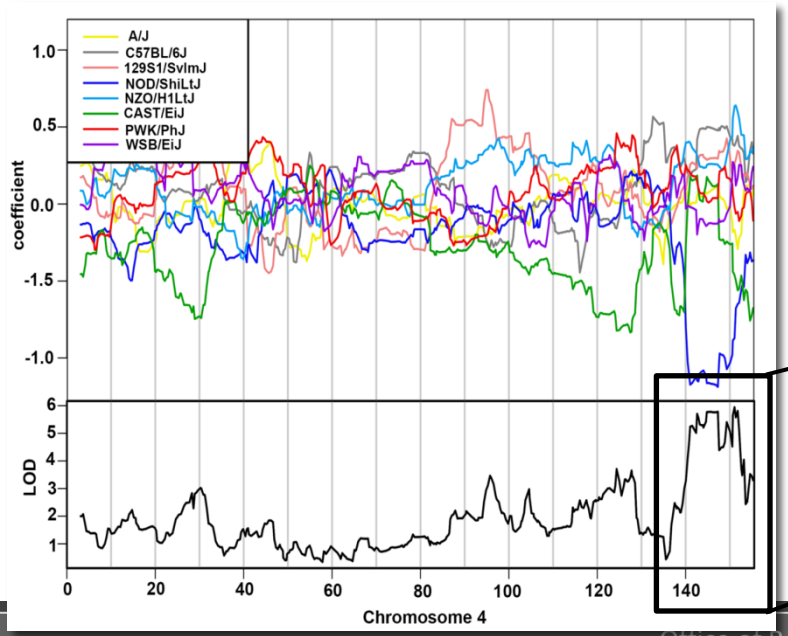
Studies of epigallocatechin gallate in ~300 female DO mice

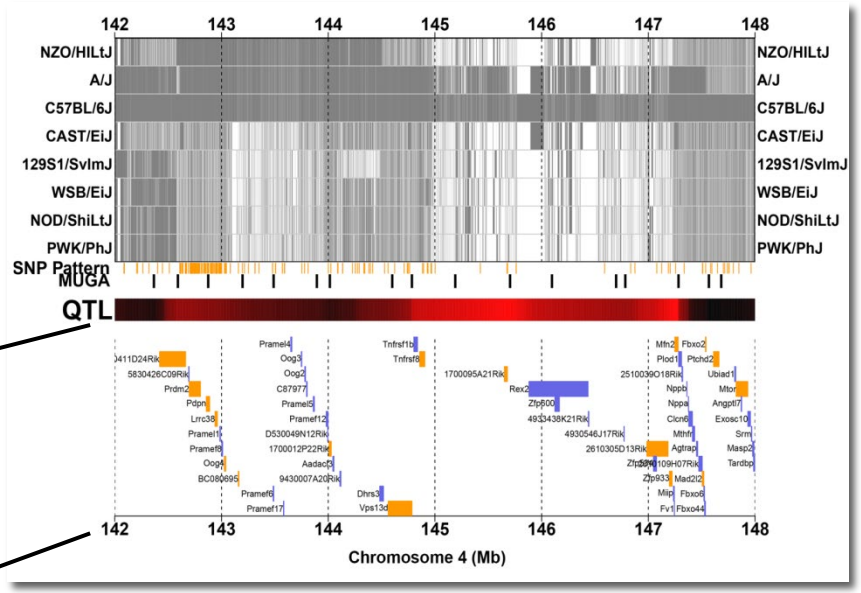


Church et al. *Food and Chem Tox*. 2015

QTL on Chr 4 in mice for zileuton hepatotoxity







Church et al. *Food and Chem Tox*. 2015

Translation of mouse genetic associations to humans for green tea extract DILI

Table 1. Confirmation of candidate quantitative trait genes in 15 clinical EGCG case samples.

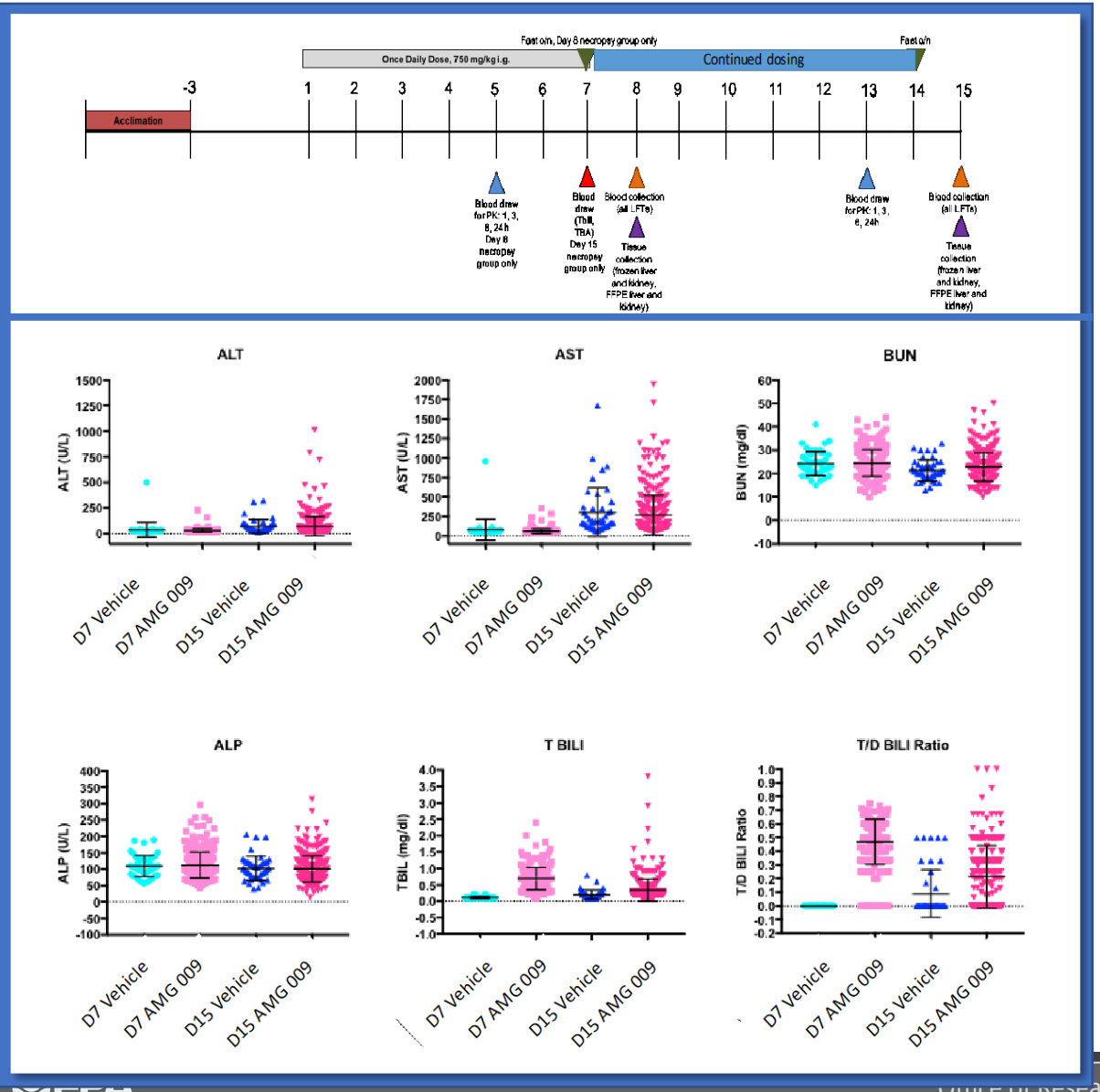
Gene Symbol	SNP ID (Array)	Gene Name	Chromosome	Position	P value for clinical association	Risk/Protective allele	Effect
PER3	exm10762	period circadian clock 3	1	7887234	0.004937	T/C	Missense (R/W)
MFN2	exm15928	mitofusin 2	1	12069692	0.0067	A/G	Missense (I/V)
VPS13D	exm16480	vacuolar protein sorting 13 homolog D (S. cerevisiae)	1	12343493	0.043064	A/T	Missense (R/S)

Mitofusin 2, involved in mitochondrial regulation and maintenance, may contribute to susceptibility to EGCG-induced liver injury by herbal supplement use.

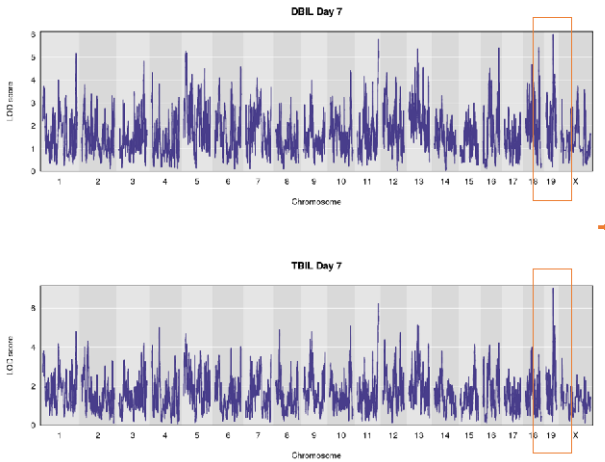
Church et al. *Food and Chem Tox*. 2015

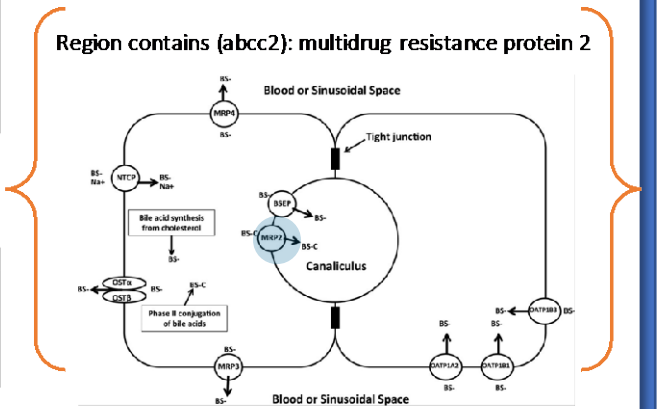
Genetic susceptibility studies in the DO – AMG 009

Genetic analysis reveals role for efflux transporter MRP2 in bilirubin increases due to AMG009.



