EPA United States Environmental Protection Agency

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Toxicokinetic New Approach Methodologies (NAMs)

In vitro measurement of key determinants of toxicokinetics

Barbara A. Wetmore

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

Office of Research and Development
Center for Computational Toxicology and Exposure

August 13, 2021

EPA United States Environmental Protection Agency

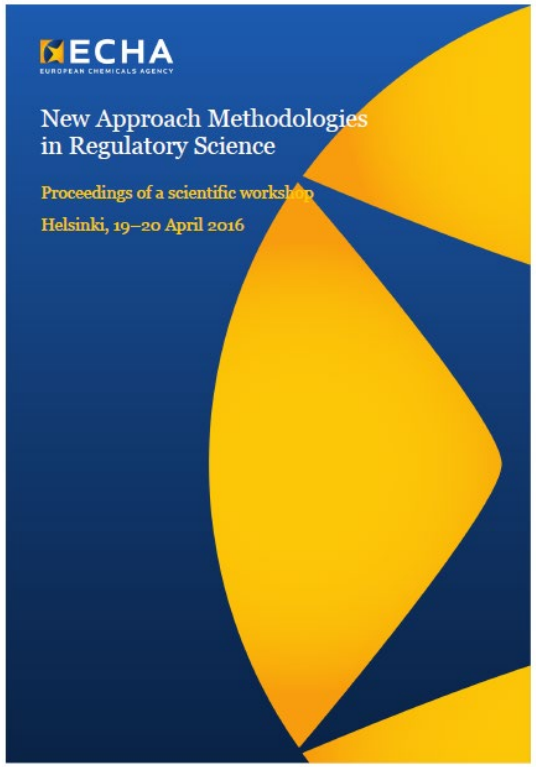
Overview

- New Approach Methods (NAMs) and Toxicokinetic NAMs
- Introducing *in vitro-in vivo* extrapolation (IVIVE)
- *In vitro* toxicokinetic assays
  - types, by tissue or parameter
  - experimental considerations
- Incorporating population variability
- Wrap-Up

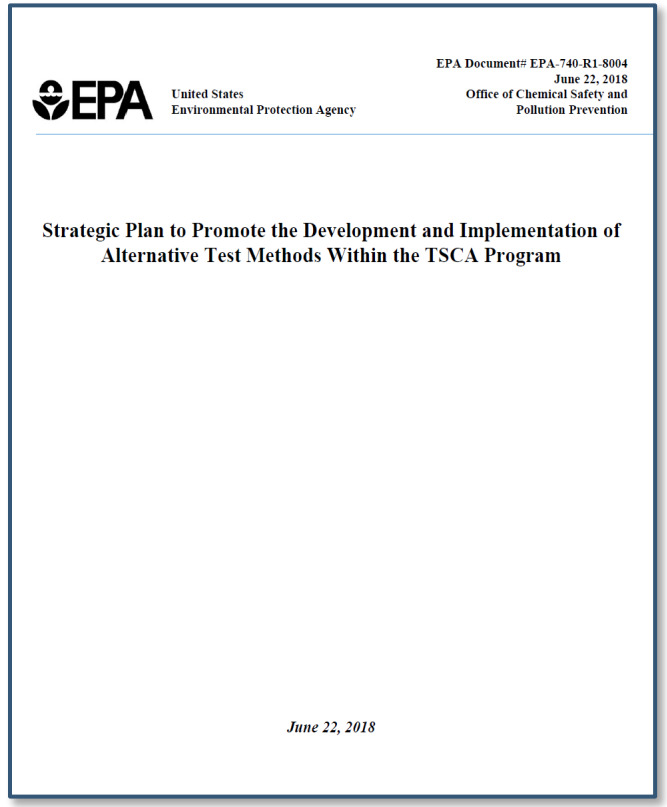
Abbreviations

ADR: adverse drug reaction
AF: aqueous fraction
BBB: blood brain barrier
C_{ss}: steady-state concentration
Cl_h: hepatic clearance
Cl_{int}: intrinsic clearance
Cl_r: renal clearance
F_u: fraction unbound
CNS: central nervous system
GFR: glomerular filtration rate
HK_{AF}: human toxicokinetic adjustment factor
HT: high throughput
HTTK: high throughput toxicokinetics
IV: intravenous
IVIVC: in vitro-in vivo correlation
IVIVE: *in vitro-in vivo* extrapolation
NAMs: new approach methods
NHANES: National Health and Nutrition Examination Survey
P_{app}: apparent permeability
PFAS: per and polyfluoroalkyl substances
PO: per os (i.e., by mouth)
POD: point of departure
PTFE: polytetrafluoroethylene
QSAR: quantitative structure-activity relationship
Q_l: hepatic blood flow
RED: rapid equilibrium dialysis
TSCA: Toxic Substances Control Act
TK: toxicokinetics
V_d: Volume of distribution

New Approach Methods (NAMs)



- Commonly defined to include *in silico* approaches, *in chemico* and *in vitro* assays, as well as inclusion of information from the exposure of chemicals in the context of hazard assessment.
- Recently defined in the EPA's TSCA Alternative Toxicity Strategy as:
  - *"A broadly descriptive reference to any technology, methodology, approach, or combination thereof that can be used to provide information on chemical hazard and risk assessment that avoids the use of intact animals."*


EPA United States Environmental Protection Agency

EPA Document# EPA-740-R1-8004
June 22, 2018
Office of Chemical Safety and Pollution Prevention

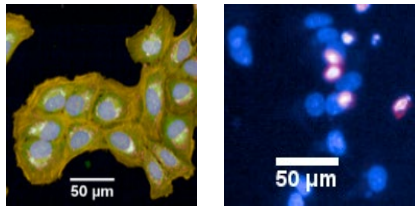
Strategic Plan to Promote the Development and Implementation of Alternative Test Methods Within the TSCA Program

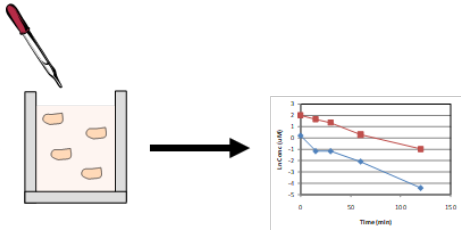
June 22, 2018

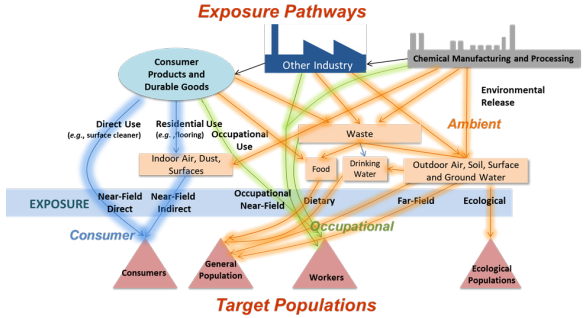
https://echa.europa.eu/documents/10162/22816069/scientific_ws_proceedings_en.pdf

https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/alternative-test-methods-and-strategies-reduce

NAMs in Risk Evaluation







In silico (e.g., QSAR and Read-across)

- Estimate effects and doses
- Consensus exposure modeling

In vitro assays

- Broad / screening (transcriptomics, phenotypic profiling)
- Targeted (receptors, enzymes)
- In vitro PODs, modes/mechanisms of action

In vitro Toxicokinetics

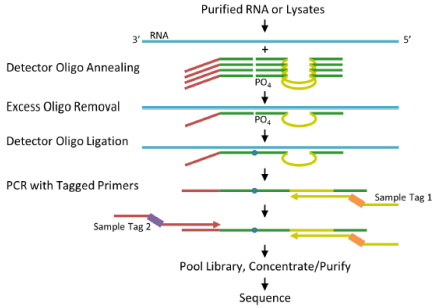
- Allows conversion of an in vitro POD to in vivo

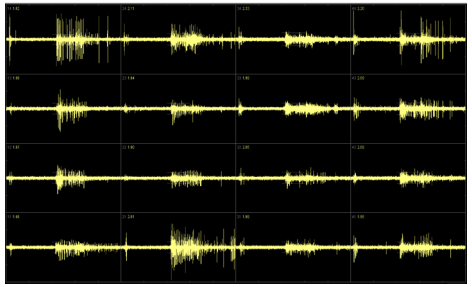
High-Throughput Exposure Measurements

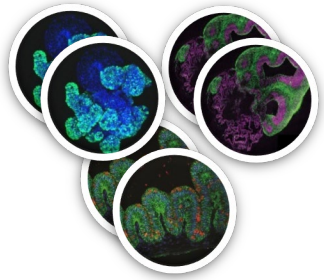
- To fill data gaps in monitoring data

Computer modeling

- Hazard models to integrate multiple in silico and in vitro data streams
- Exposure models to increase information on different pathways of exposure



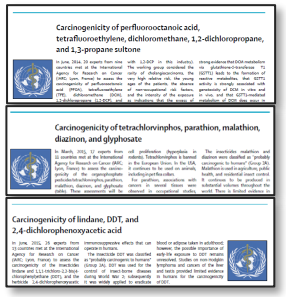




Where can NAMs "fit" in Risk Assessment?

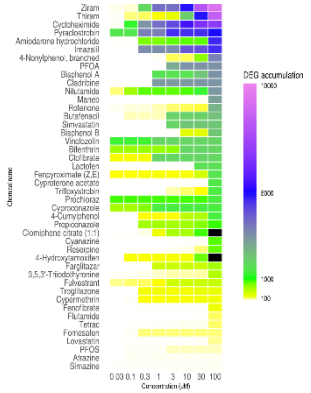
- Hazard characterization
- Dose-Response
- Exposure assessment

Provide Mechanistic Support for Hazard Identification



IARC Monographs
110, 112, 113

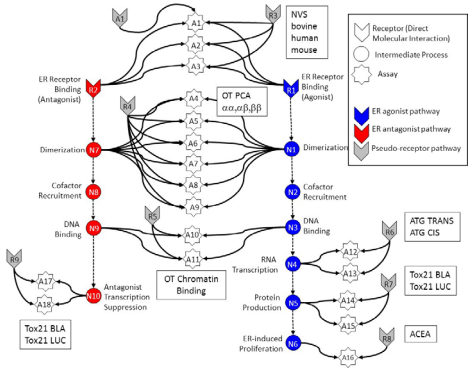
Tiered testing with High-throughput screening



Bundy et al unpublished

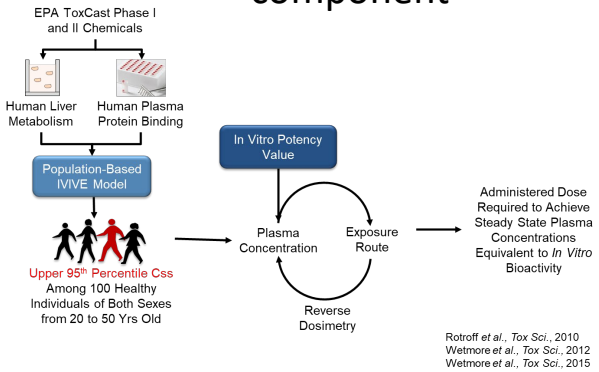
…and more!

