

TOXICOLOGICAL RESEARCH PROGRAM IN PERFLUORINATED CHEMISTRIES

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3M Company

Value of Legacy Fluorochemical Toxicological Research

- Association of Chemistry with 3M
- Reduced Uncertainty in Risk Assessment
- Credibility in the Health Science Field
- Causal Perspective for:
 - Employee medical surveillance
 - Epidemiological investigation
- **Defensive Barriers to Litigation**
- Application to Current and New Products

Causal Perspective for Epidemiology

The Environment and Disease: Association or Causation?¹

¹Hill (1965) Proc Royal Soc Med 58, 295-300.

Bradford-Hill Criteria

- Strength
- Consistency
- Specificity
- Temporality
- Biological Gradient
- Plausibility
- Coherence
- Experiment
- Analogy

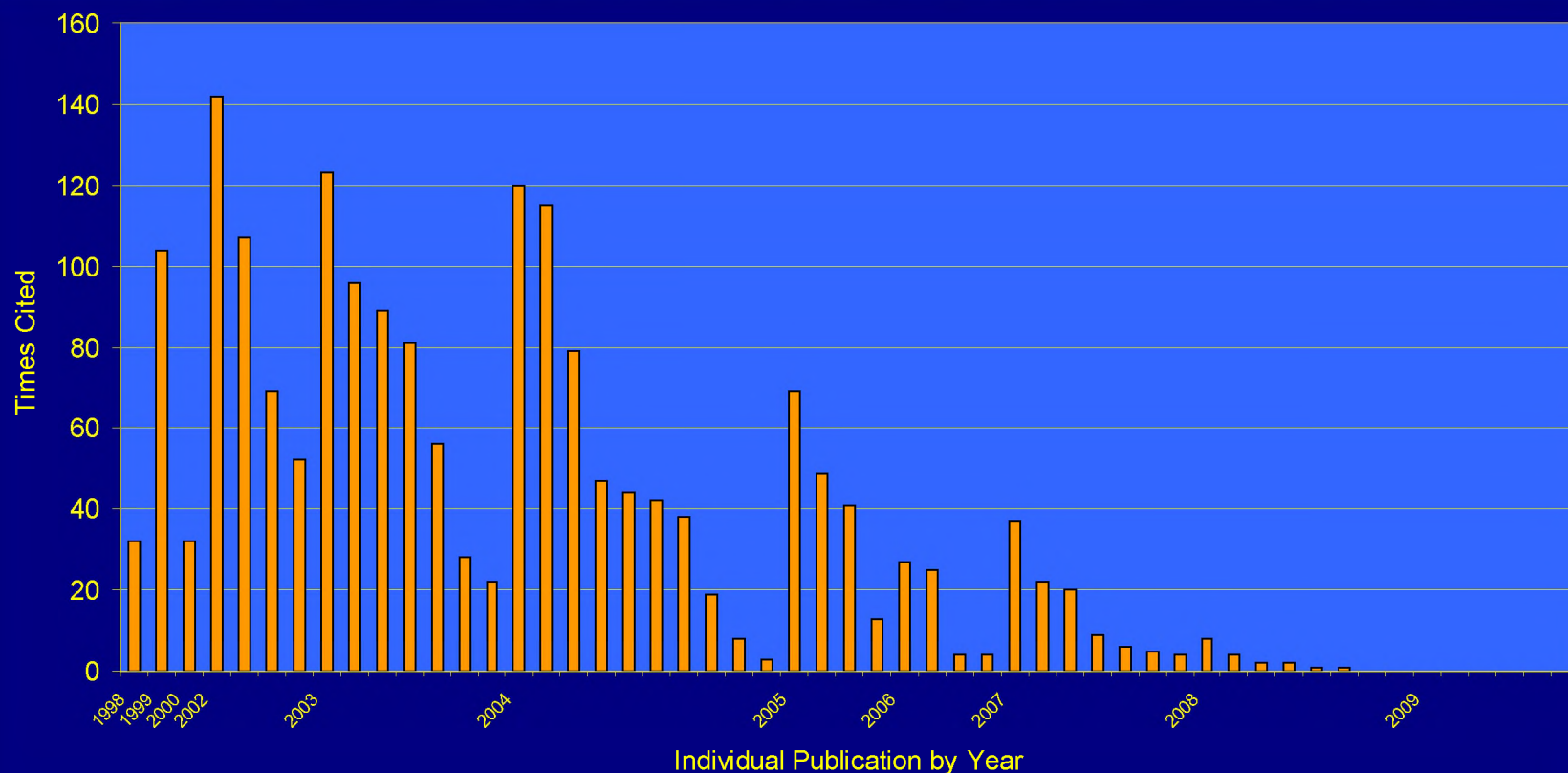
This area has become increasingly important as new epidemiological studies are released.