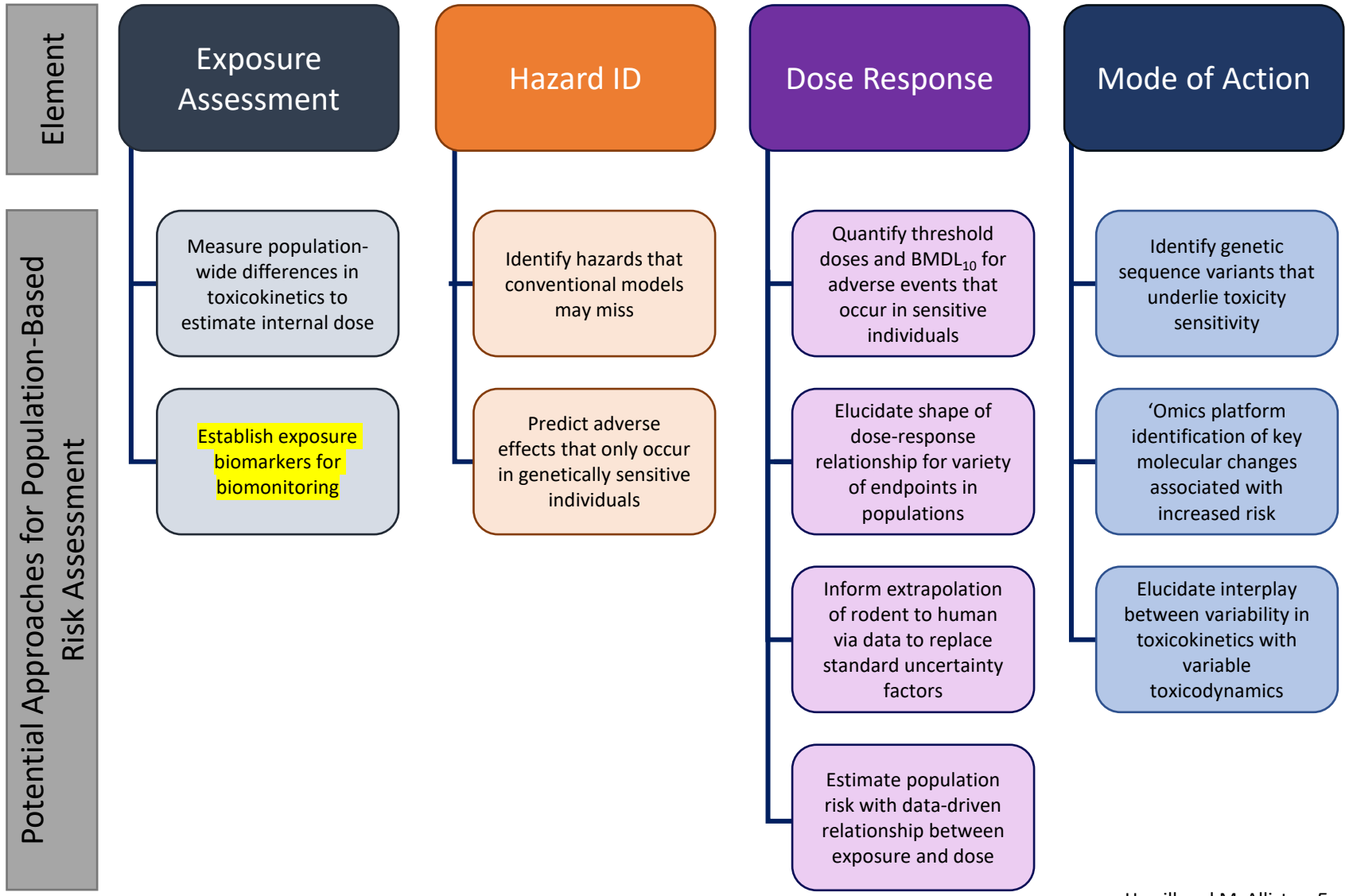
Susceptibility Mode of Action





A. Harrill lab, unpublished data.

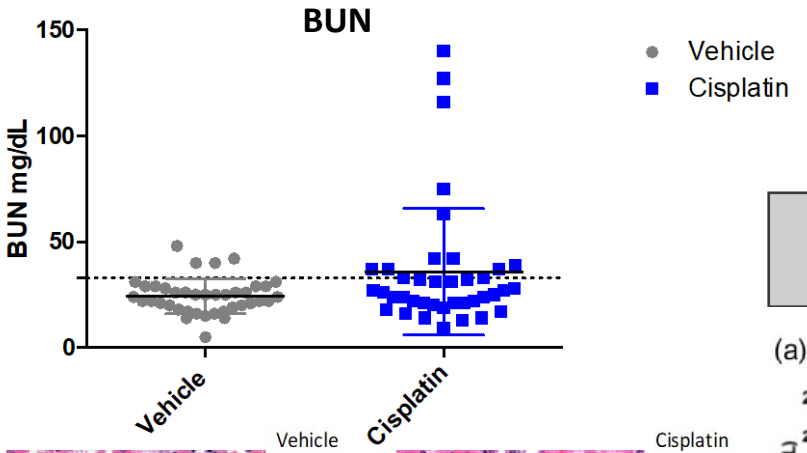
Establishing & Evaluating Biomarkers

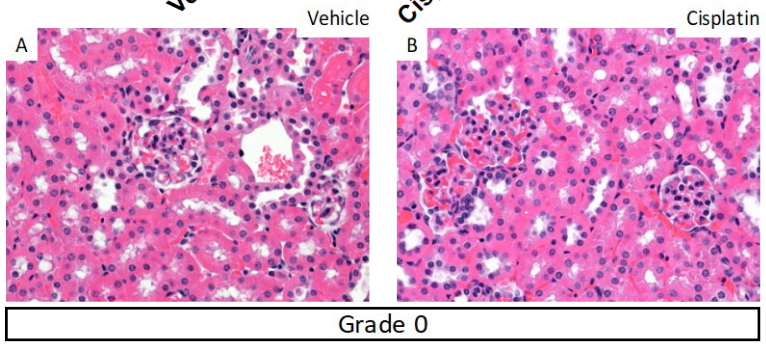


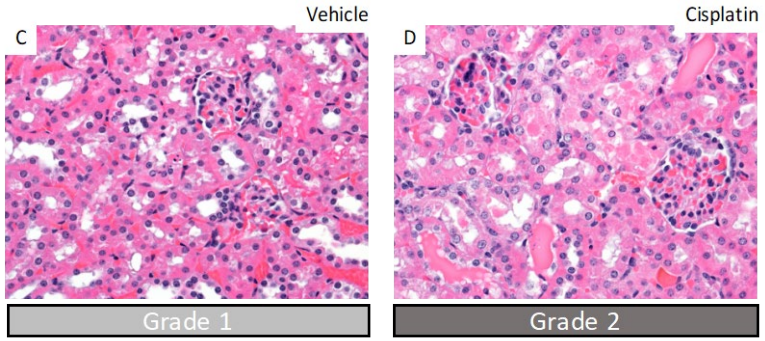
Harrill and McAllister, *Environmental Health Perspectives* 2017

Biomarker sensitivity studies in the DO (Kidney)

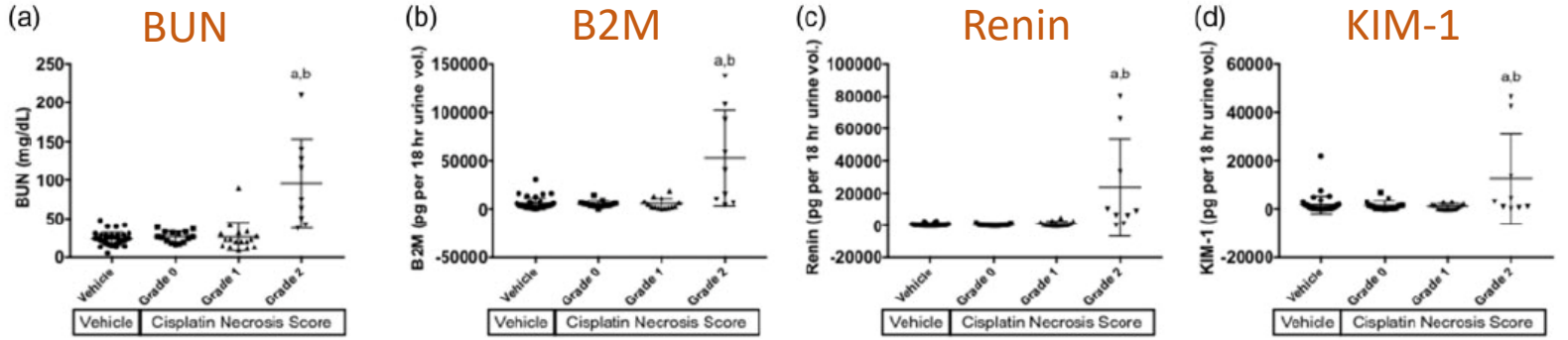
Modeling patient susceptibility to cisplatin-induced kidney injury – benchmark biomarkers to underlying pathology