Prioritization of Chemicals for Further Testing



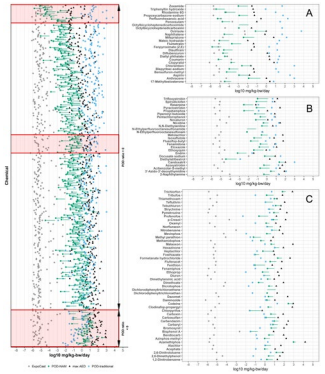
Judson et al., 2015

High-throughput toxicokinetic component



Rotroff *et al.*, *Tox Sci.*, 2010
Wetmore *et al.*, *Tox Sci.*, 2012
Wetmore *et al.*, *Tox Sci.*, 2015

In vitro point-of-departure development from NAMs



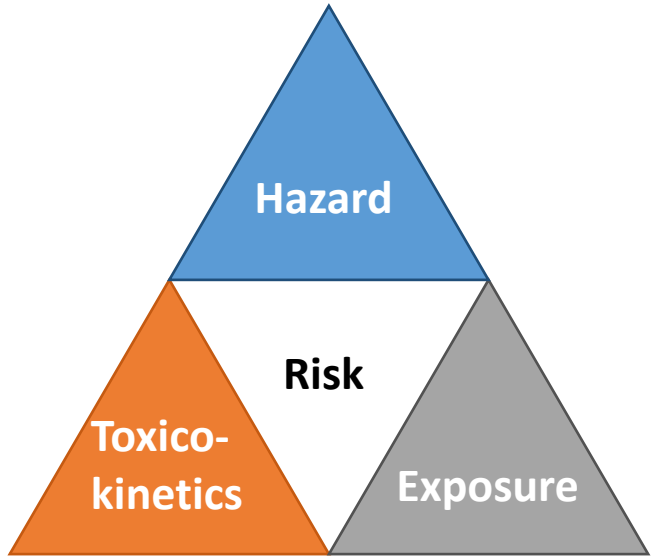
Paul-Friedman et al, 2020

Toxicokinetic NAMs

"Acceptance and use of in vitro *data for hazard identification is limited by uncertainties associated with exposure characterization and metabolism"*

Many *in vitro* systems:

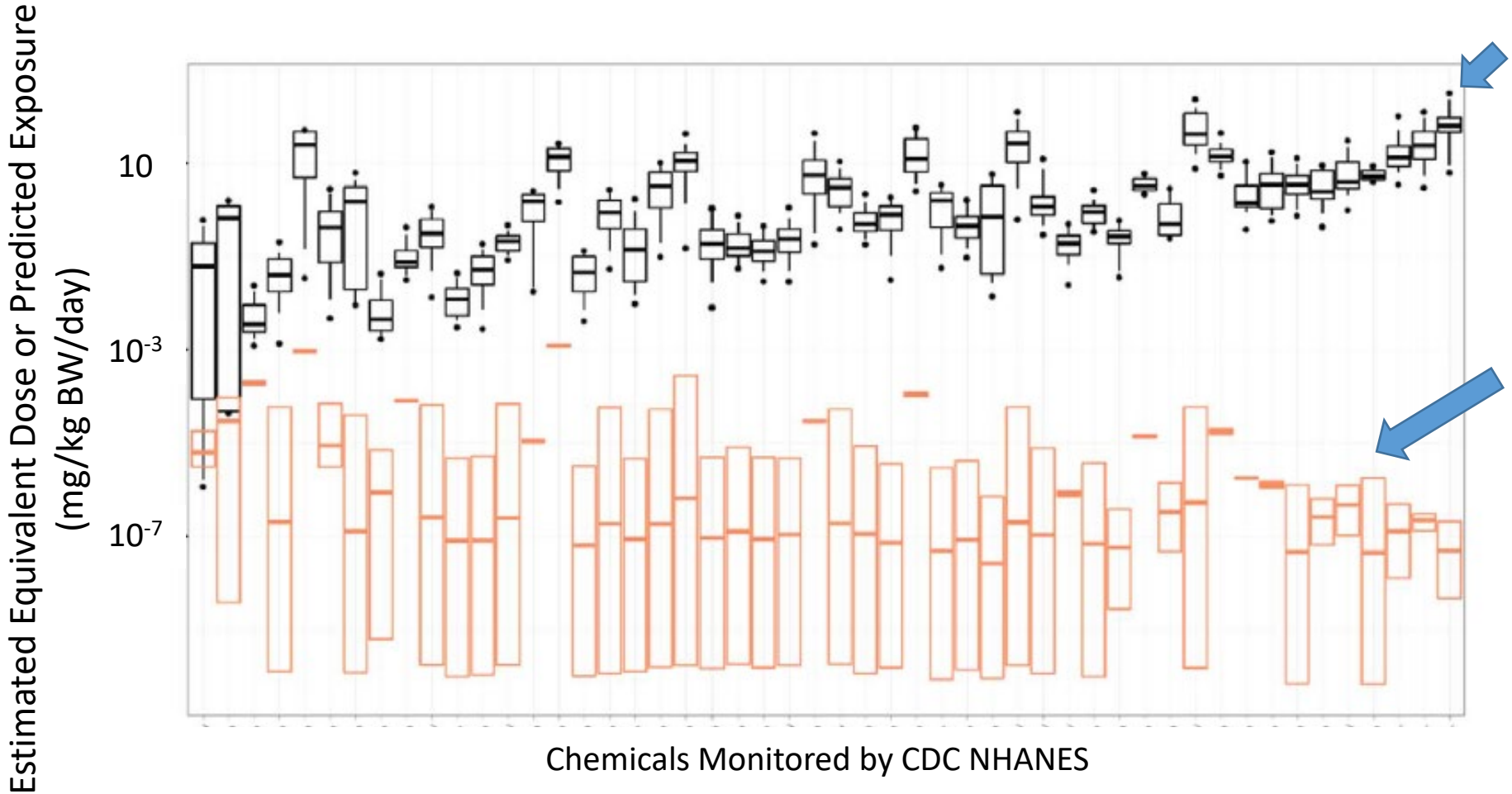
- lack consideration of biotransformation capabilities
  - Overestimation of hazard for chemicals rapidly cleared *in vivo*
  - Underestimation of hazard for chemicals bioactivated *in vivo*
- lack consideration of exposure route
- lack consideration of susceptible populations / life stages
- *In vitro* potency estimates are often not adjusted for chemical availability in the *in vitro* system (ie, *in vitro* disposition)



*"A Proof-of-Concept Case Study Integrating Publicly Available Information to Screen Candidates for Chemical Prioritization under TSCA"

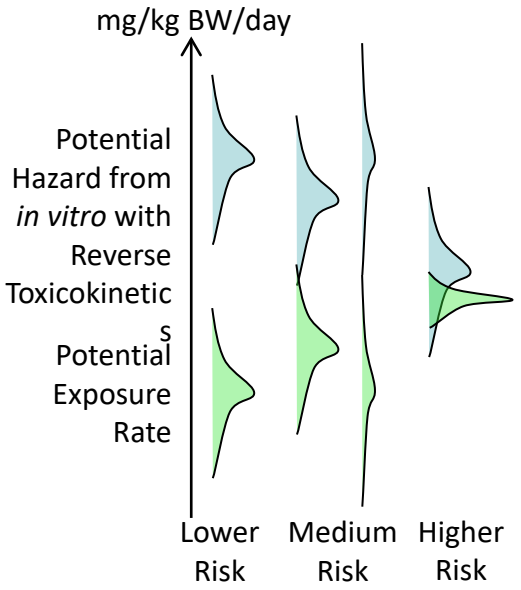
NAMs for Prioritization

Integrating Hazard, TK, and Exposure



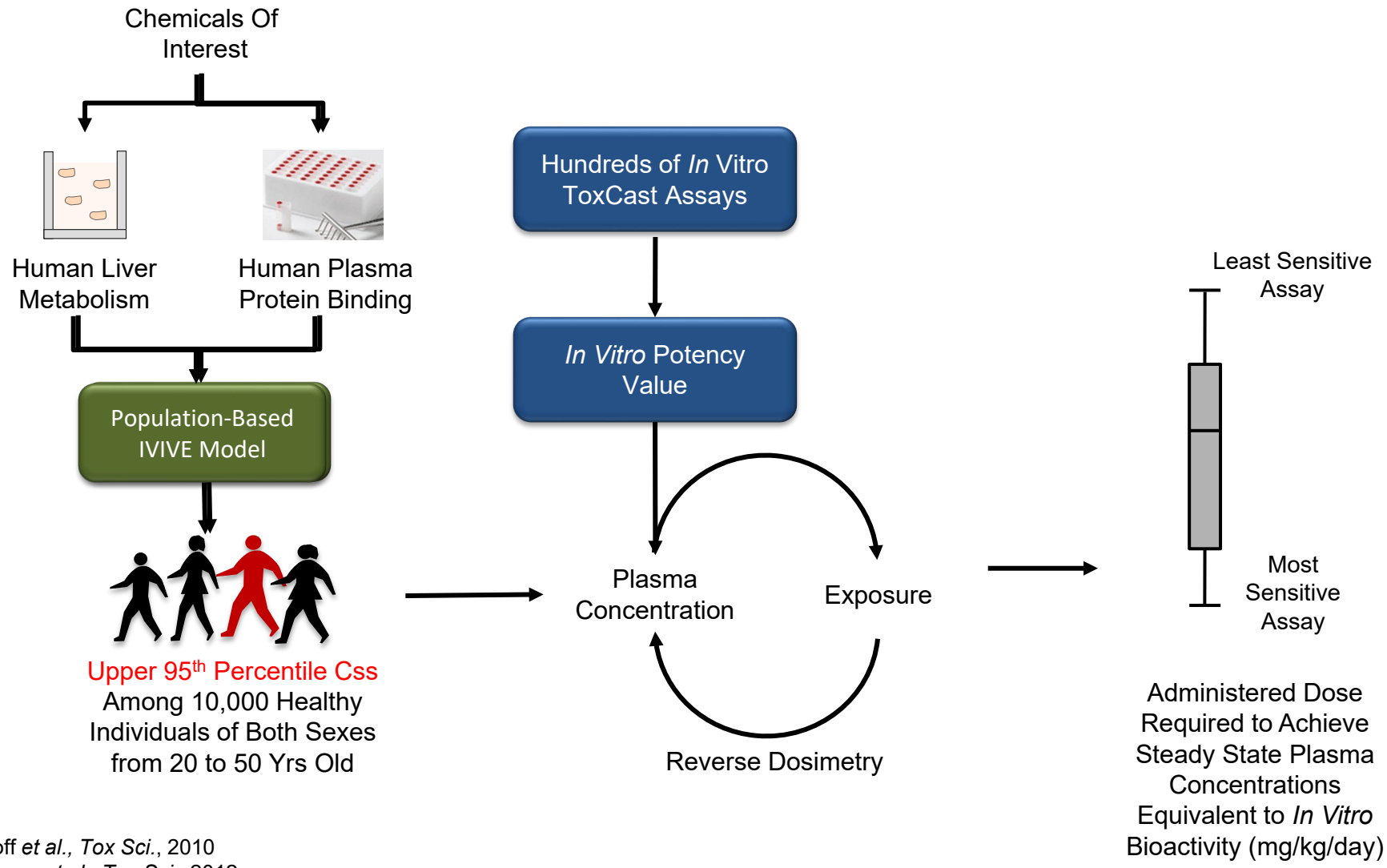
High throughput *in vitro* screening can be used to estimate doses needed to cause bioactivity

Exposure intake rates can be inferred from biomarkers



Wambaugh *et al.*, 2014
Wetmore *et al.*, 2015
Ring *et al.* (2017)
And others…

Integrating Human Dosimetry and Exposure with the ToxCast *In Vitro* Assays



Rotroff *et al., Tox Sci.*, 2010
Wetmore *et al., Tox Sci.*, 2012

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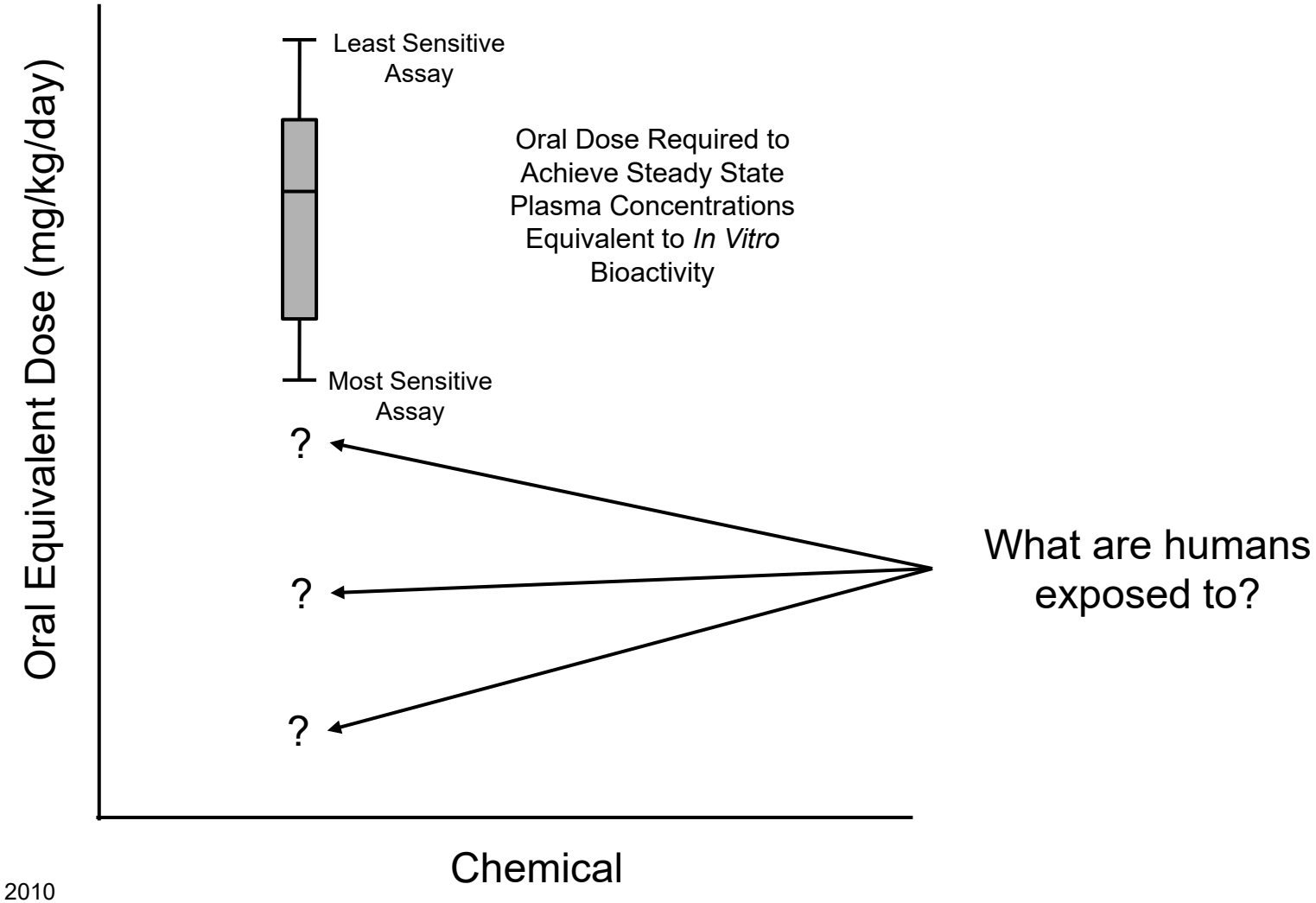
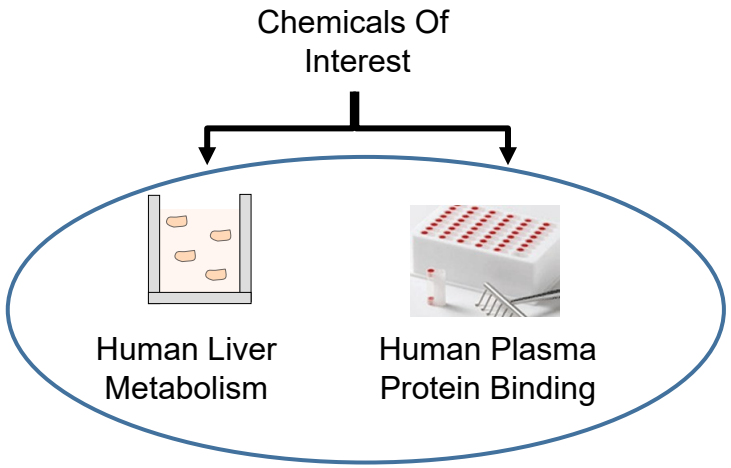