Flood of New Science

- Frequency of new scientific papers has increased.
- Appreciation of the whole field by the newer authors is obviously limited.
- Increasing attempts to associate effects with general population exposures.

3M Publication Impact

- 54 3M-authored, peer-reviewed fluorochemical papers cited 1804 times in scientific literature.



Two Broad Areas of Research

- Pharmacodynamics
 - Biochemical interactions
 - Biochemical and physiological responses
 - Adaptive or pathological
- Pharmacokinetics
 - Absorption, distribution, metabolism, excretion

Current Research Strategies

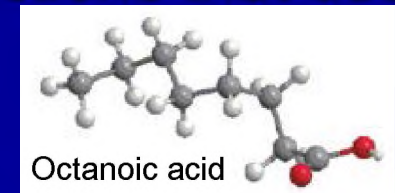
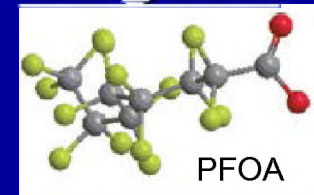
- Internal 3M research
 - Pharmacodynamics and pharmacokinetics
- Collaborative research
 - E.g., USEPA NHEERL, Universities
- Contract research
 - E.g., TNO
- 3M-sponsored university research
 - U of MN, Stockholm U, UKMC, U of Houston, Penn State

Chemical and Physical Properties

- Perfluorinated alkyls (PFAs)
 - Exceptionally stable
 - Non-reactive
 - Solubility varies
 - Amphiphilic, “organic” acids with low pKa
 - Essentially dissociated under most conditions
 - Surface active
 - Low Van der Waal’s forces in carbon chain

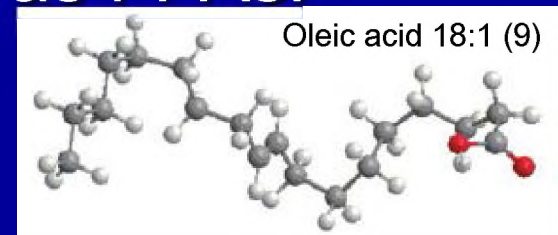
Physical/Chemical Determinants

- Resemble free fatty acids (FFAs); *although...*
 - Non-reactive
 - Not metabolized
 - Do not enter into the biochemical reactions that use fatty acids as substrate.



- *However*, PFAs may present as FFAs.

- Transporters
- Receptors
- Carrier proteins



Biological Interactions of PFAs

- Expected interactions
 - Biological membranes
 - Organic anion transport processes
 - Induction, competition
 - Protein ionic binding sites
 - Competition with endogenous substrates (e.g., FFA, hormones)
 - Activation of biochemical processes
 - Nuclear receptor activation (e.g., PPAR α)

Pharmacodynamics

Responses of Laboratory Animals To Perfluorinated Alkyls (PFAs)