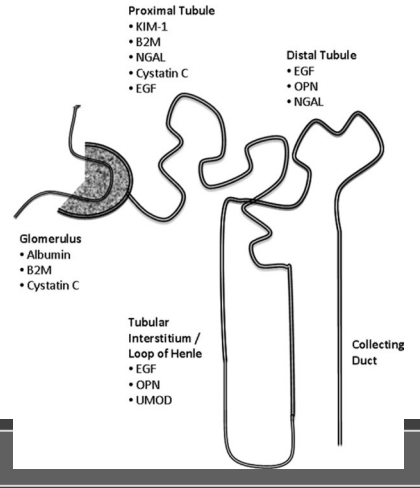




Evaluate Biomarker Performance



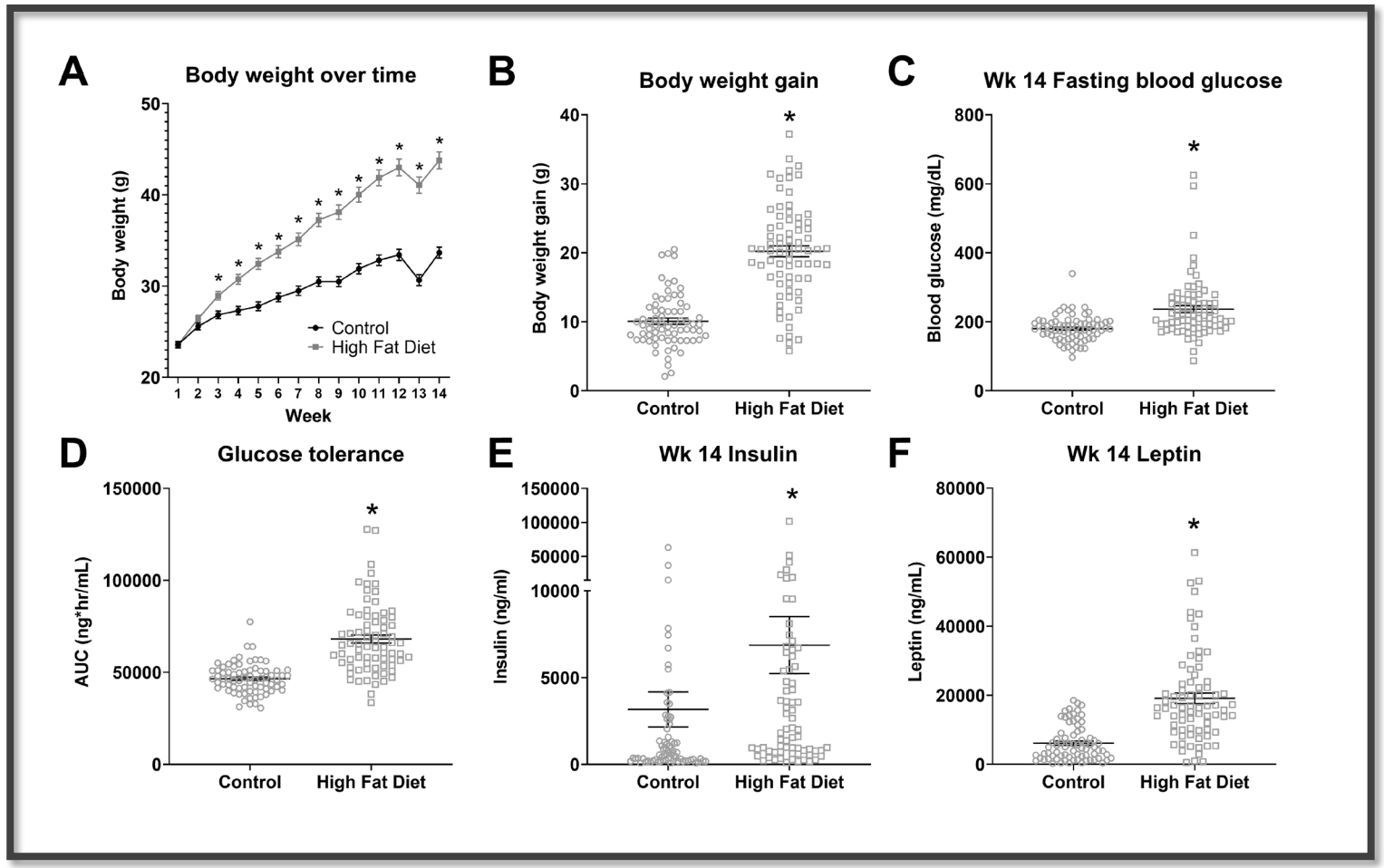
More severe kidney pathology

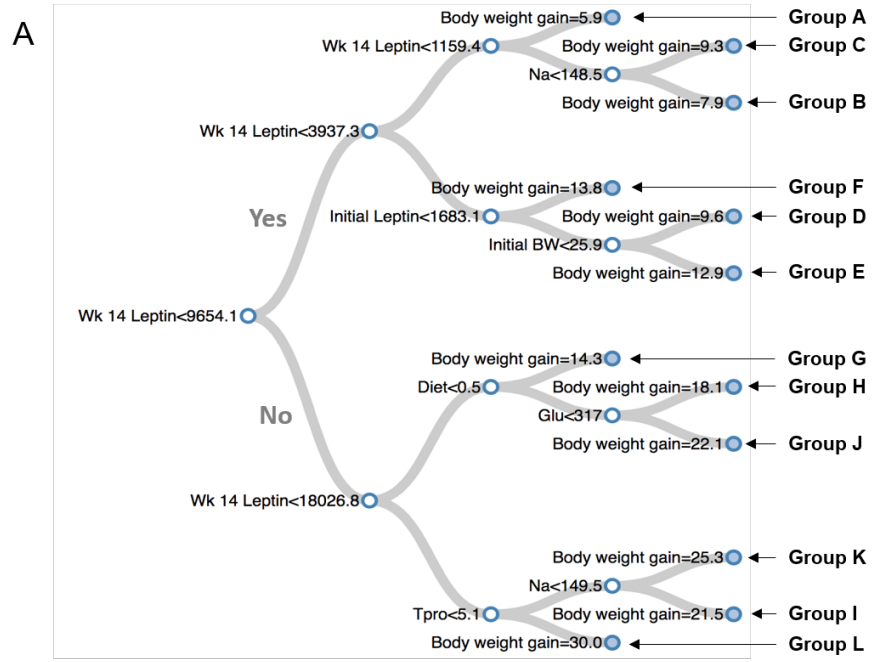


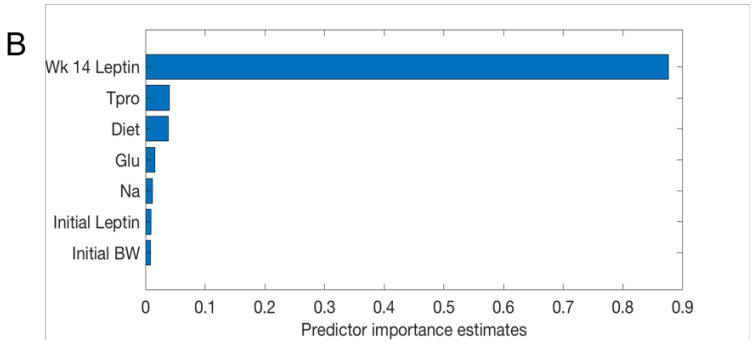
A. Harrill et al. Exp Biol. Med 2017.

NTP/NIEHS study: metabolic syndrome biomarkers

75 male DO mice on control or high fat diet for 14 weeks





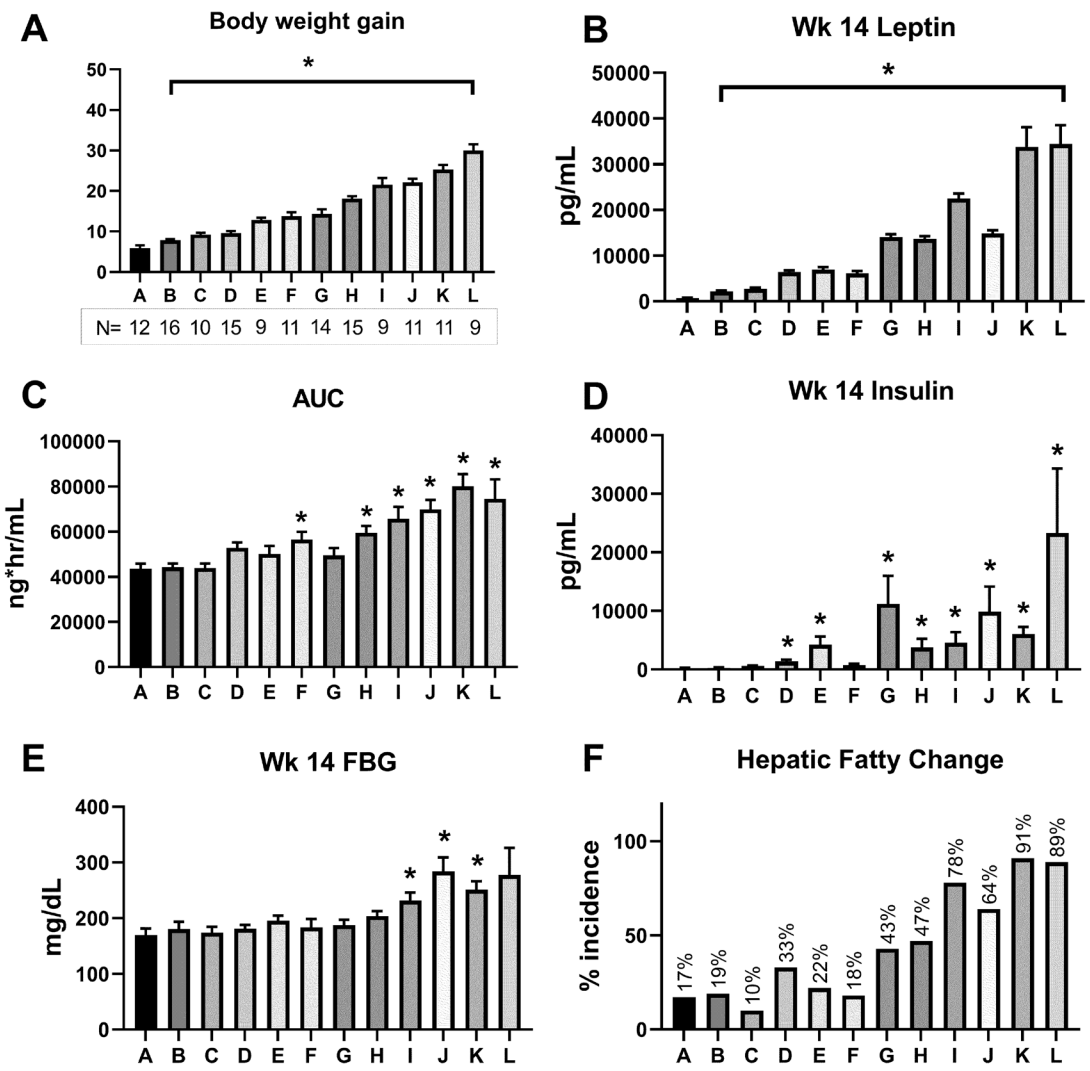


DNTP collaboration with DIR: Leping Li, Yuanyuan Li, Keith Shockley, Kevin Gerrish Lab w/ NTP trainees Mimi Huang, Dahea You, Natalie Bell

Mimi Huang et al., unpublished data

CART classification of metabolic biomarkers to predict weight gain

Group	# animals in group	% animals on HFD	Average body weight gain (g)	Difference in group average body weight gain compared to Group A (g)
A	*12*	25	*5.9*	*NA*
B	16	0	7.9	2.0
C	10	20	9.3	3.4
D	15	27	9.6	3.8
E	9	22	12.9	7.0
F	11	55	13.7	7.9
G	14	0	14.3	8.4
H	15	100	18.1	12.2
I	9	89	21.5	15.7
J	11	100	22.1	16.3
K	11	100	25.3	19.4
L	9	100	30.0	24.2