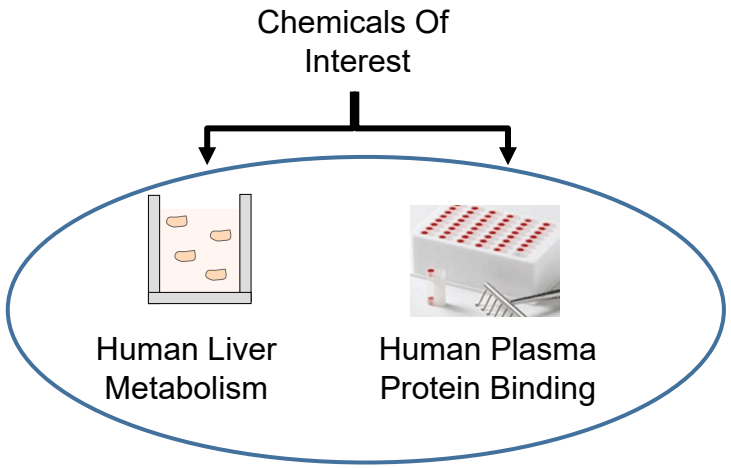
Rotroff *et al., Tox Sci.*, 2010
Wetmore *et al., Tox Sci.,* 2012

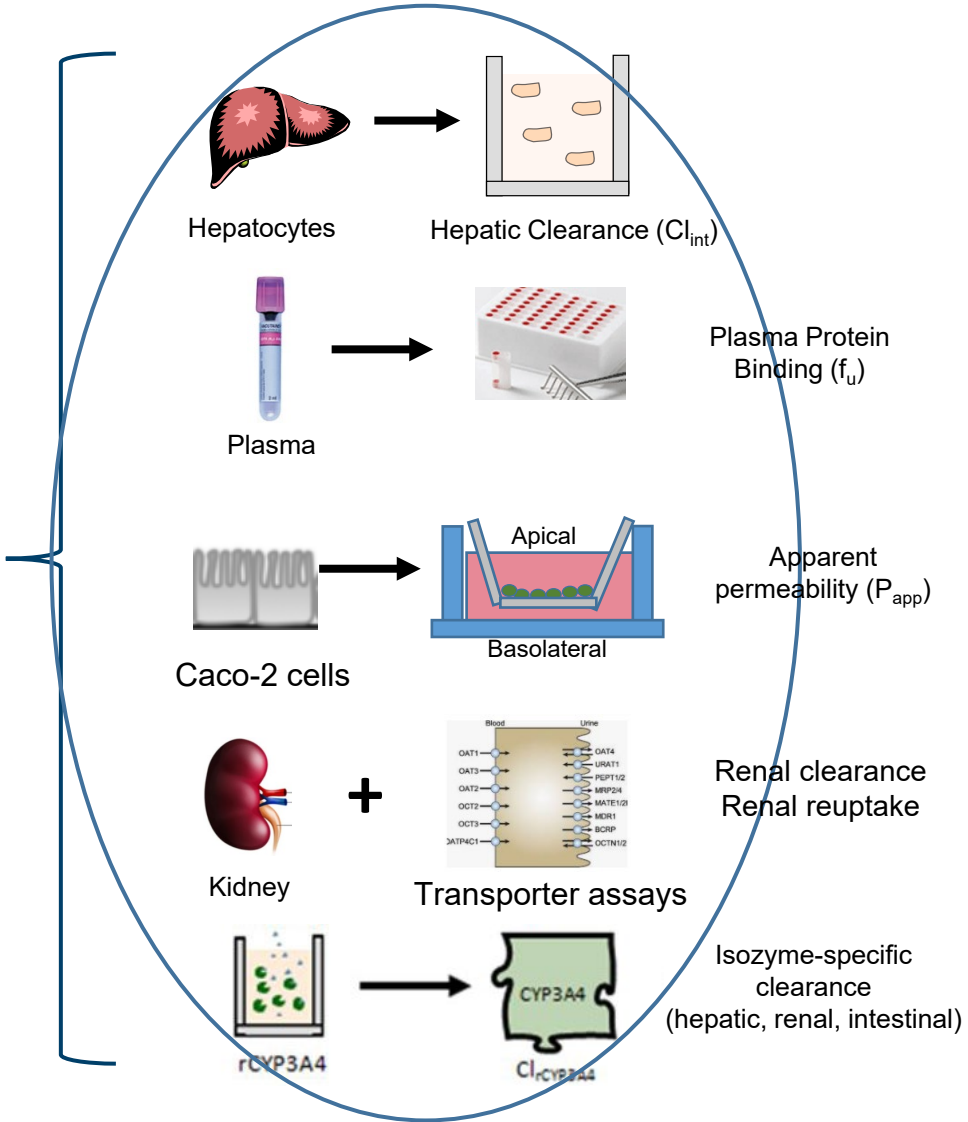
So…how do we get from here…



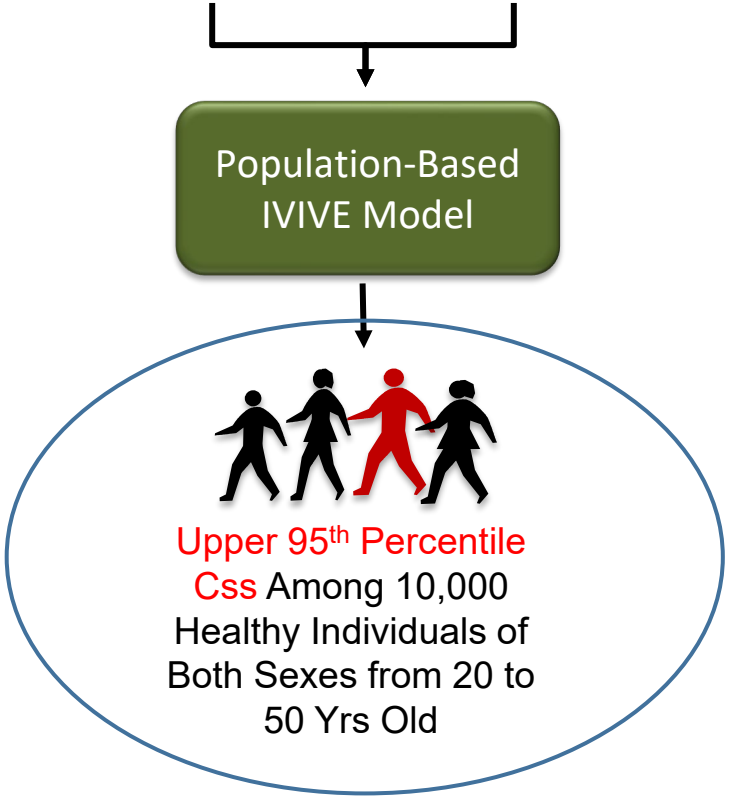
So…how do we get from here…

(which really is evolving to this…)

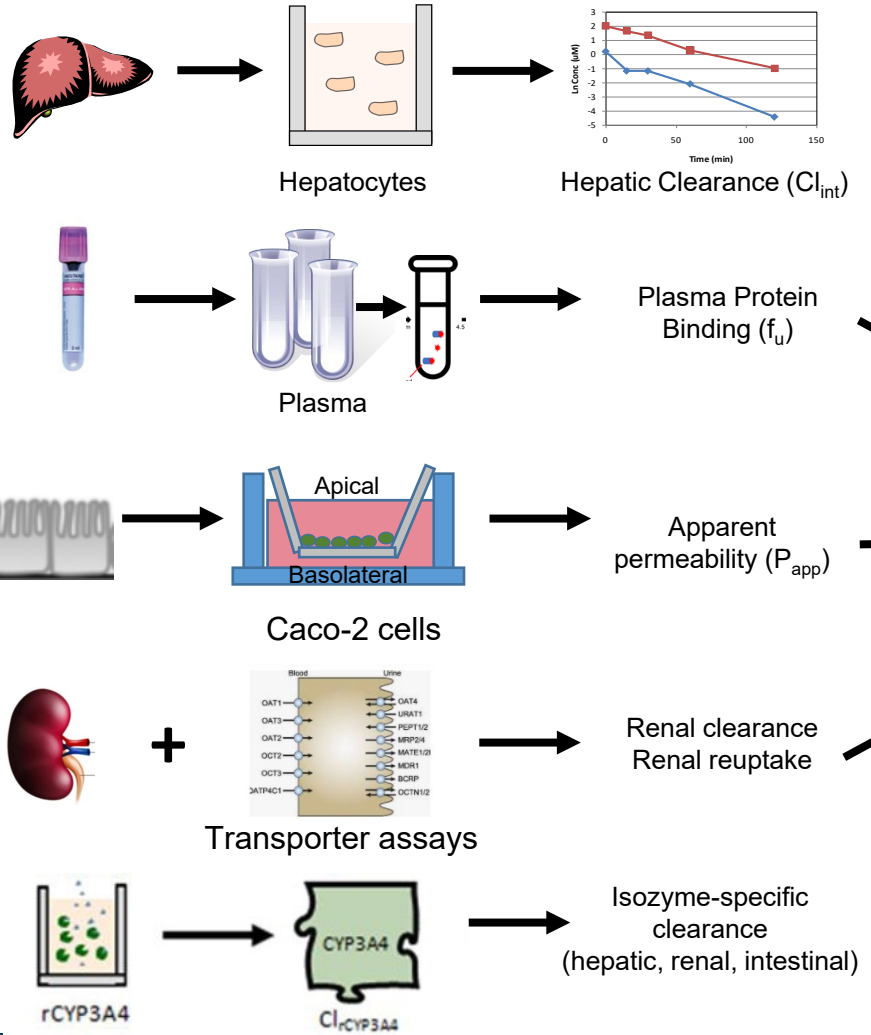




To *here*?...