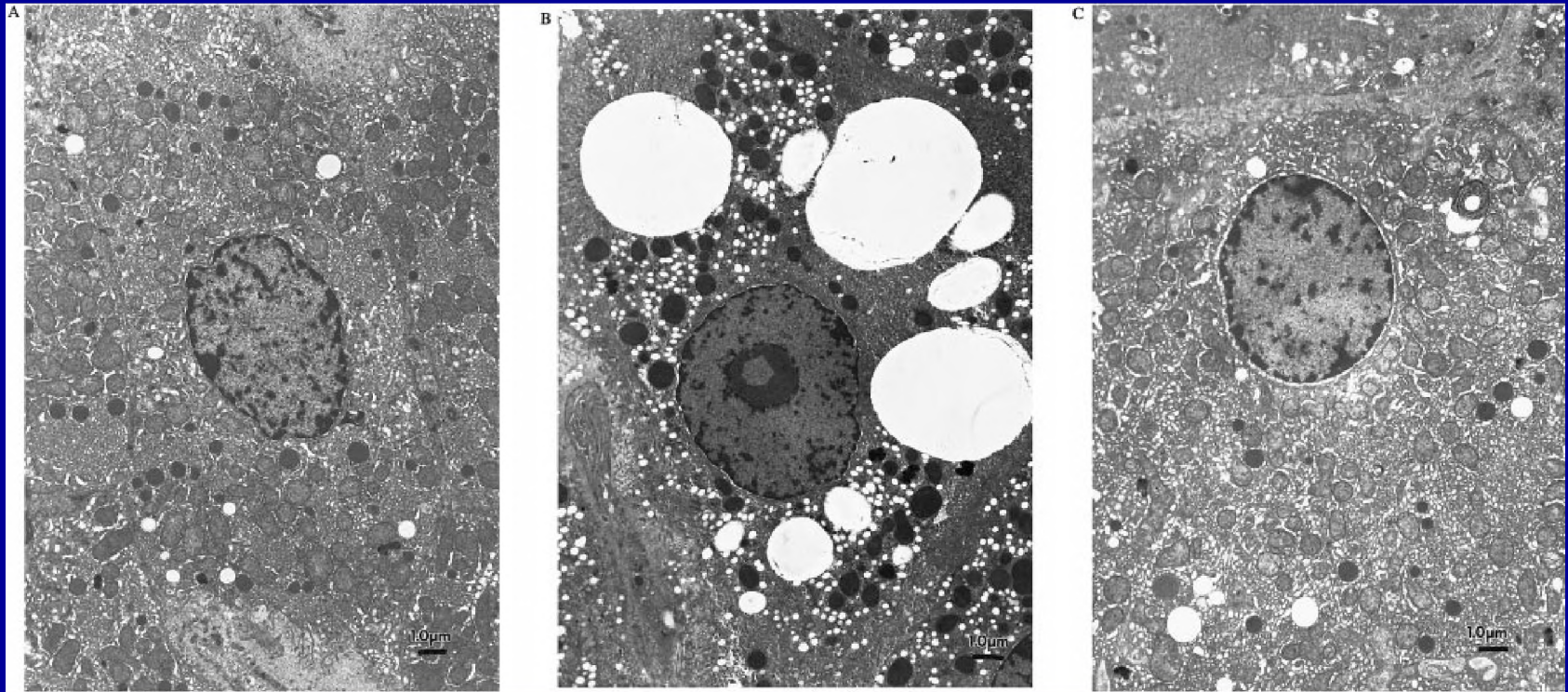
- Liver function and health
- Serum lipid chemistry
- Body-weight change
- Tumorigenesis
- Reproduction/Development
- Immune system
- Nervous system
- Endocrine system (hormones)

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Monkey Liver at 0.75 mg/kg/d K⁺PFOS (human equivalent dose = 53 mg/d)