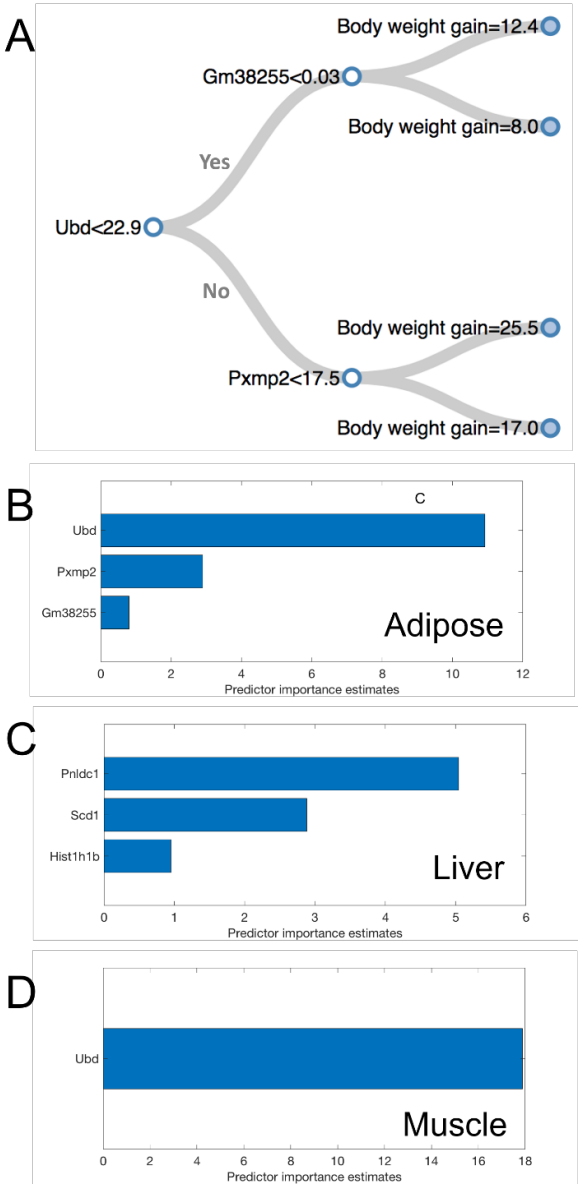
Mimi Huang et al., unpublished data

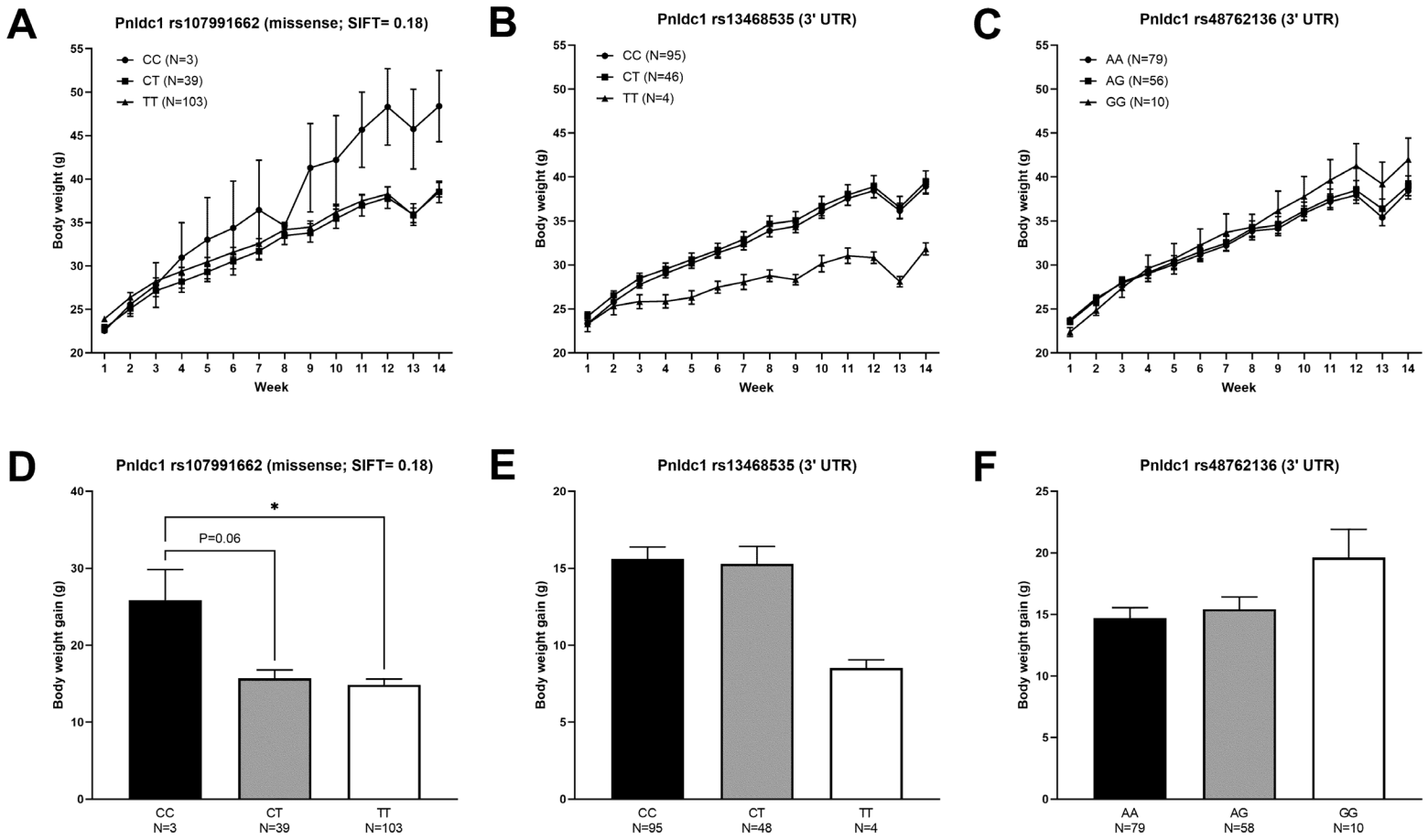
RNA-Seq: DEGs expressed in 23 metabolically active tissues

Group	Difference in between average body weight gain compared to Group A (g)	Adipose-DEG	Liver-DEG	Muscle-DEG
A	*NA*	*NA*	*NA*	*NA*
B	2.0	8	1	0
C	3.4	6	9	5
D	3.8	35	1	0
E	7.0	83	8	12
F	7.9	39	2	2
G	8.4	139	4	0
H	12.2	118	1	0
I	15.7	486	2	1
J	16.3	905	2	0
K	19.4	1510	6	0
L	24.2	3408	16	73

Mimi Huang et al., unpublished data

CART analysis of RNASeq Data → Genetic predisposition

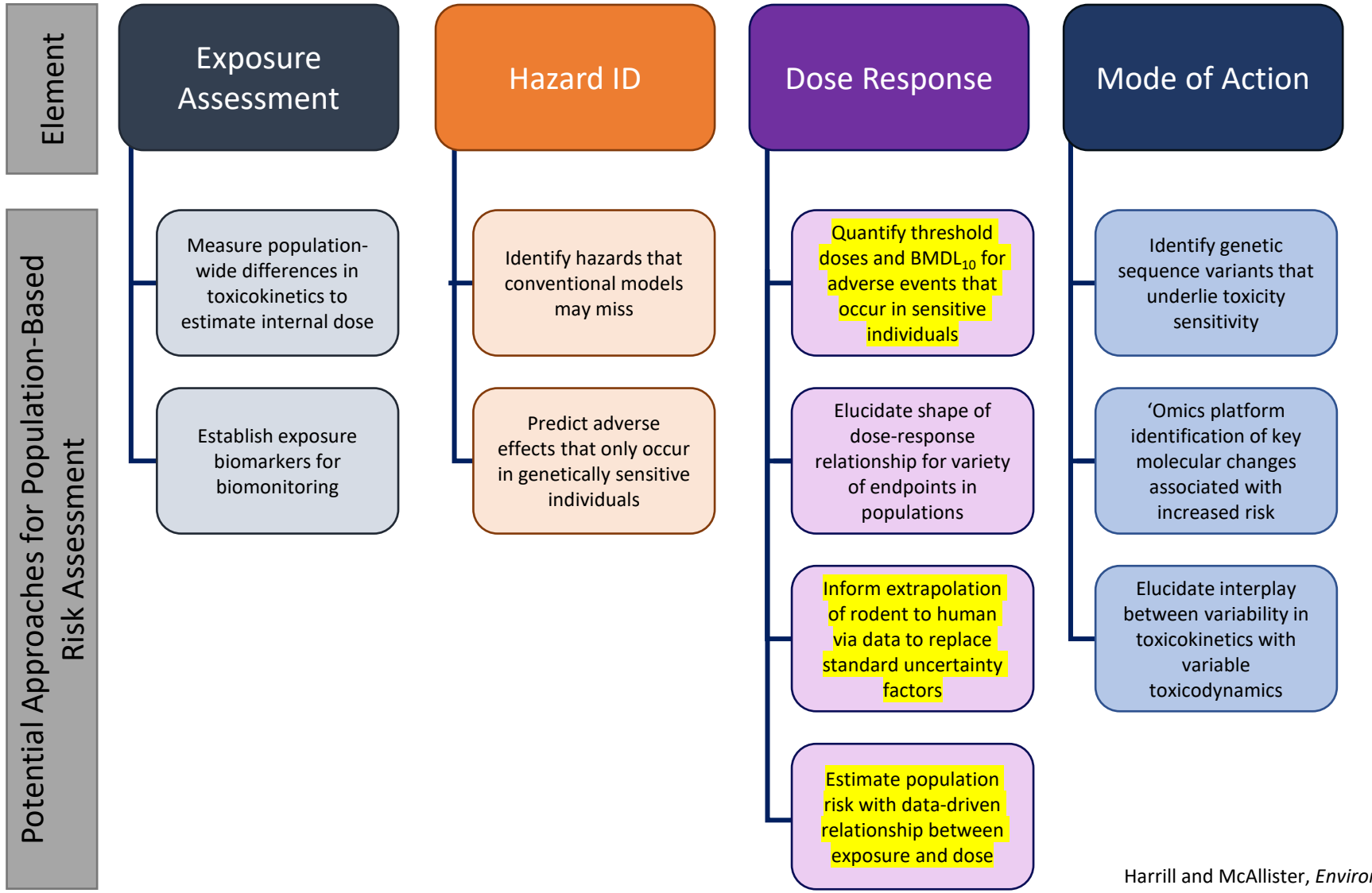




Missense SNP homozygous C/C in coding region of deadenylase gene Pnldc1 is associated with increased weight gain over 14 weeks, irrespective of diet given, and may be **a step toward identifying a genetic biomarker**.

Mimi Huang et al., unpublished data

Population-based dose response requires a higher throughput / in vitro approach

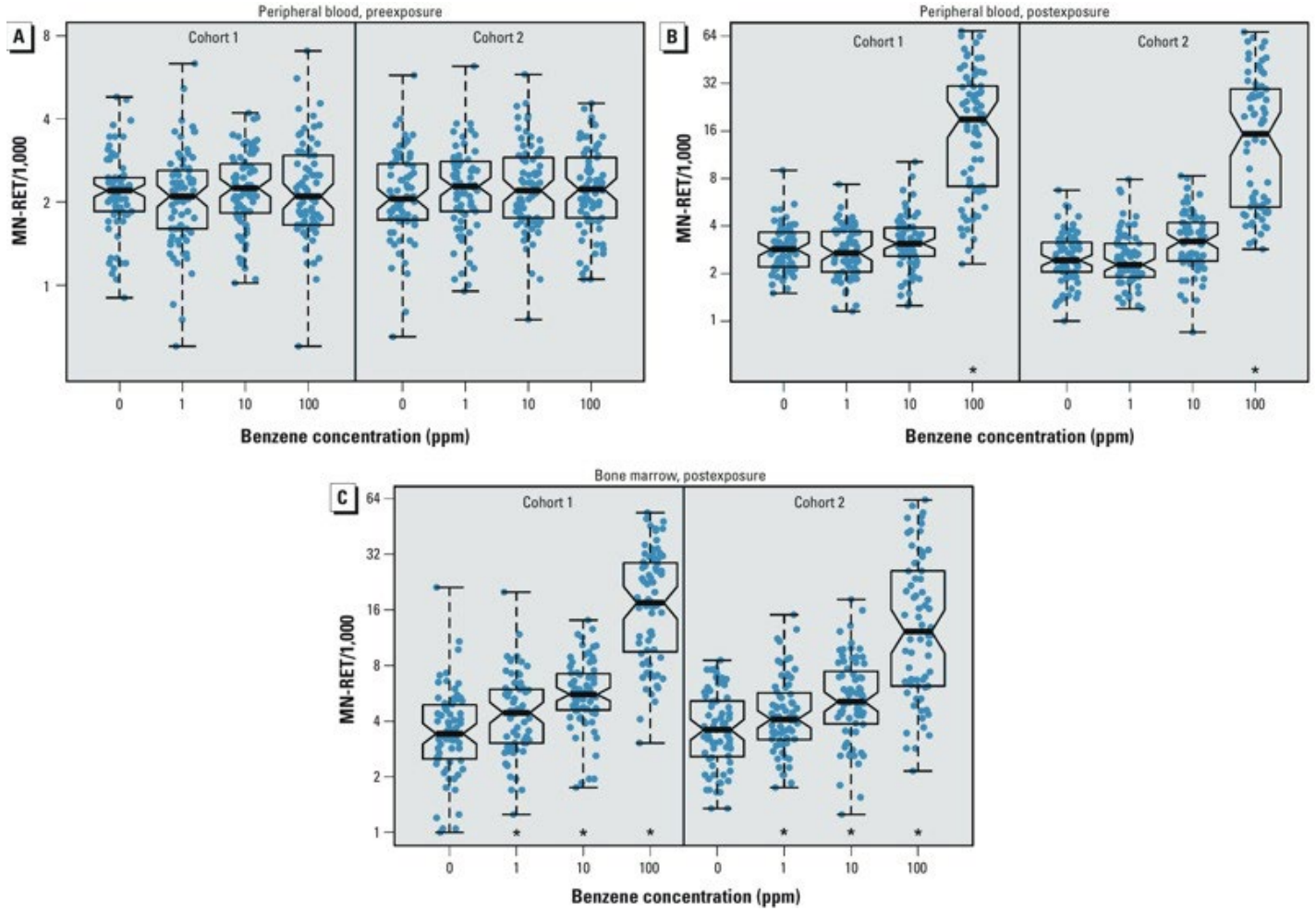


Harrill and McAllister, *Environmental Health Perspectives* 2017

Dose response studies have been done in vivo by NTP

Human $BMCL_{10}$ 7.2 ppm (44 subjects), < 1 ppm FX

DO $BMCL_{10}$: 0.205 ppm ; B6C3F1 $BMCL_{10}$: 3.12 ppm



DO mice, by virtue of including diversity, better predict the human POD.

French *et al. EHP* 2015.