Population-Based
IVIVE Model
Upper 95th Percentile
Css Among 10,000
Healthy Individuals of
Both Sexes from 20 to
50 Yrs Old

In Vitro-In Vivo Extrapolation (IVIVE)
In Vitro Toxicokinetic Assays



IVIVE Incorporates:

Chemical Specific Information
- physicochemical properties
- organ-specific TK
- plasma protein binding, non-specific binding

IVIVE

Internal Concentration Predictions Given a Set Administered Dose

Rotroff *et al., Tox Sci.*, 2010
Wetmore *et al., Tox Sci.,* 2012
Wetmore *et al.*, Tox Sci., 2014
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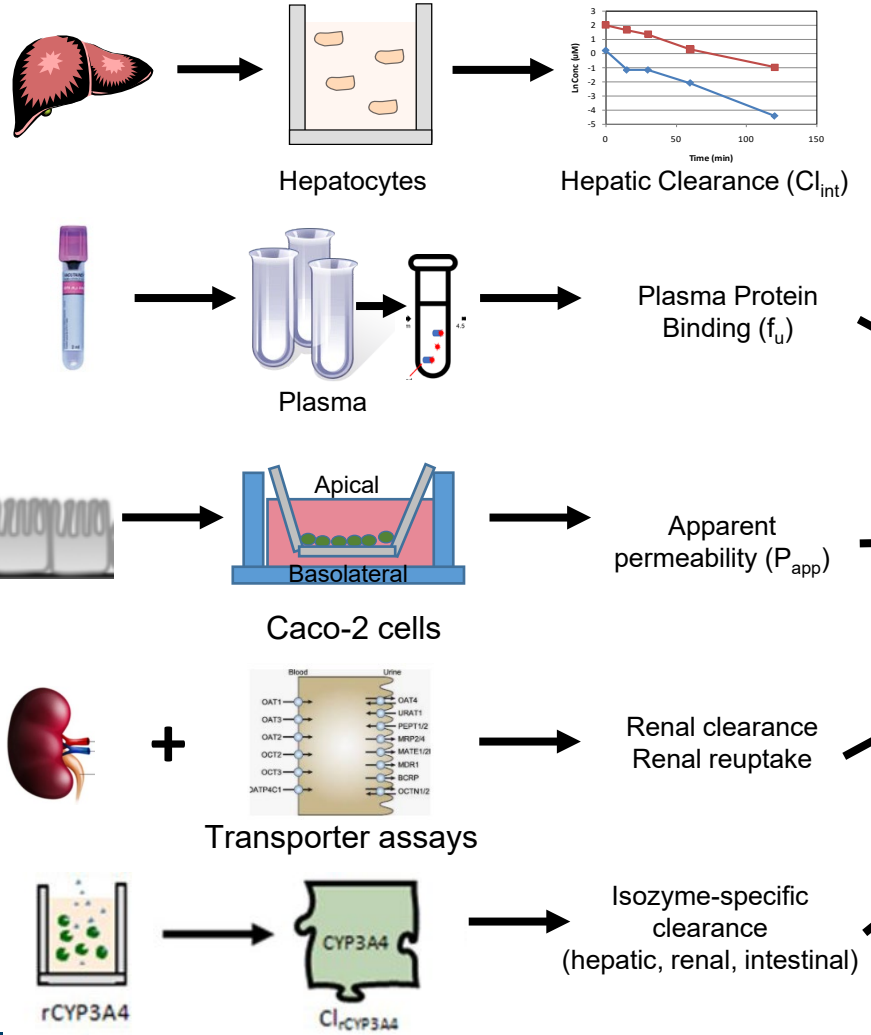
Wambaugh *et al.*,*Tox Sci.,* 2015
Wambaugh *et al., Tox Sci.* 2019

In Vitro-In Vivo Extrapolation (IVIVE)



IVIVE Incorporates:

Chemical Specific Information
- physicochemical properties
- organ-specific TK
- plasma protein binding, non-specific binding

IVIVE

Internal Concentration Predictions Given a Set Administered Dose

Underpinned by:

Physiology

Organ-specific info
- (in vitro and in vivo -> for scaling/extrapolation)

Population
- Incorporation of physiologic and TK differences to quantitate population and life-stage TK variability

-- IVIVE in a HT Environment --
Modeling *In Vivo* Pharmacokinetics Using *In Vitro* Assays

In Vitro - In Vivo Extrapolation

$$[Conc]_{SS} = \frac{\text{Dose Rate} * \text{Body Weight}}{CL_{WholeBody}}$$

$$CL_{WholeBody} = CL_R + CL_H$$

- 100% Oral bioavailability assumed for both CL_R and CL_H
- Kinetics are assumed to be linear

- CL_R: renal clearance (L/hr)
- $CL_{H:}$ hepatic clearance (L/hr)
- $Cl_{int:}$ intrinsic clearance (L/hr)
- GFR: glomerular filtration rate (L/hr)
- F_{uB}: fraction unbound in blood
- Q_L: hepatic blood flow (L/hr)
- HPGL: hepatocytes per gram liver
- $V_{L:}$ volume of liver (g)

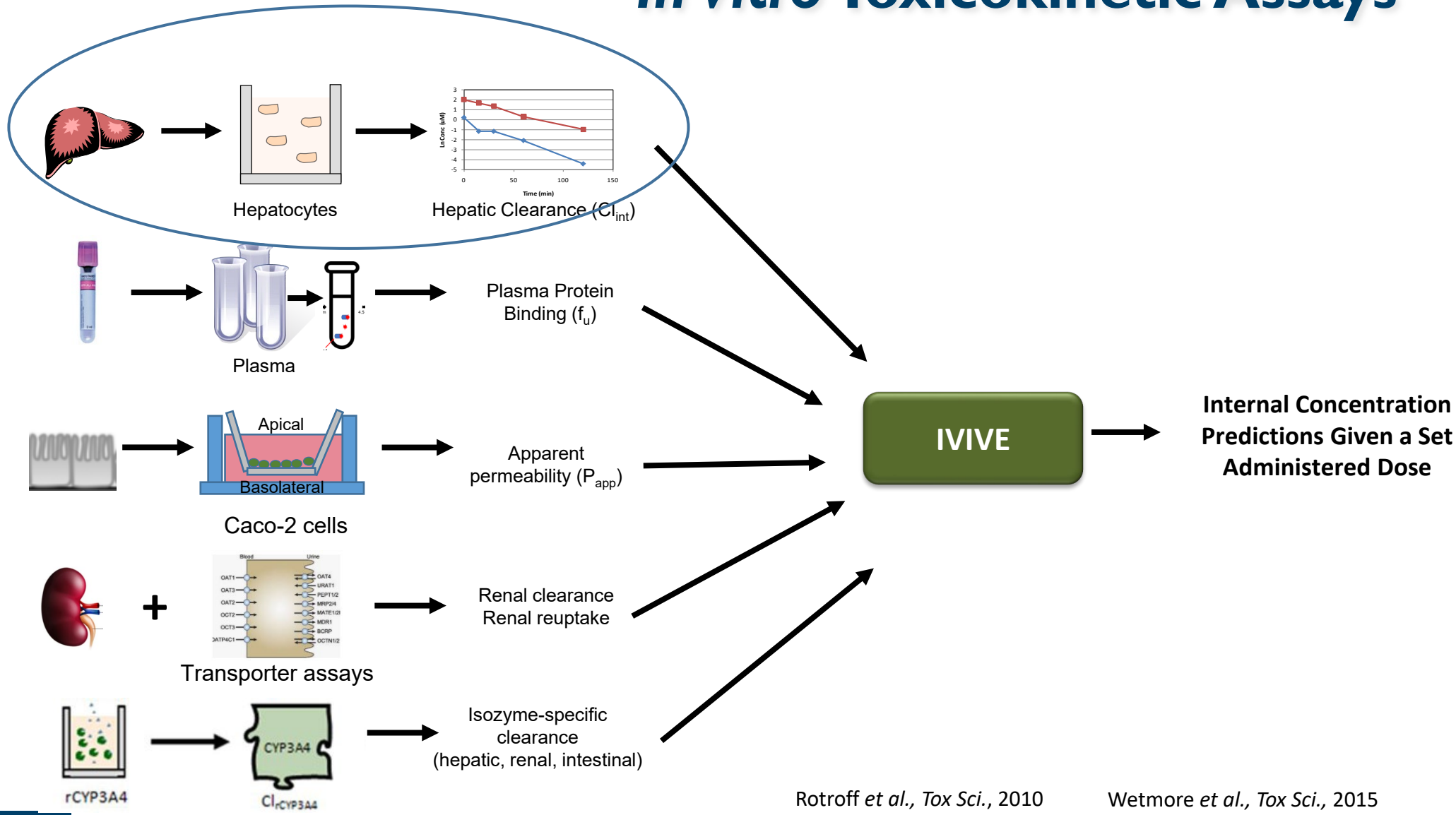
$$CL_R = F_{UB} * GFR$$ *where GFR ≈ 6.7 L/hr*

$$CL_H = \frac{F_{UB} * Q_L * CL_{Int}}{Q_L + F_{UB} * CL_{Int}}$$ *where Q_L ≈ 90 L/hr*

$$CL_{Int} = HPGL * V_L * CL_{invitro}$$ *where HPGL ≈ 137 million cells/g*

V_L ≈ 1820 g

In Vitro-In Vivo Extrapolation (IVIVE)
In Vitro Toxicokinetic Assays



Rotroff *et al., Tox Sci.*, 2010
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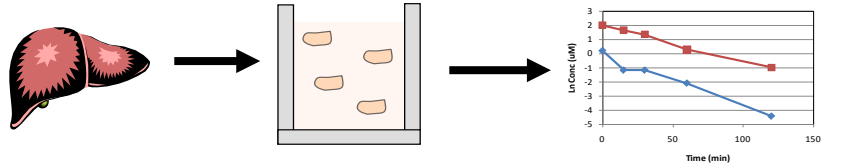
Wetmore *et al.*, Tox Sci., 2014
Wetmore *et al., Tox Sci.,* 2015
Wambaugh *et al.*,*Tox Sci.,* 2015
Wambaugh *et al., Tox Sci.* 2019

Hepatic Clearance

- Main metabolic pathway of xenobiotic clearance in body
- Several *In vitro* systems in use for measurement:
  - hepatocytes, microsomes, S9 fractions, HepaRG cells

Hepatocyte suspensions currently considered the gold standard in screening efforts



- Physiologically relevant; Full complement of enzymes present
- Custom pools can be created across multiple donors
- Historical data available to evaluate performance and reproducibility
- Viability and activity maintained in culture out to 4 hr
- Scaling factors well established in IVIVE

Con: Not sufficiently sensitive to derive clearance rates for slowly metabolized compounds

Hepatic Clearance

Experimental Considerations

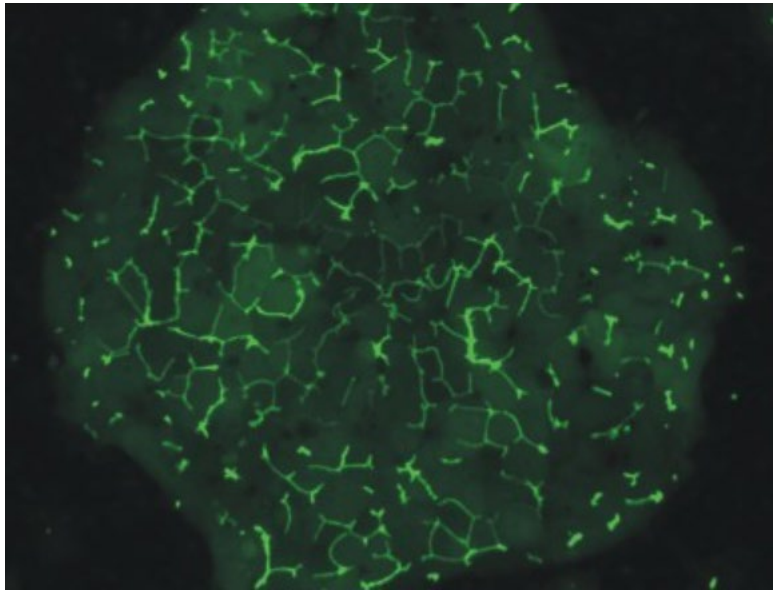
- Select a substrate concentration << K_m to ensure Cl_{int} not saturated
- Donor pools typically used to incorporate a range of metabolism (ie, to control for donor-specific outlier effects)
- Standardized protocols exist for a reason. Use them. And clearly describe them.
- Include reference compounds to ensure assay performance is reproducible
- Include media and metabolically inactivated controls to evaluate compound stability/mass balance
- Unbound intrinsic clearance rates (Cl_{uint}) corrected for non-specific binding in the assay are required to correct for assay-specific artifacts

→ Chemicals non-specifically bound in the assay are not available to be cleared; but they are quantitated during analytical measurement. Adjustments are required or the Clint value will be underestimated.

$$Cl_{uint} = Cl_{int} / f_{uinc}$$

Hepatic Clearance
Low Turnover Compounds

- HepaRG spheroids
  - Emerging technology
  - Hepatoma cell line, 1 donor
  - How to handle IVIVE scaling?
- Plated hepatocytes (48 hr time-frame)
- HepatoPac – proprietary micropatterned hepatocyte co-culture
  - Pros:
    - Highly functional; most physiologically relevant
    - maintained in culture for 28 days
    - Highly characterized donor information available
  - Cons:
    - Expensive to run multiple donors



Transport of fluorescent dye via MRP-2 indicates functional and robust network of bile canniliculi in Hepatopac culture

Hepatic Clearance
Low Turnover Compounds

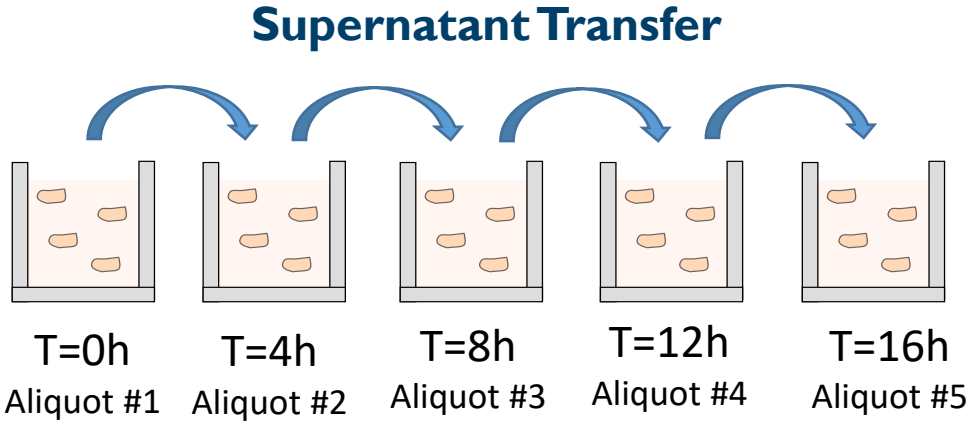
Hepatocyte Relay Method

Pros:

- Any lot of pooled hepatocytes
- # of relays flexible (up to 5)
- Good correlation with human *in vivo* data
- Suitable to generate metabolites for metabolite ID

Cons:

- Uses 4-5x the hepatocytes as in std assay
- Labor Intensive
- Dilution factor consideration
- Compound loss across transfers



Thaw new aliquots for each 4 hr period

Di *et al.,* 2012, Drug Metab Dispos.
Di *et al.,* 2013, Drug Metab Dispos.
Ballard *et al.,* 2014, Drug Metab. Dispos.
Peng *et al.,* 2016, *Drug Metab Letters*

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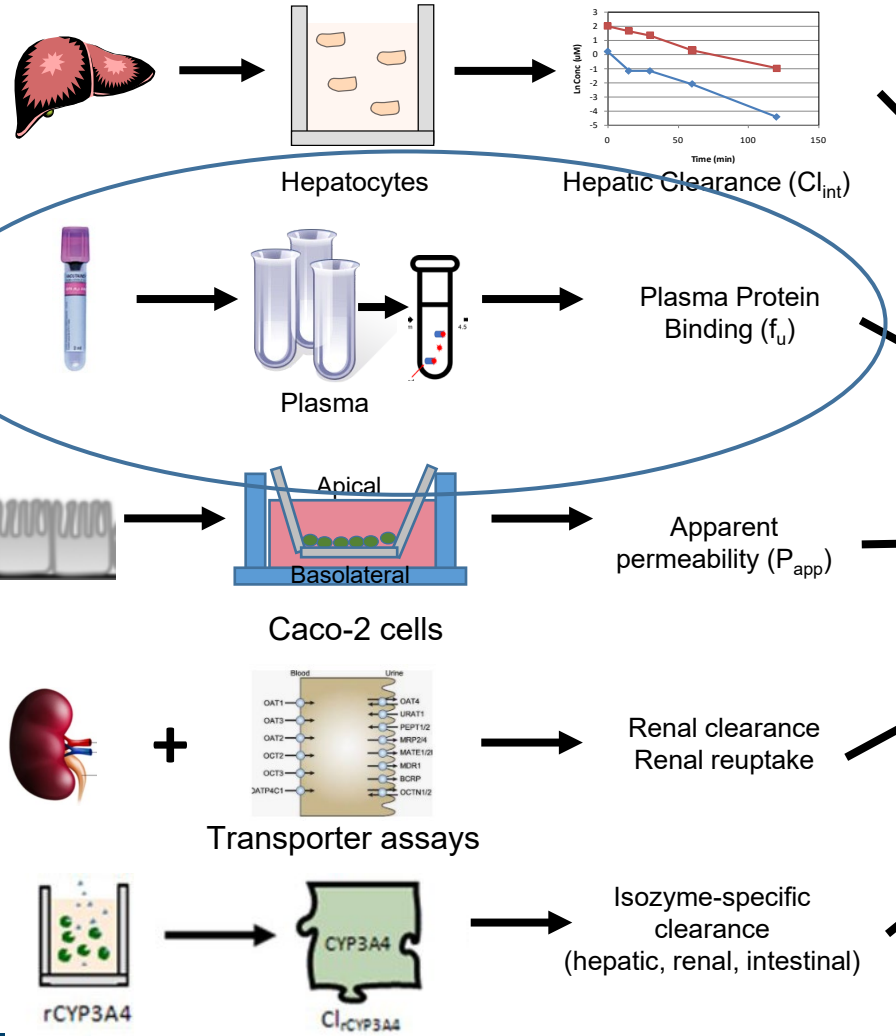
In Vitro Toxicokinetic Assays



IVIVE

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Rotroff *et al., Tox Sci.*, 2010
Wetmore *et al., Tox Sci.,* 2012
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Wetmore *et al., Tox Sci.,* 2015
Wambaugh *et al.*,*Tox Sci.,* 2015
Wambaugh *et al., Tox Sci.* 2019

Plasma Protein Binding

- Knowledge of chemical binding a key metric in TK evaluations

  Unbound chemical is:

  - free to elicit effect / therapeutic activity
  - available to be metabolized (cleared; or bioactivated)
  - available to be transported across membranes
  - Importance can be seen in application in C_{ss} equations:

$$[Conc]_{SS} = \frac{\text{Dose Rate} * \text{Body Weight}}{CL_{WholeBody}}$$

$$CL_H = \frac{f_U * Q_L * CL_{Int}}{Q_L + f_U * CL_{Int}}$$

where $Q_L \approx 90$ L/hr

$$CL_R = f_U * GFR$$

where GFR ≈ 6.7 L/hr

Cl_H: hepatic clearance	Q_L: hepatic blood flow	Cl_R: renal clearance
f_u: fraction unbound	Cl_{int}: intrinsic clearance	GFR: glomerular filtration rate

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Plasma Protein Binding
Ultrafiltration; Solid-Phase Microextraction

Available Methods:

- **Ultrafiltration** (commonly used technique in protein purification efforts)
  - molecular weight cut-off filter employed; chemical-spiked plasma added to upper chamber
  - Separation achieved by centrifugation; free chemical flows through filter; plasma-bound is retained
  - Individual tubes and high-throughput (HT) plate available
  - Cons: Inconsistent recoveries observed across HT plate; potential for non-specific binding to membrane; hence not truly high-throughput
- **Solid-Phase Microextraction (SPME)**
  - Method of choice for smaller-scale, case study projects focused on lipophilic compounds
  - Cons: method optimization precludes this from being a viable technique in HT space

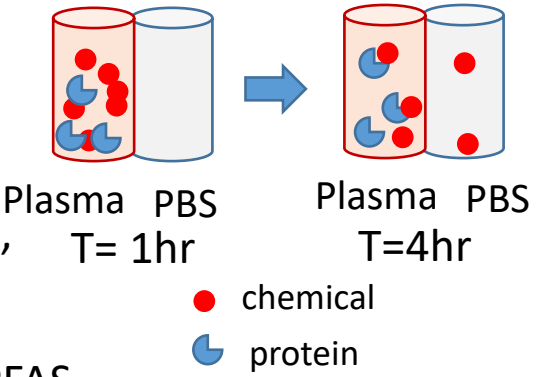
Plasma Protein Binding
- Rapid Equilibrium Dialysis -

Available Methods:

- **Ultrafiltration** (commonly used technique in protein purification efforts)
  - molecular weight cut-off filter employed
  - Separation achieved by centrifugation
  - Individual tubes and high-throughput (HT) plate available
  - Cons: Inconsistent recoveries observed across "HT" plate; potential for non-specific binding to membrane; hence not truly high-throughput

- **Rapid Equilibrium Dialysis (RED) –** Gold standard approach in HTS, Pharmaceutical R&D
  - Miniaturized dialysis chambers mapped to a multi-well robotic footprint
  - Highly amenable to HT evaluations
  - Cons:
    - Assumption that chemical at equilibrium may not apply for subset of chemicals (e.g., lipophilic)
    - Non-specific binding/loss of chemical to membrane/surfaces in apparatus
    - Presence of PTFE/plastics may confound measures of certain TSCA chemicals (e.g., PFAS, phthalates)

RED Plate



Waters et al., 2008, J. Pharm. Sci.
Wetmore et al., 2012, Toxicol. Sci.

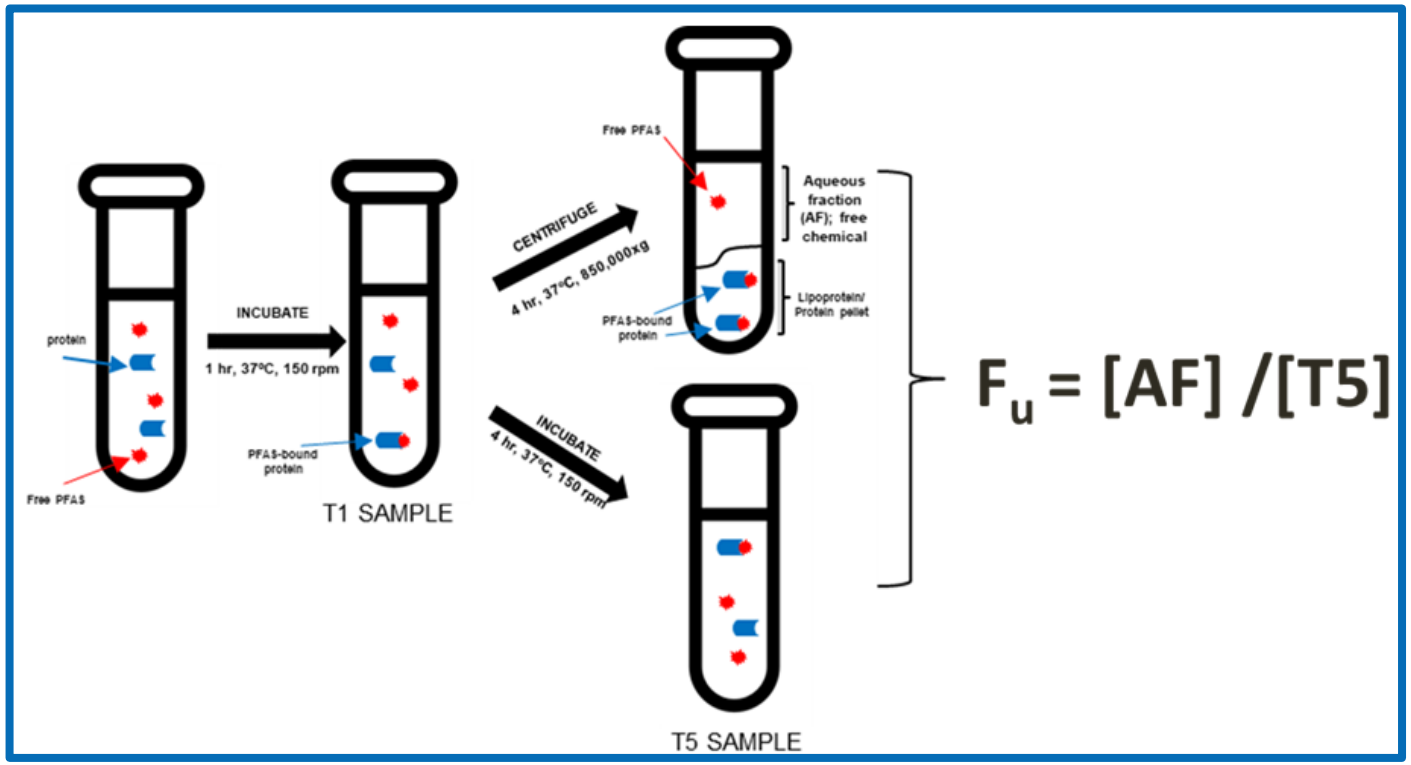
Plasma Protein Binding - Ultracentrifugation -

Available Methods:

- Ultrafiltration
- Solid-Phase Microextraction
- Rapid Equilibrium Dialysis (RED)
- **Ultracentrifugation**
  - Membrane-free approach (no binding concerns)
  - Ultracentrifugation (850,000xg) separates plasma into aqueous, protein fractions

Cons:

- Specialized instrumentation required
- Not particularly HT (but cassette format can be used for some chemicals)