Tox21: Cross-Partner Project to translate DO to in vitro

Developmental neurotoxicity is a critical area for NTP and a new Health Effect Initiative (HEI)

NTP + EPA
CPP#7

- Goals:
  - Quantify dose-response relationship in neurotox across individuals
  - Calculate chemical-specific toxicodynamic variability factors
  - Understand mechanisms of toxicity in sensitive subpopulations

Protect *this* kid

AND *these* kids, by using population-based toxicity models

Evidence DO mice can detect human-relevant neurotox variation

JWH-018 (spice/K2) Tetrad

Variation in development of tolerance and incidence of seizure in female DO mice

Neurohospitalist. 2011 Oct; 1(4): 182–186. PMCID: PMC3726077
doi: 10.1177/1941874411417977

The Secret "Spice": An Undetectable Toxic Cause of Seizure

Adam de Havenon, MD,[1] Brian Chin, MD,[2] Karen C. Thomas, PharmD, PhD,[3] and Pegah Afra, MD[1]

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Abstract

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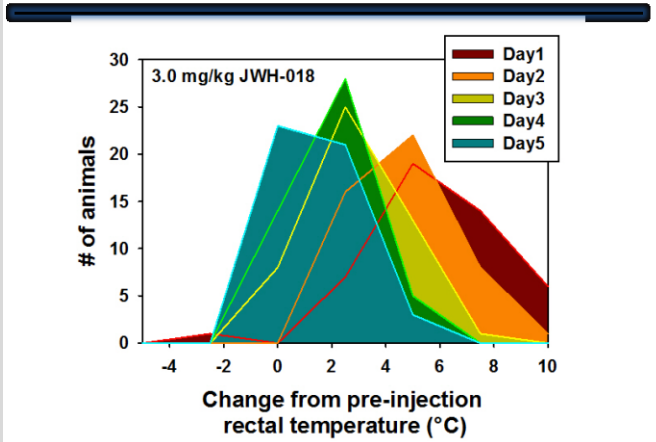
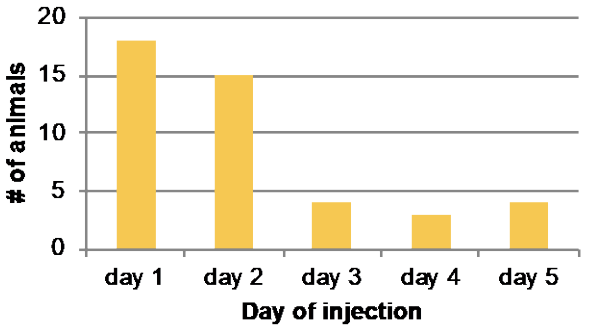
Neurologists and emergency department physicians are frequently involved in the comprehensive evaluation of a first generalized seizure. An important aspect of this evaluation is a detailed history which can identify a provoked seizure secondary to drug toxicity and hence avoid unnecessary treatment with antiepileptic drugs. "Spice" is an umbrella term for a variety of synthetic cannabinoid products whose inhalation has been associated with an increasing number of toxic side effects resulting in emergency department visits. These side effects (including psychosis, tachyarrhythmia, and seizures) are not typically seen with marijuana (*Cannabis*

Change in body temperature



Convulsions per day (not rated)



N=50

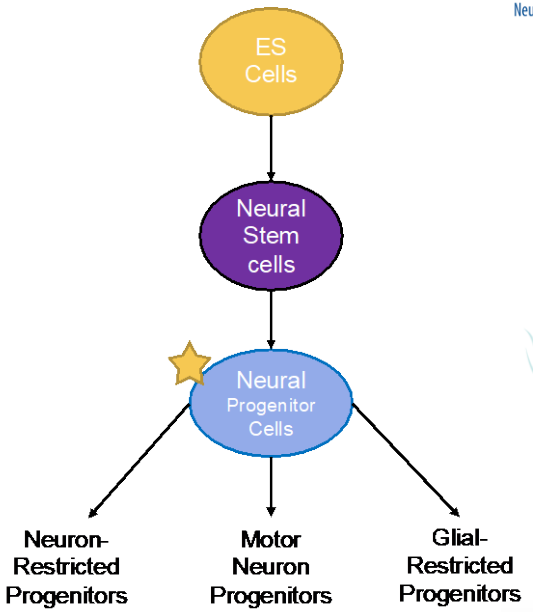
Convulsions observed in some DOs – this has never been reported with conventional mouse strains (Balb/c) for this dose of JWH-018

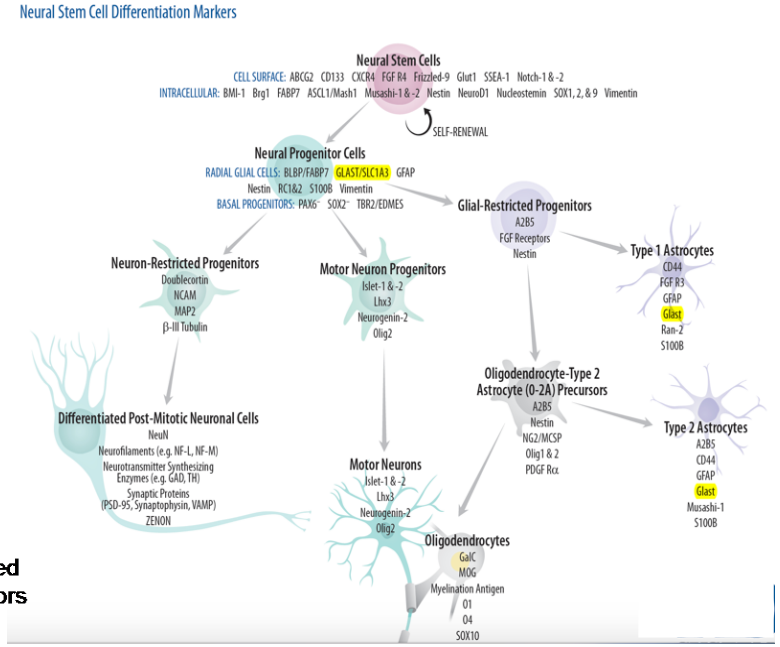
A. Harrill and Fantegrossi, unpublished data (UAMS)

200 DO NPC lines created (M/F) by Predictive Biology

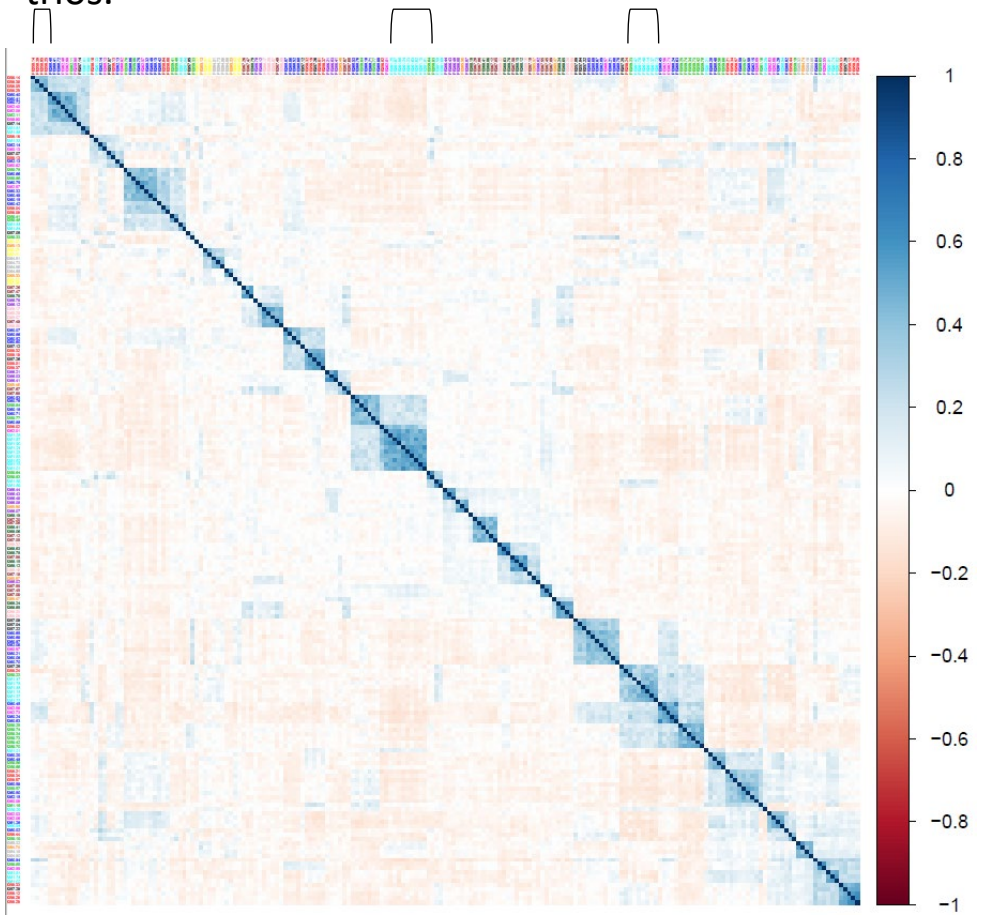
Genetic characterization of DO cell lines:

- ES Lines: Whole genome sequence, baseline RNA-seq
- NPC lines: baseline RNA-seq



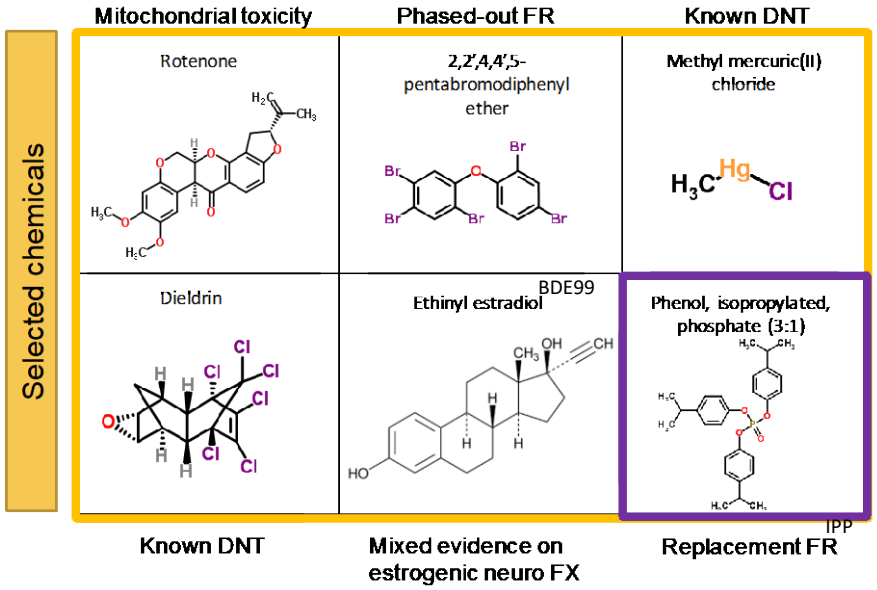


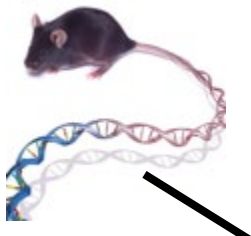
Most of the genetic relatedness structure is within subsets of cell lines derived from the same mating trios.