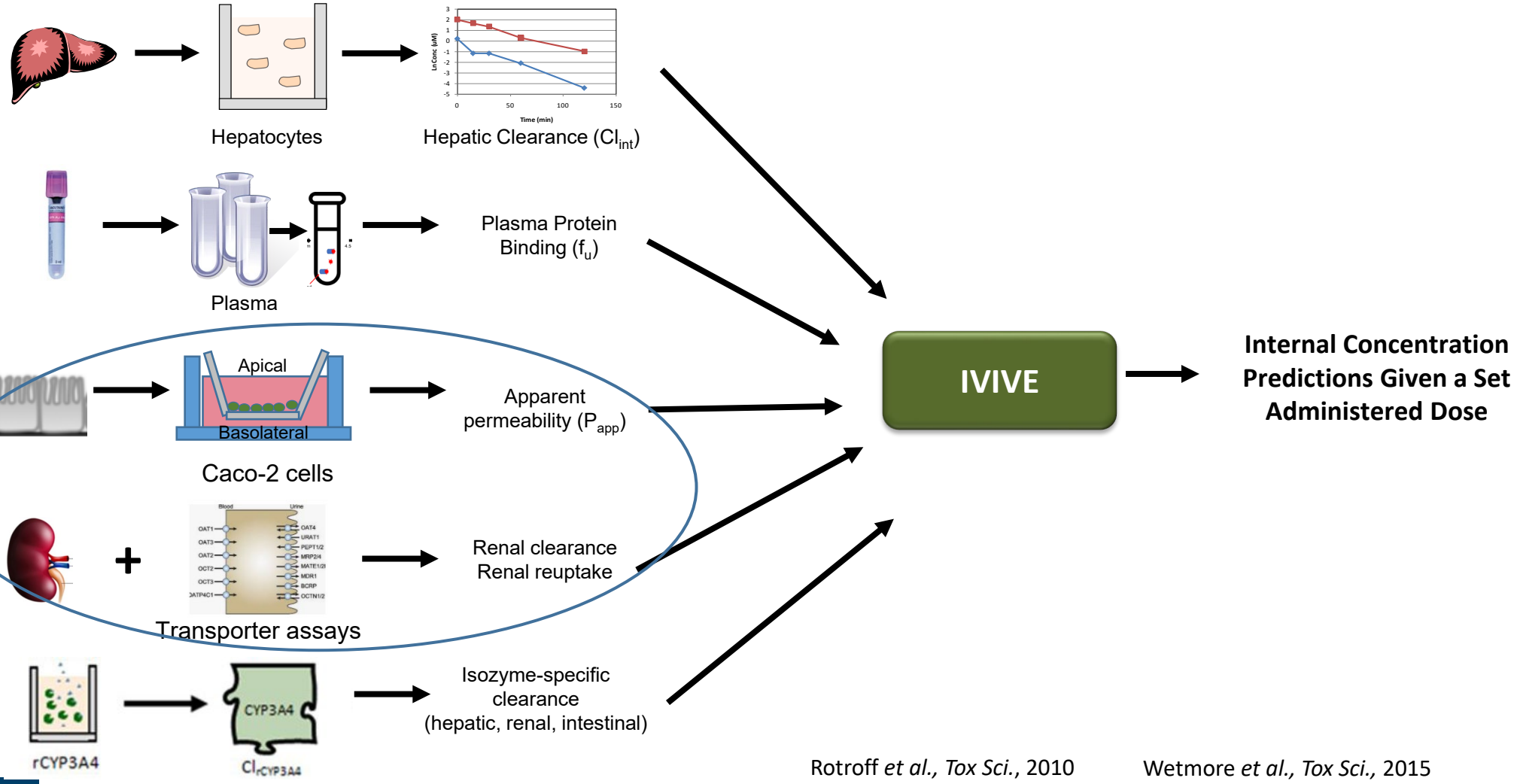
Kieltyka *et al.*, 2016, J. Pharm Res.
Brockman *et al.*, 2018, J. Med. Chem.

F_u: fraction unbound
AF: aqueous fraction
T5: Time 5 hr sample

In Vitro Toxicokinetic Assays
Binding and Stability Considerations

- Focus on non-drug chemicals has reinforced need for:
  - Inclusion of stability controls in all assays
  - Inclusion of reference compounds
  - Inclusion of equilibrium controls
  - Consider if mass balance is an important to track in your work
- Lipophilic compounds present challenges
  - may confound membrane-based evaluations (e.g., RED)
  - Greater rates of non-specific binding in assays affect mass balance, *in vitro* disposition
  - Use of experimental data preferred over QSARs, which typically fail at high $LogP_{ows}$

In Vitro-In Vivo Extrapolation (IVIVE)
In Vitro Toxicokinetic Assays

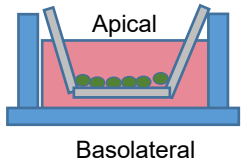


Rotroff *et al., Tox Sci.*, 2010
Wetmore *et al., Tox Sci.,* 2012
Wetmore *et al.*, Tox Sci., 2014
Wetmore *et al., Tox Sci.,* 2015
Wambaugh *et al.*,*Tox Sci.,* 2015
Wambaugh *et al., Tox Sci.* 2019

Considering Bioavailability

- **Bioavailability**: the fraction of chemical that reaches the systemic circulation
- For IV-administered chemicals, bioavailability (F) = 100%
- F for orally administered compounds is affected by:
  - Fraction of chemical absorbed in the intestine (F_{abs})
  - Fraction of chemical that escapes intestinal metabolism (F_{gut})
  - Fraction of chemical that escapes first-pass hepatic metabolism (F_{hep}
- →*in vitro* apparent permeability (P_{app}) assays provide estimates of F_{abs}
  - Poised as the next parameter for entry into *httk*
- Extrahepatic intestinal metabolism also a key parameter to evaluate in future efforts

Apparent Permeability Assays



Cell-based systems

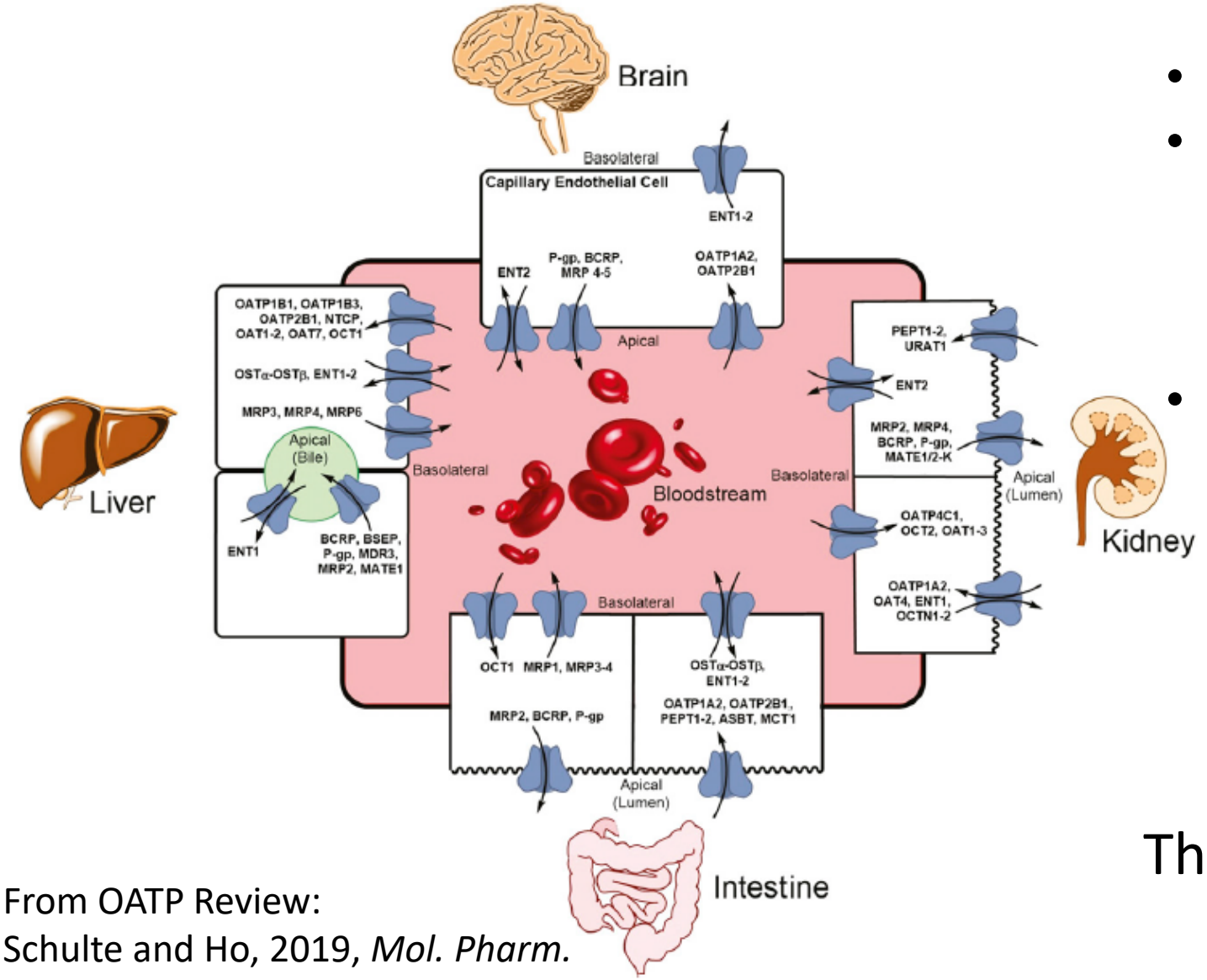
- Caco-2: Most widely accepted/used
  - Expression of relevant transporters ; long cultivation time
- MDCK-II, LLC-PK-I: low levels of endogenous transporters present; can be transfected; shorter cultivation period than Caco-2

Non cell-based system

- PAMPA (parallel permeability assay)
  - High-throughput; no transporters present

Take-home: Know your system! Various experimental conditions (e.g., pH gradients, sink conditions) can impact reproducibility of results

Considering Transporters…



- Involved in chemical efflux and uptake
- Play roles in multiple organs
  - Blood-brain-barrier
  - Renal re-uptake (PFAS) /clearance
  - Hepatic uptake
- Emerging field
  - Functions, substrates still unknown
  - Abundances for many still unknown
  - Ontogenies even less well understood
  - More likely to be discovered

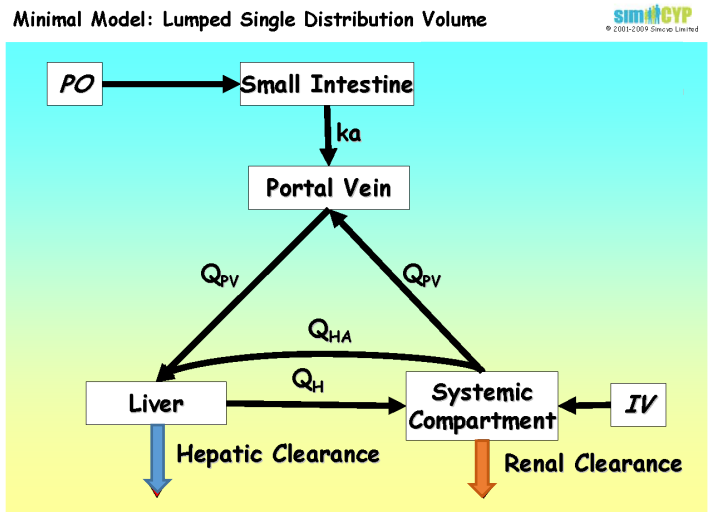
The next frontier in TK-IVIVE??

From OATP Review:
Schulte and Ho, 2019, *Mol. Pharm.*

- Incorporating Population Variability -

Incorporating Variability in IVIVE

Jamei *et al*. (2009)



$$C_{ss} = \frac{\text{oral dose rate}}{(GFR * F_{ub}) + \left(Q_l * F_{ub} * \frac{Cl_{int}}{Q_l + F_{ub} * Cl_{int}}\right)}$$

(Passive) Renal Clearance

Hepatic Clearance (Metabolism)