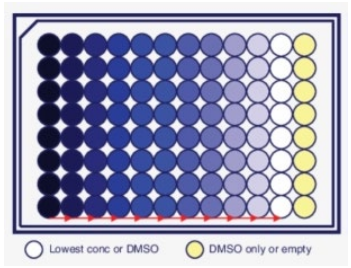
Pilot study – dose response of cytotoxicity

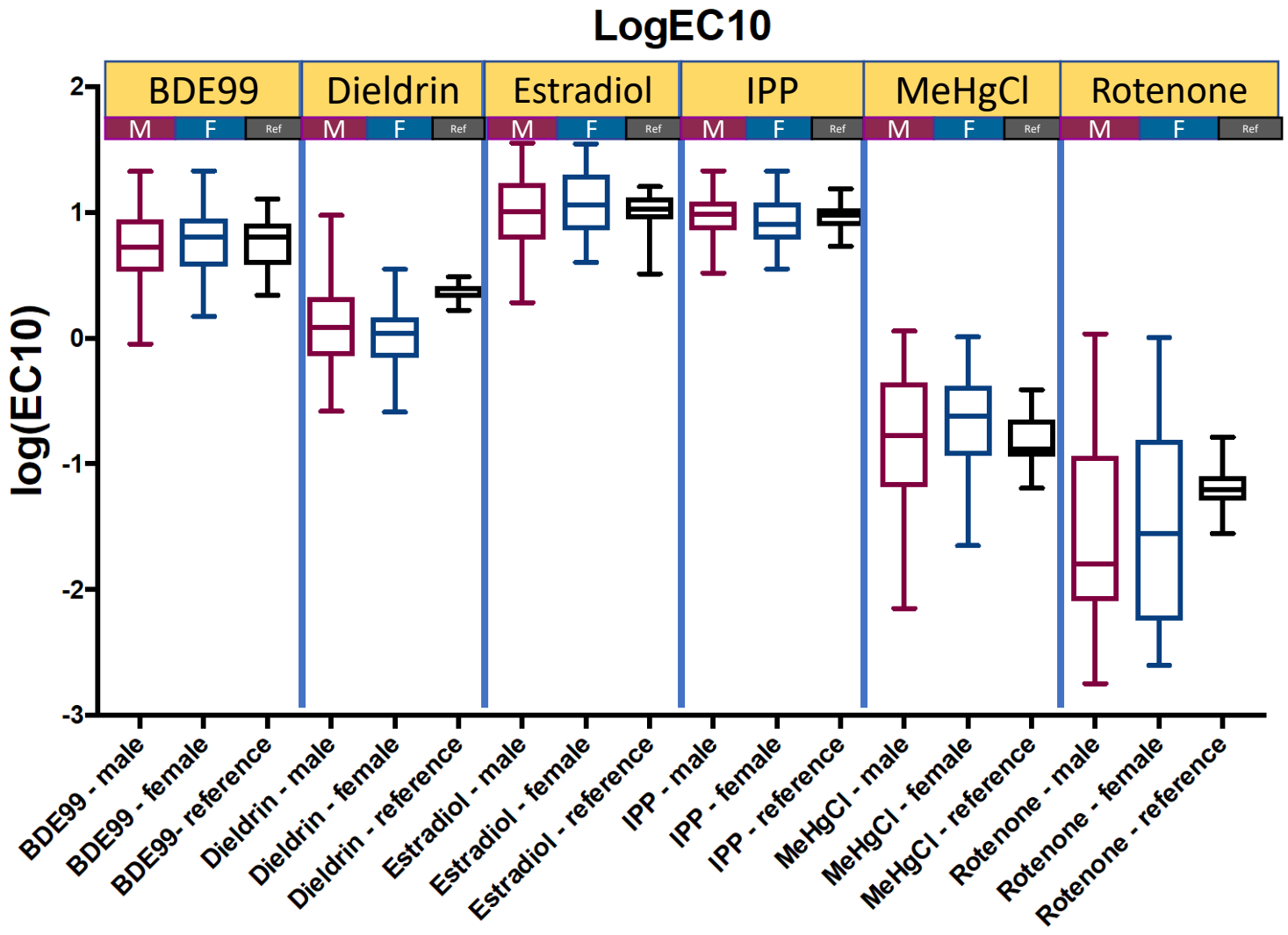
No obvious sex differences in the EC10 range for the 6 chemicals tested



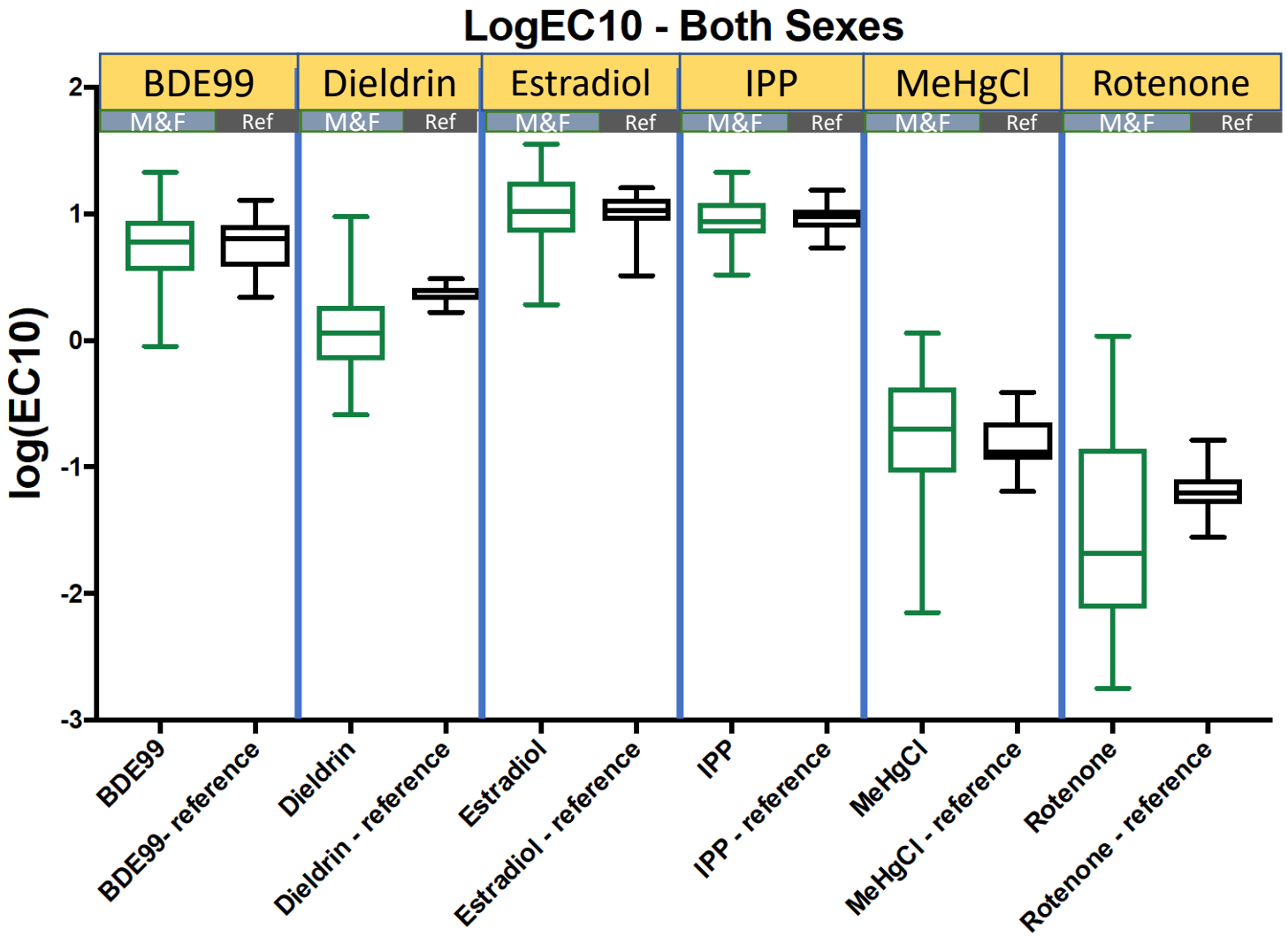


Identical NPC
12-dose response plates
6 chemicals; alamar blue @ 114 h





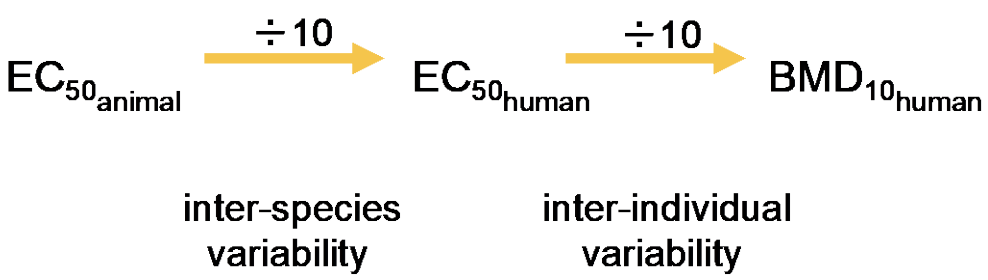
Grouping the sexes into single population: Alamar Blue 114 h



Quantifying toxicodynamic variability from population data

For a given chemical, we can quantify:

- **Variability:** The observable diversity in biological sensitivity or response, and in exposure parameters
- **Uncertainty:** Imperfect knowledge concerning the present or future state of an organism, system, or population under consideration

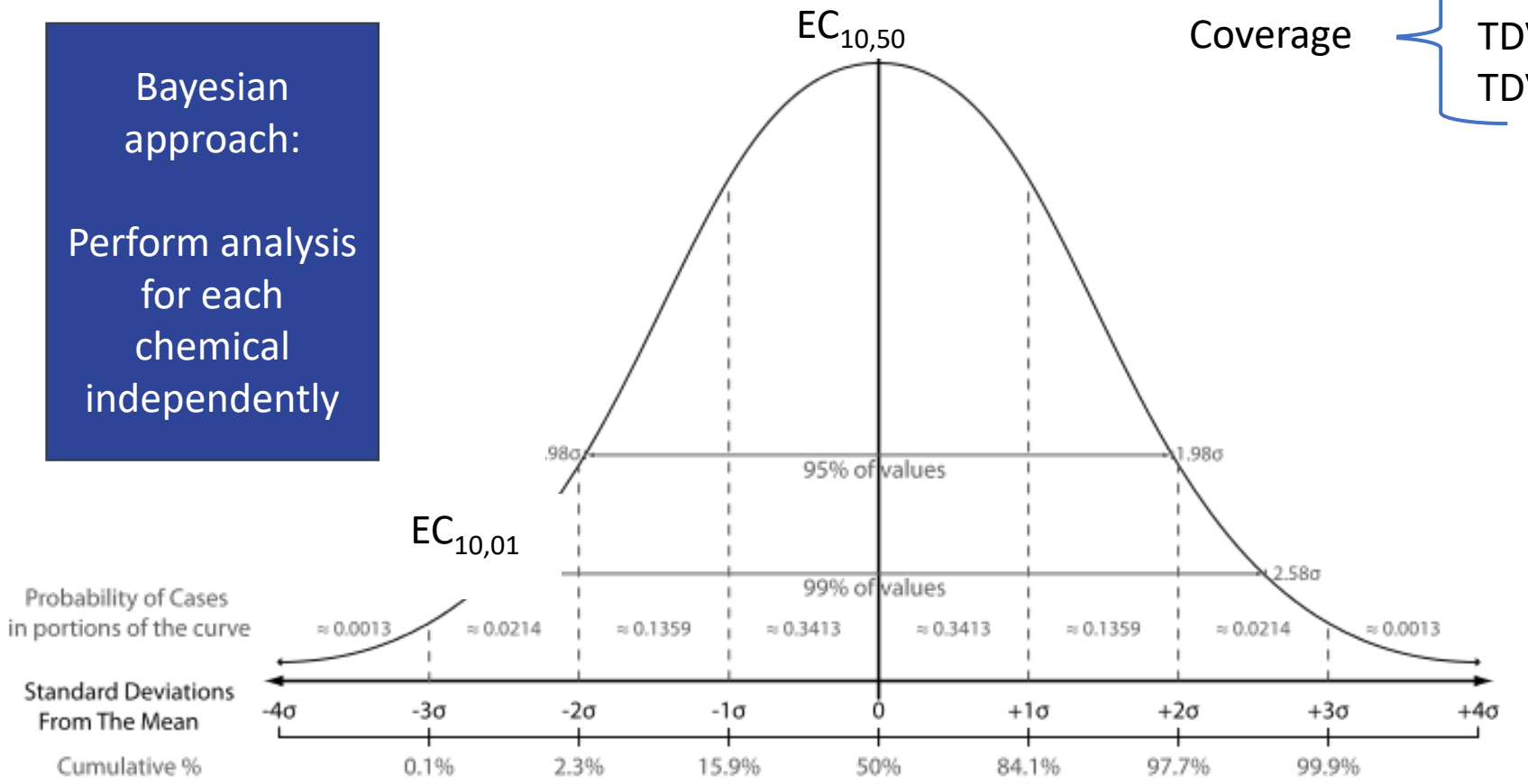


The default fixed uncertainty factor for toxicodynamic variability is $10^{1/2}$
Corresponds to:
TDVF = 3.16

Using population data to quantify toxicodynamic variability

Bayesian approach:

Perform analysis for each chemical independently



Coverage

Toxicodynamic Variability Factor

$TDVF_{01} = (EC_{10,50} / EC_{10,01})$

$TDVF_{05} = (EC_{10,50} / EC_{10,05})$

WHO/IPCS 2005

(Pilot) Data-driven TDVFs of DO NPCs for Chemical Cytotoxicity

- Bayesian approach to determine chemical-specific variability factor

Chemical	TDVF05 (90% CI)	
	DO Mouse NPCs	Human LCLs[1]
IPP	1.71 (1.60, 1.86)	-
Estradiol	1.82 (1.66, 2.05)	-
BDE 99	2.39 (2.00, 2.96)	-
Dieldrin	2.80 (2.42, 3.33)	3.76
Default factor = 3.16		
Rotenone	11.2 (7.51, 19.1)	-
MeHgCl	26.9 (10.3, 109)	16.03

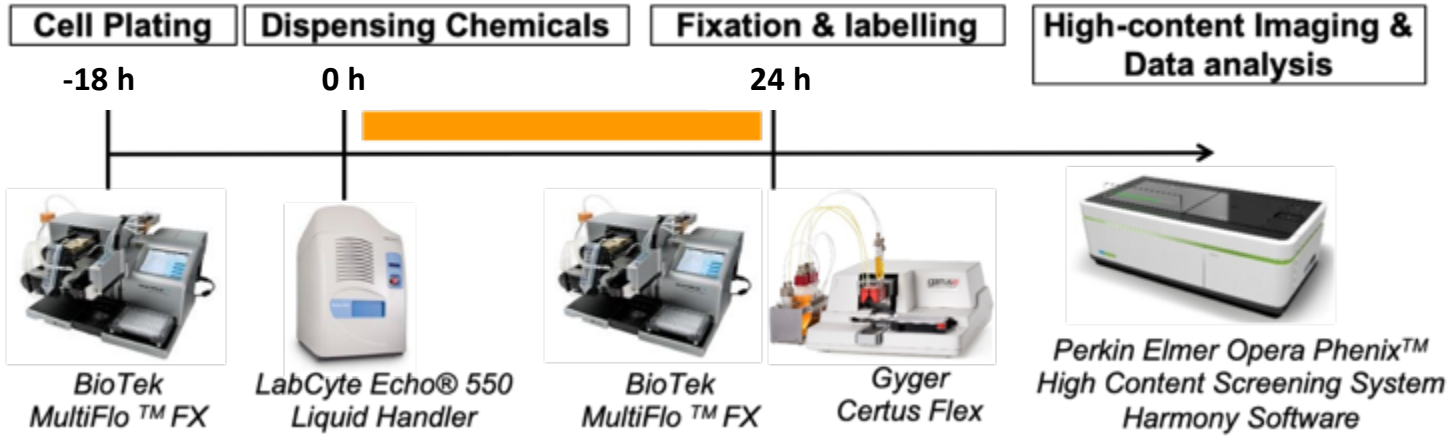
- LCL: Lymphoblastoid cell lines
- Sample size: Human >1000 individuals vs. Mouse < 200 individuals
  - DO mouse NPCs have highly randomized polymorphisms throughout genome.

[1] Chiu WA, et al. ALTEX, 2017. 34(3): 377-388

Phase 2: Phenotypic Profiling via Cell Painting (High-Content Imaging)

- Visualize sub-cytotoxic effects: understand mechanism and susceptibility

No antibodies!



Fluorescent Labels
DNA: H-33342
RNA: SYTO14
ER: Concanavalin A-488
Actin: Phalloidin-568
Golgi + Membrane: wheat germ agglutinin (WGA) -555
Mitochondria: MitoTracker

Analysis Steps

1,300 morphometric endpoints

↓

Cell-level data

Normalize to DMSO control

↓

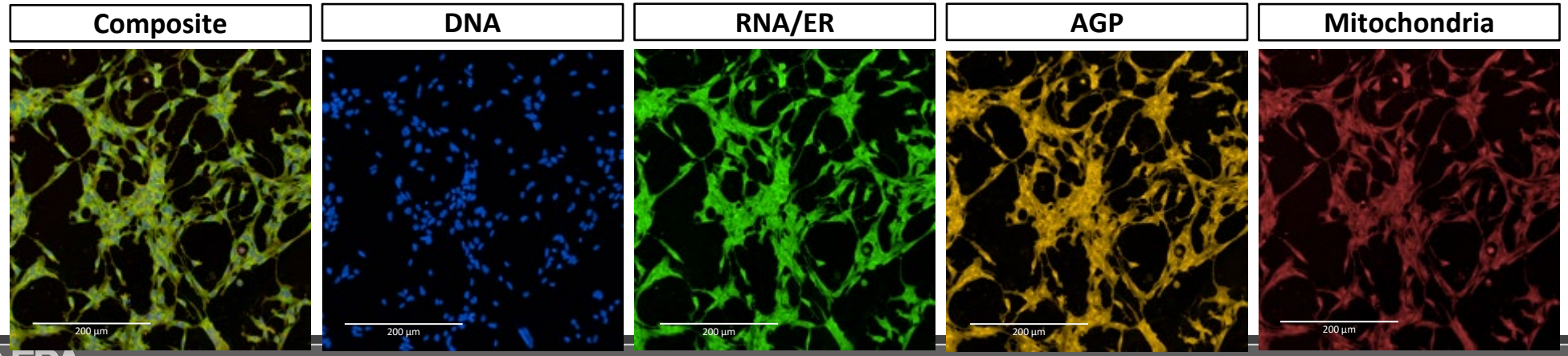
Normalized cell-level data

Aggregate and pool data

↓

Normalized data from pooled wells

- Untreated PB361.63 DO NPCs, 20X water immersion objective



Test Chemical	Concentration (uM)	
	Lowest	Highest
BDE99	0.0002	20
Dieldrin	0.00025	25
IPP	0.0005	50
MeHgCl	0.00002	2
Rotenone	0.0002	20
5-Fluorouracil	0.0002	20
Hexachlorophene	0.0002	20
Captan	0.0002	20
Tebuconazole	0.0002	20
p-nitrosodiphenylamine	0.0002	20
Bisphenol A	0.0002	20
Saccharin	0.001	100

Fluorescent Labels
DNA: H-33342
RNA: SYTO14
ER: Concanavalin A-488
Actin: Phalloidin-568
Golgi + Membrane: Wheat Germ Agglutinin - 555
Mitochondria: MitoTracker

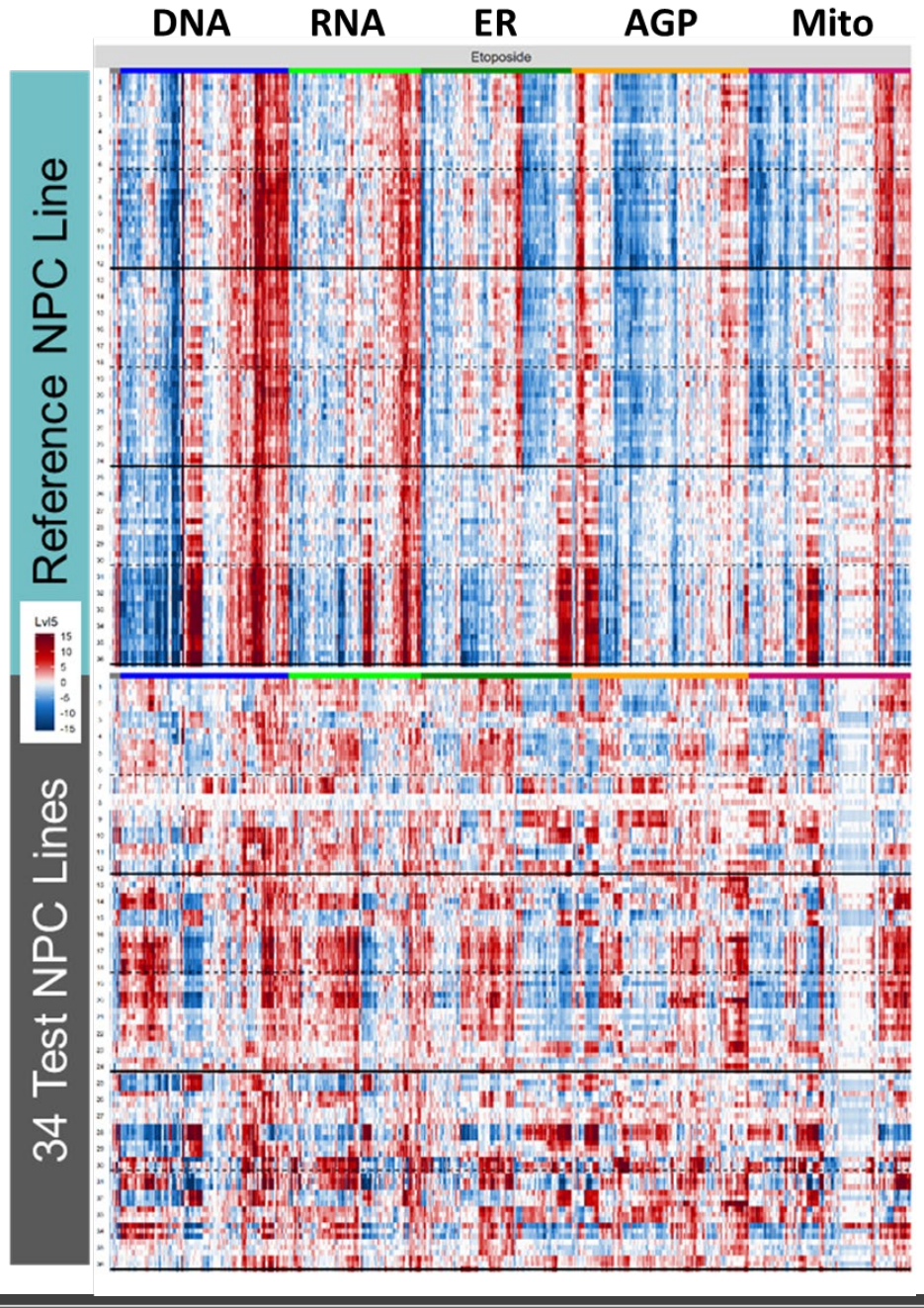
Cell Lines: 98 Diversity Outbred neural progenitor cell lines (male and female). Reference cell line included on every test plate. All conditions in triplicate wells.