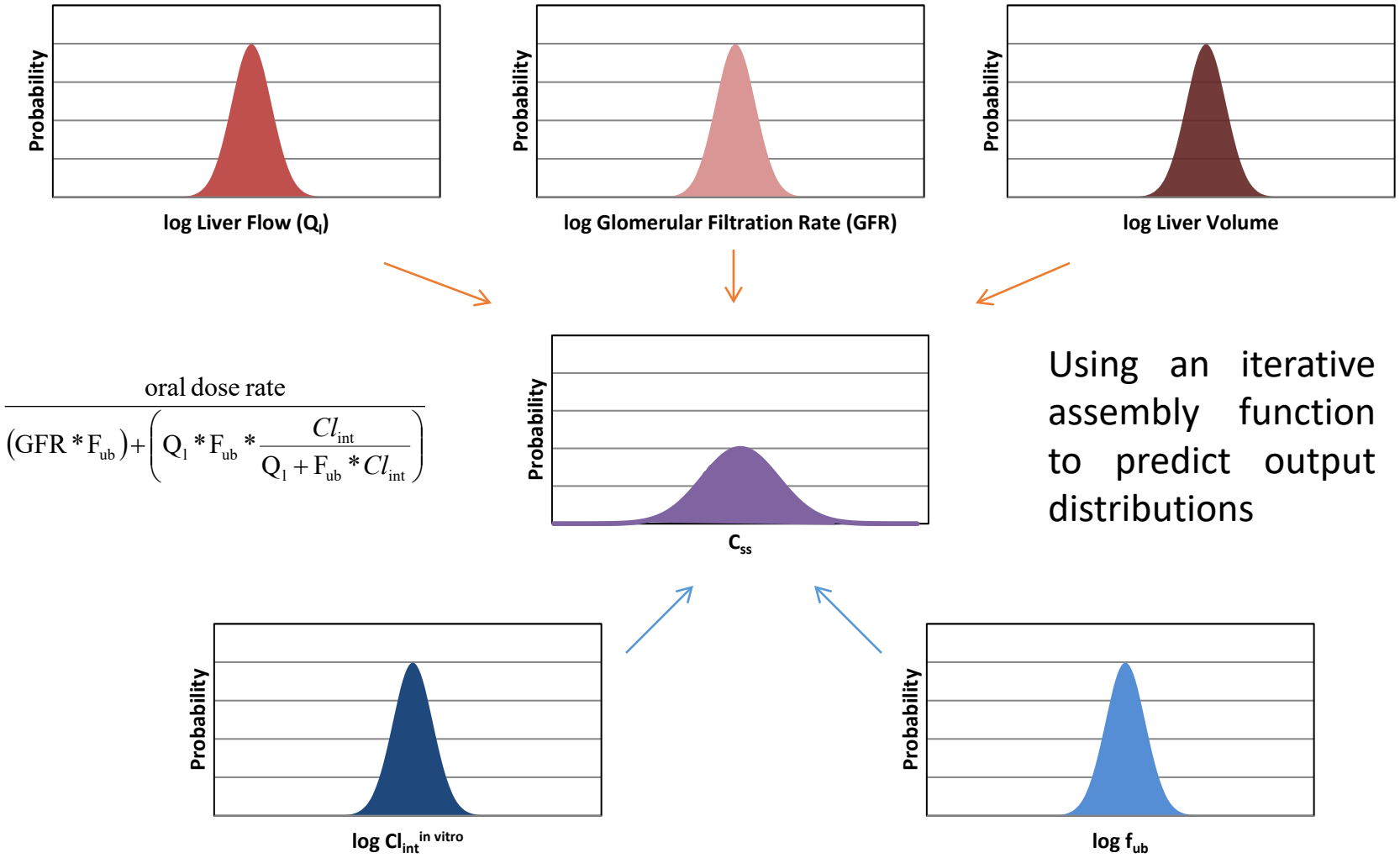
- *In vitro* clearance (µL/min/10^6 hepatocytes) is scaled to a whole organ clearance using the density of hepatocytes per gram of liver and the volume of the liver (which varies between individuals)
- Glomerular filtration rate (GFR) and blood flow to the liver (Q_l) both vary from individual to individual
- Further assume that measured HTTK parameters have 30% coefficient of variation

EPA United States Environmental Protection Agency

Monte Carlo Simulations to Characterize TK Variability

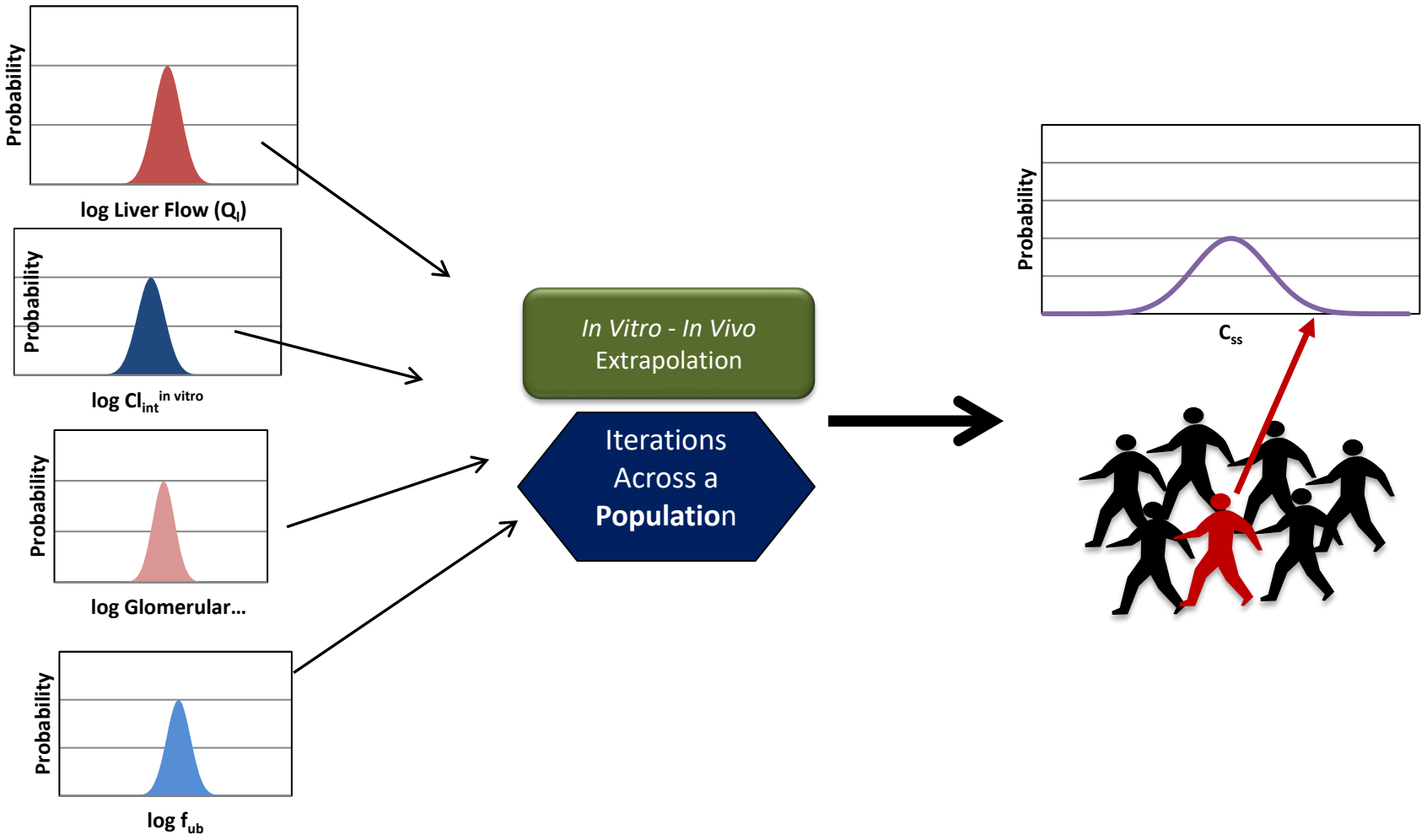
- Builds models of possible outcomes
- Substitutes a range of values for any factor that has inherent uncertainty (using probability distributions)
- Simulations are performed over and over, each time using a different set of random values from the probability functions
- Depending on number of uncertainties and ranges specified, a simulation could involve tens of thousands of recalculations before it is complete.
- Outputs are distributions of possible outcome values

Monte Carlo (MC) Approach to Simulating Variability



Wetmore *et al*. (2012)

Monte Carlo Simulation in IVIVE



Distributions based on known populations

Sources of TK Variability

— Across each of the ADME drivers of TK —

- **Absorption** (route considered (e.g., dermal, etc.); also - intestinal, hepatic, renal; transporters
  - — Physiologic, genetic, ontogenetic
- **Distribution** tissue partitioning
  - — Physiologic, ontogenetic
- **Metabolism** hepatic, extrahepatic
  - — Physiologic, genetic, ontogenetic
- **Excretion** hepatobiliary, renal - glomerular filtration/secretion/resorption
  - — Physiologic, ontogenetic

Physiologic Differences

- **Tissue by tissue…**

  Brain, lungs, heart, skin, liver, kidney, intestine, etc.
- **Parameter by parameter…**

  Weights, blood flow rates, metabolism, etc.
- **Systemic concerns** (red blood cells, blood volume)
- **Tissue-specific concerns**

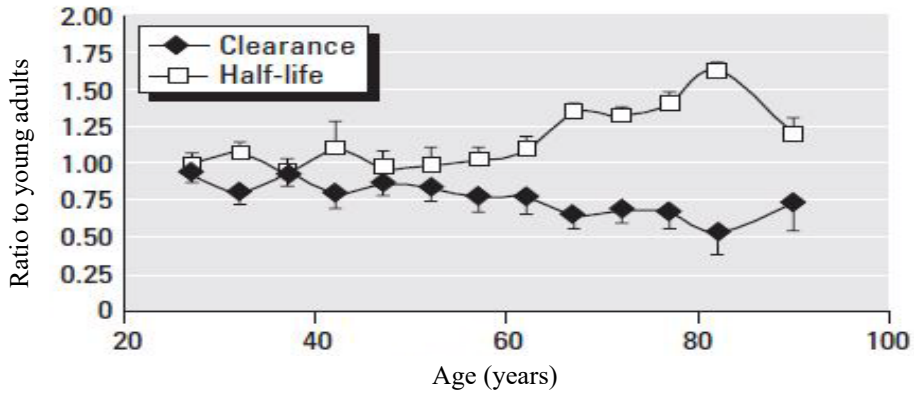
  Blood-brain barrier, capillary size (lungs, heart), etc.
- **Drivers:** Life-Stage, Lifestyle, Ethnic, Sex, Disease

➢ **All ADME Processes impacted**

Contributors to TK Variability

Contributors to Variability	Effect Window	Extent of Effect	Frequency
Physiologic (e.g., tissue weights, blood flow rates)	All life stages Early; Late greatest	Moderate	All populations and lifestages
Ontogenetic (differing abundances of enzymes, transporters)	Early Life Stages	Can be significant	All within relevant lifestages
Genetic (functional differences in enzymes, transporters)	All life stages	Depends on polymorphism, functional effects	0-10% of population
Exposomic (e.g., co-exposures, lifestyle)	Throughout life	Unknown	Unknown

TK Variability in the Elderly



PK Meta-analysis:

- 4500 subjects
- across >46 substrates
- 18 to >85 years of age
- >100 subjects per age group

(except for >85; n=45)

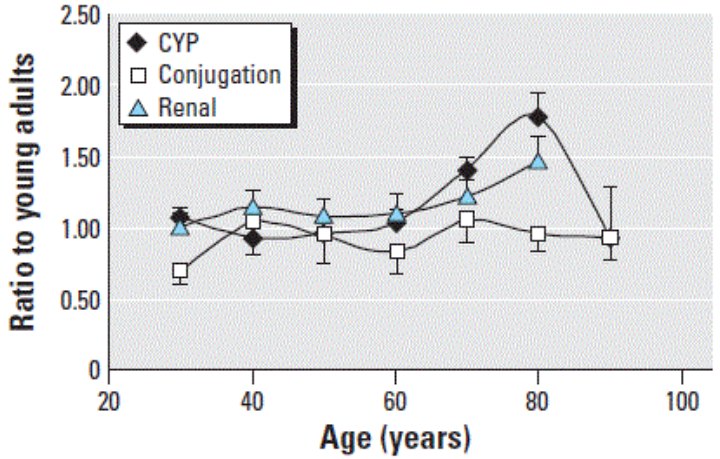
Hattis and Russ, 2003

- **Decreased cardiac outputs; tissue blood flow rates** (hepatic – 25% ↓)
- **Decline in muscle mass and body water** (up to 25% ↓)
- **Increase in body lipid content** (↑ V_d; longer $T_{1/2}$, lipophilic compounds)
- **Decrease in plasma protein binding** (15-25% ↓; higher free drug conc.)