Exposure: 12 chemicals were tested across cell lines, with concentration ranges empirically determined in pilot experiments. These included priority compounds for the NTP and EPA for developmental neurotoxicity testing and putative negative control saccharin. Vehicle: DMSO 0.1%

Assay Control Chemicals: Etoposide, berberine chloride, and rapamycin were included on each plate.

Cell Painting: Cells were fixed and labeled 24 h post-exposure according to Bray et al. 2016 and updated in Nyffeler et al. 2020. Images were acquired using the Opera Phenix. Cells were segmented and cell compartments were profiled (1300 features).

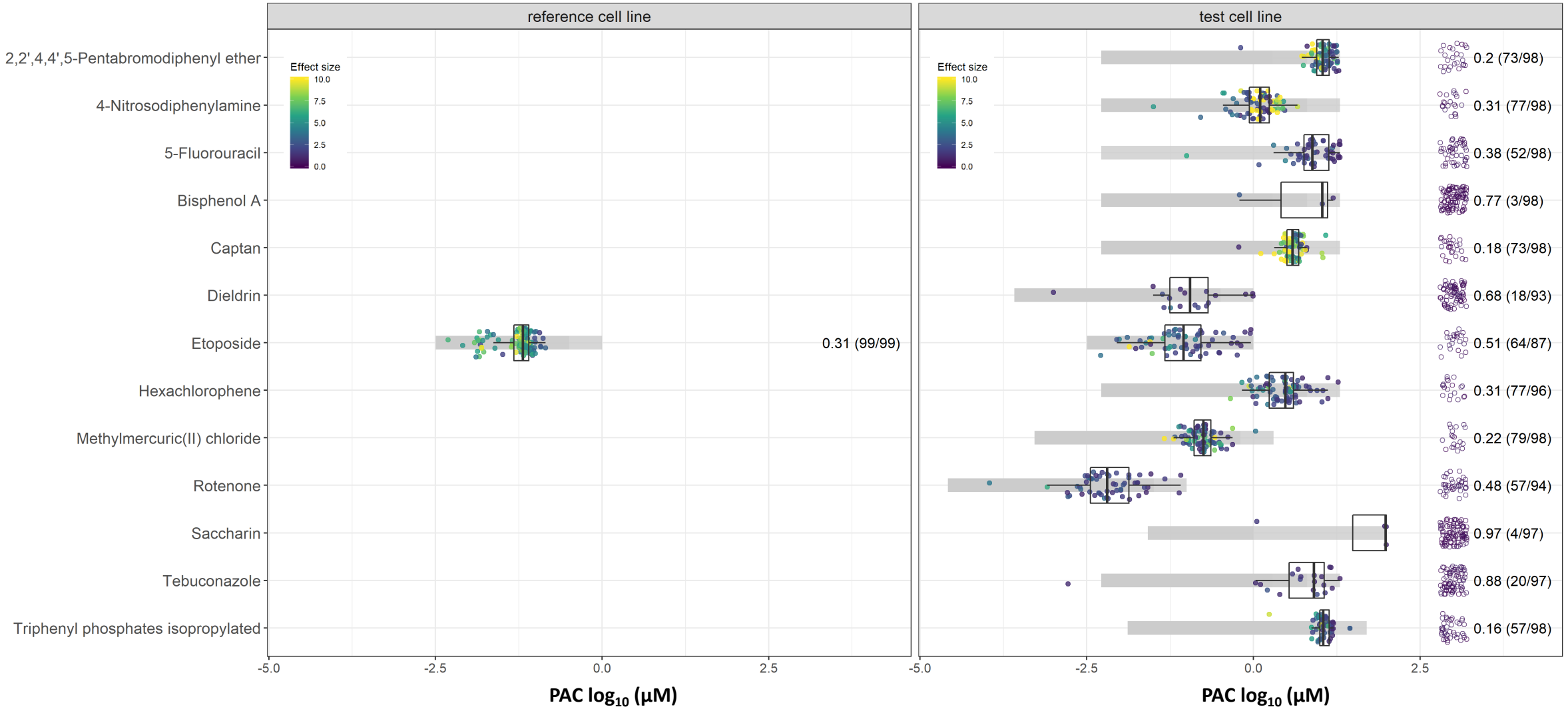
Analysis: Global Mahalanobis distance and concentration-response modeling for potency estimates.

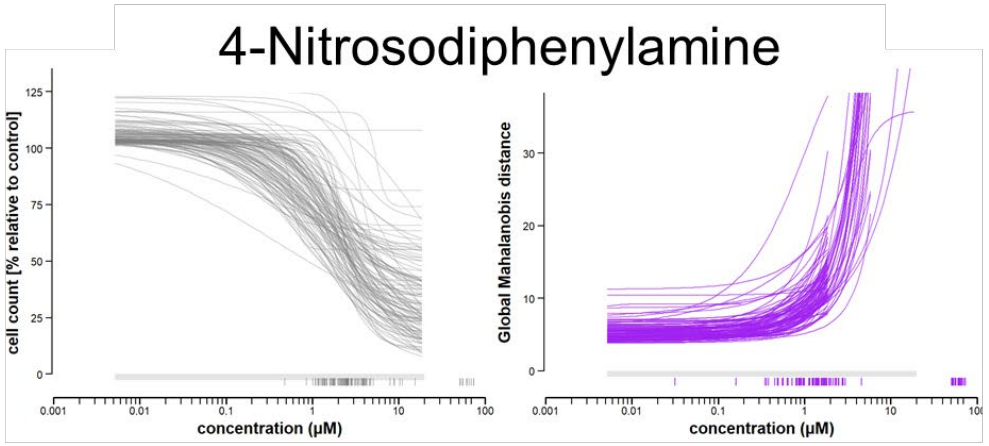


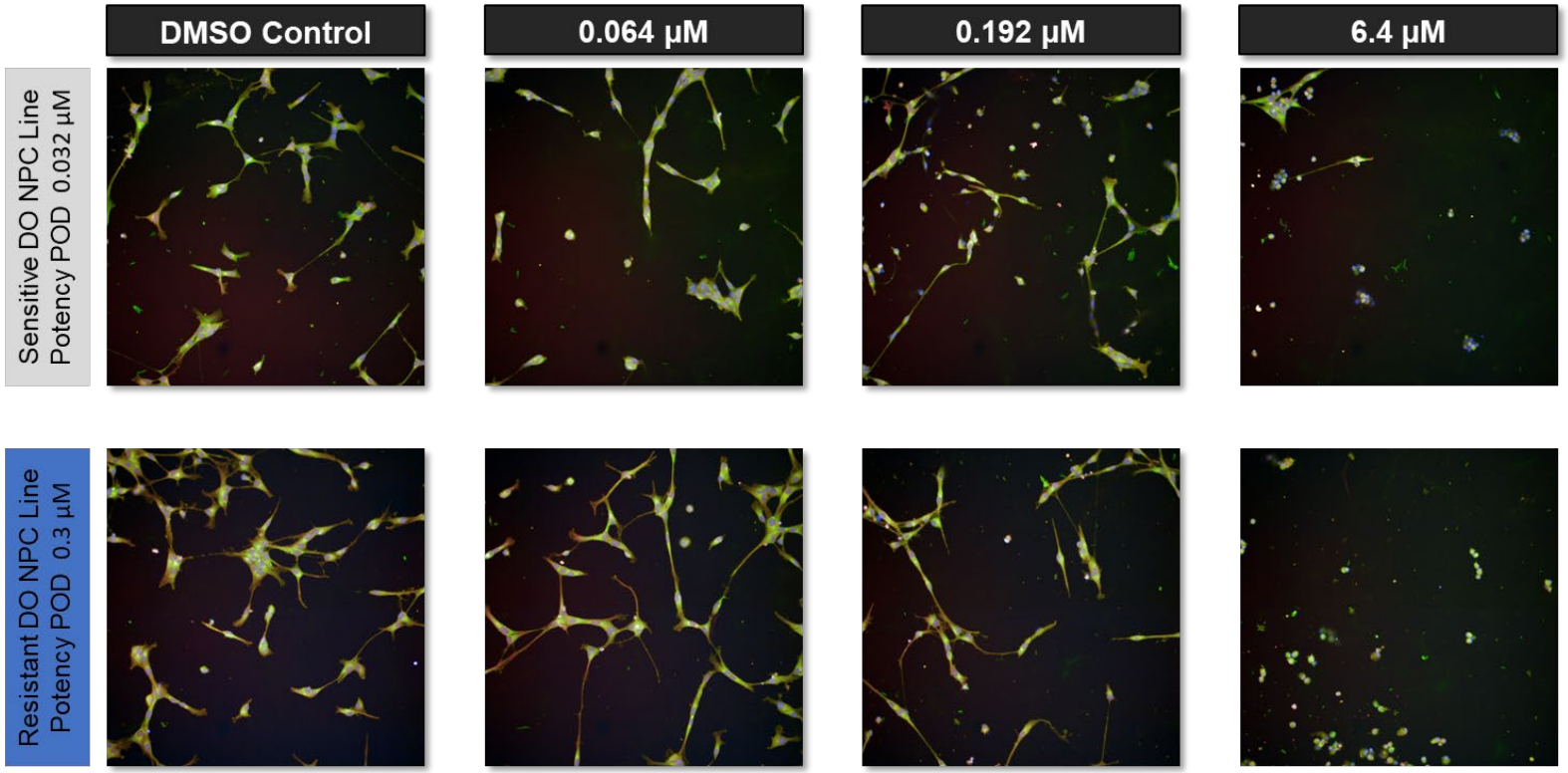
Inter-individual variation in affected cellular domains: Etoposide

- Heatmap indicates the biological effect size at 1 µM etoposide, with row numbers corresponding to test plates.
- Reference cell line (PB361.14) is included on every test cell plate as an experimental control. Figure displays a subset of 34 DO NPC lines.
- Affected intracellular compartments are consistent for reference cell line, but differ across test cell lines. **This suggests that test cell lines have differential responses associated with etoposide.**

Inter-individual variation in biological potency across chemicals (98 test cell lines)







Inter-individual variation in dose response: 4-NDA

- Next Steps: Running last set of 12 plates in September to increase sample size.
- Working with NTP/Sciome to perform Bayesian analysis of toxicodynamic variability
- Exploring inter-individual differences in MOAs.

Conclusions

- Finalize dose-response analysis of cell-painting data
- Determine MOA differences in sensitive and resistant lines for each chemical
- Calculate uncertainty factors for each chemical

- **Summary**: DO mice allow for analysis of population-variability that impacts our understanding of potential human susceptibilities

Acknowledgements – an Ode to Trainees!

The Hamner Institutes

Merrie Mosedale

Rachel Church

Lisa Kurtz

Rohit Singhal

Jessica Brown

Maria Davis

Veronica Adams

UAMS

Haixia Lin

Julia Tobacyk

Lascelles Lyn-Cook Jr.

Shamiso Ngongoni

Laura Abbott

DNTP

Dahea You

Mimi Huang

Natalie Bell

Extra

Diversity Outbred: Background of population diversity

DO are a genetic reshuffling of genes from 8 founder strains

- 129S1/SvImJ – Sensitive uterine response to estrogens, high serum cholesterol
- A/J –resistant to cigarette smoke induced emphysema; late onset muscle disease (homozygous mutation)
- C57BL/6J – refractory to many tumors, high susceptibility to diet-induced obesity/ T2D, atherosclerosis, high incidence of eye abnormalities
- NOD/ShiLtJ – autoimmune T1D, defects in Ag presentation, impaired wound healing
- NZO/HILtJ – obesity on standard diet and T2D
- CAST/EiJ – highly genetically divergent from other strains, improved neuron axonal regeneration, fast and highly active
- PWK/PhJ – Highly genetically divergent from other strains, docile
- WSB/EiJ – Highly active, wild temperament, age-related autosomal dominant deafness