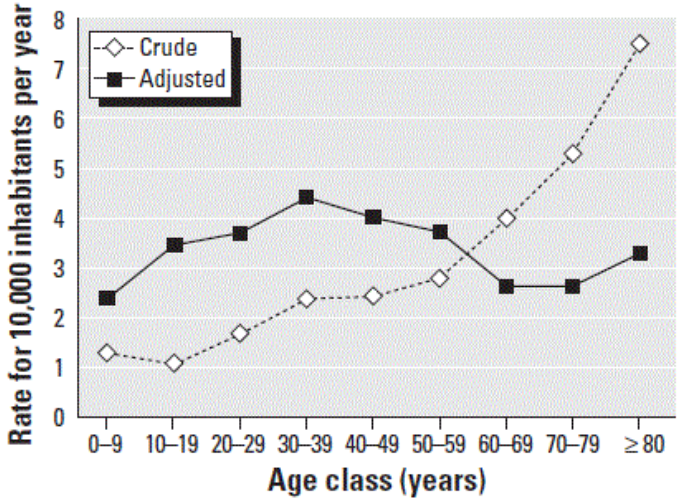
-- Figures from this and next slide from Ginsberg *et al.*, 2005, *Environ. Health Persp.*, 113, 1243-49. --

TK Variability in the Elderly

$T_{1/2}$s for Different Clearance Mechanisms



Age-specific Rates of ADRs

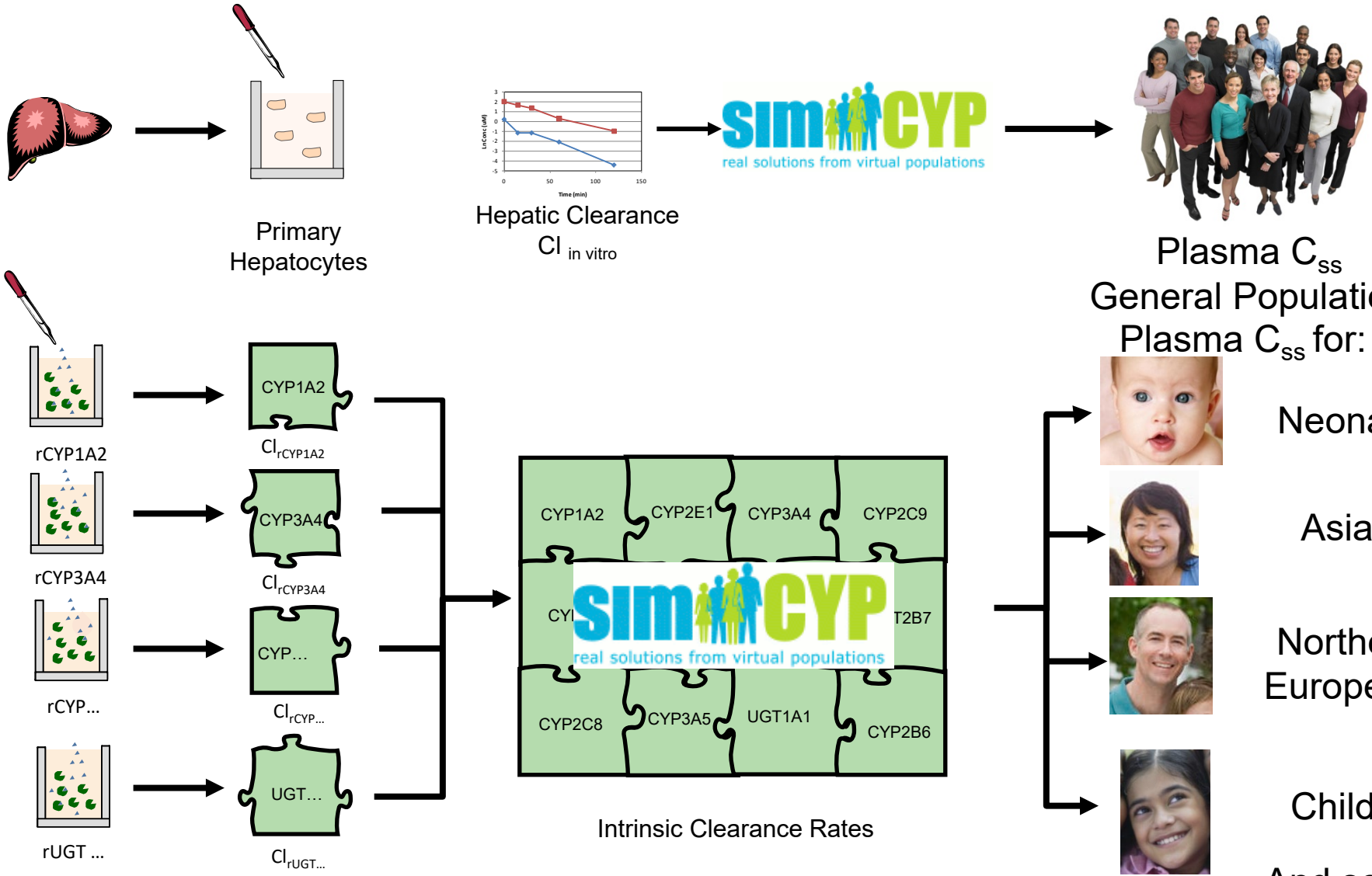


- **↓ in renal clearance, glomerular filtration rate**
- **↓ in hepatic clearance** (↓ liver size, P450 content, bile flow, blood flow)
- **Higher disease rates**
  - Polypharmacy (>65; 2-6 prescriptions; 1-3 over-the-counter)
  - ADRs– challenge at any age group, but heightened in elderly

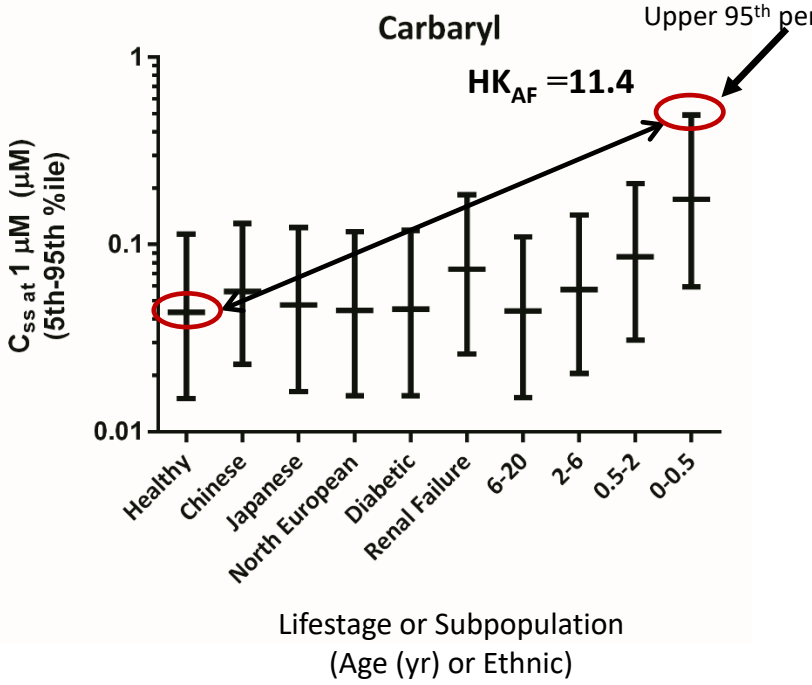
TK Variability in Children

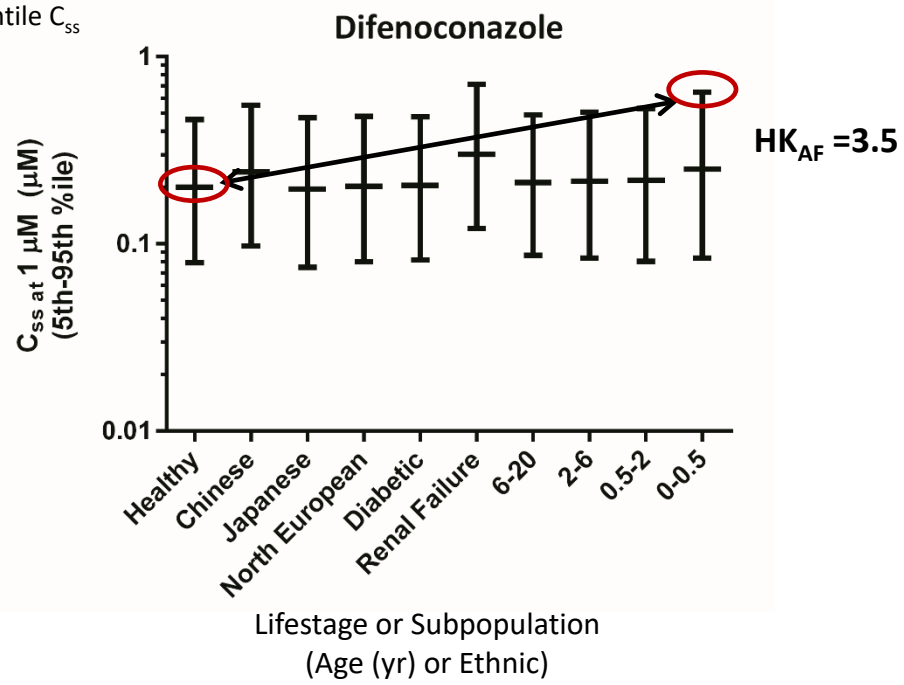
Developmental Feature	Relevant Life-Stage	Impact on TK
Body composition: lower lipid, greater water content	Birth through 3 months	↓ partitioning and retention of lipid-soluble cmpds ↑ V_d for water soluble cmpds
Larger liver:body weight ratio	Birth through 6 yr (largest ratios, birth-2yr)	↑ Hepatic extraction/metabolite clearance ↑ potential metabolic activation
Immature Phase I/II enzyme functionality	Birth through 1 yr (largest differences in first 2 months)	↓ metabolic clearance, activation ↓ removal of activated metabolites
Larger brain:body weight ratio; greater CNS blood flow; higher BBB permeability	Birth through 6 yr (largest differences in first 2 yr)	↑ CNS exposure, particularly for water soluble agents normally impeded by BBB
Immature renal function	Birth through 2 months	↓ elimination of renally cleared chemicals/metabolites
Limited serum protein binding capacity	Birth through 3 months	↑ potential, free toxicant ↑ distribution of chemicals normally bound/unavailable

In Vitro Assays to Quantitate TK Variability



Comparison of C_{ss} Values Derived Across Multiple Lifestages and Subpopulations





HK_{AF}: human toxicokinetic adjustment factor

Wrap-Up

- Despite the linkages with HT and computational approaches, development of the experimental TK assays and supporting information to support IVIVE came after years – decades – of effort, optimization, and verification
- To facilitate adoption of these approaches, clearly written, well detailed methods with descriptions of approaches, scalars used, etc. is paramount.
- Given the complexity of these approaches, education and outreach is key.
- Contact me at Wetmore.barbara@epa.gov with *any* questions.

EPA United States Environmental Protection Agency

Questions?

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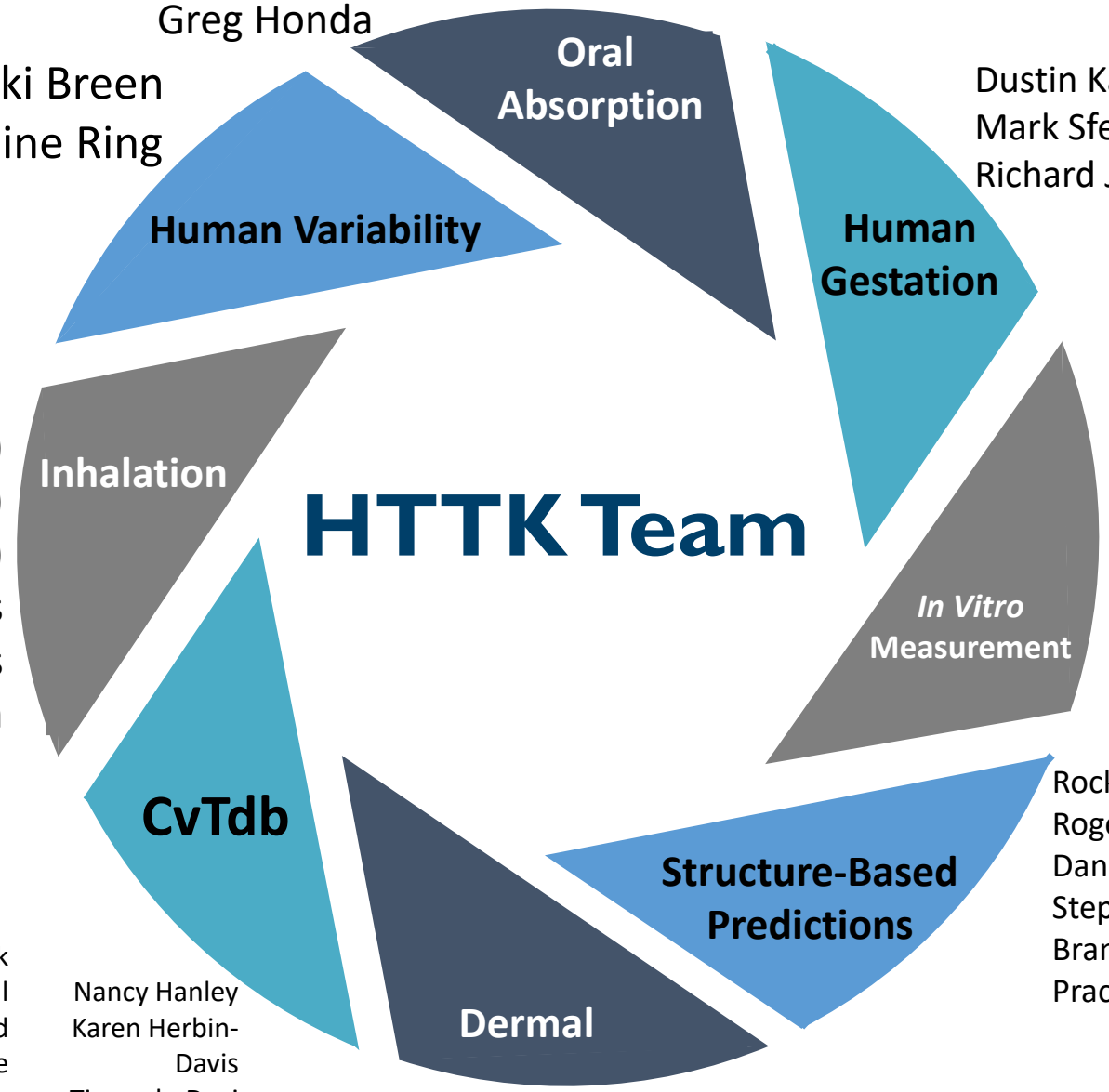

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