Electron micrographs of liver cells from six-month monkey study with K⁺PFOS¹



Male control 184 d

Male 0.75 mg/kg 184 d

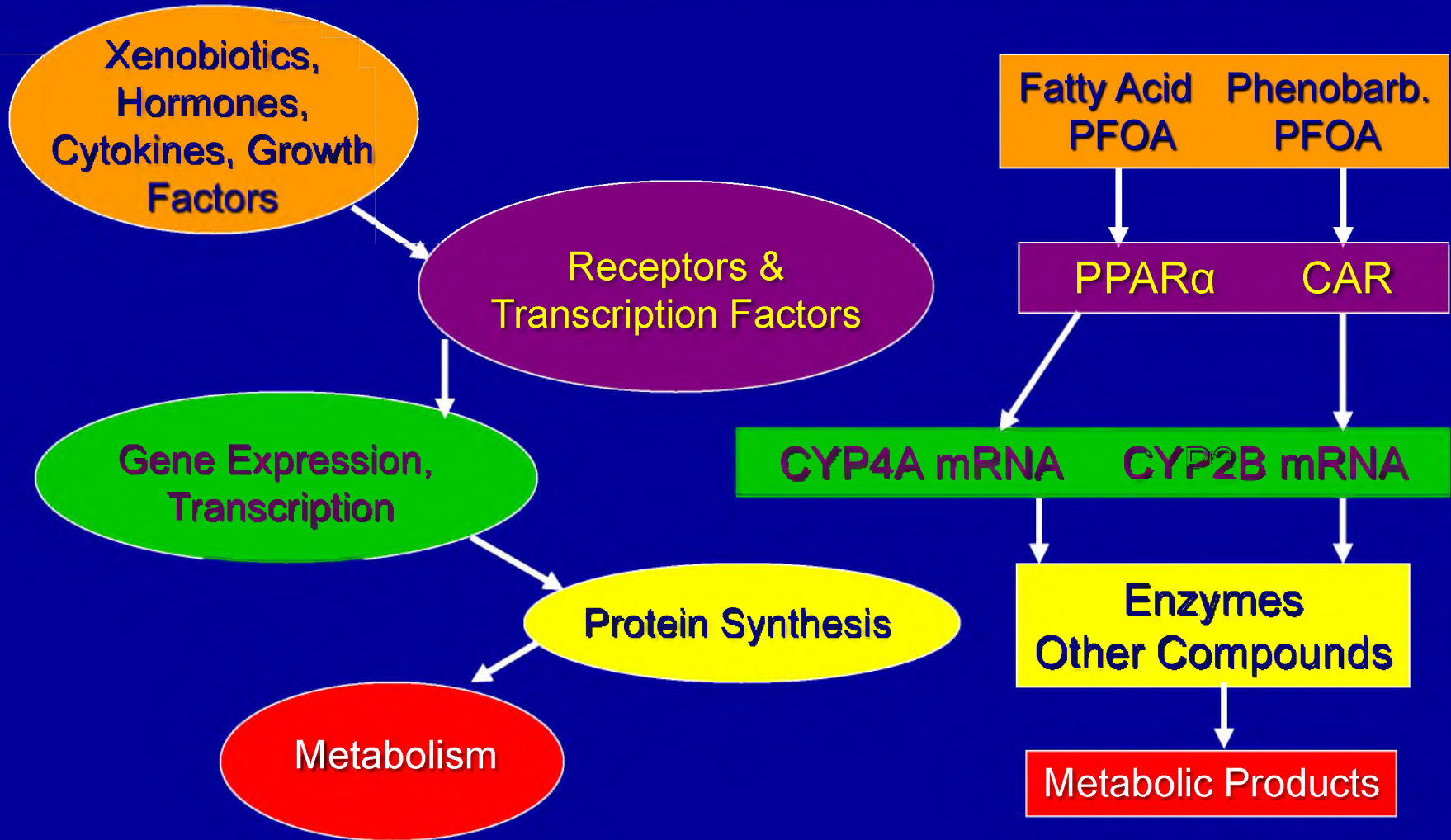
Male 0.75 mg/kg after
211 d recovery

¹Seacat et al. (2002) Toxicol Sci 68, 249-264.

Liver Effects

- Increased liver weight
 - Enlarged cells (hypertrophy)
 - Adaptation or pathological change?
 - Increased numbers of cells (hyperplasia)
 - Pathological change (hyperplasia → tumor → cancer)
- Metabolic and biochemical changes
 - e.g., increased burning of fat
- Human relevance
 - PPAR α activation
 - Other processes (e.g., CAR and PXR)
 - Adaptation vs. pathological change

Diversion #1 - Molecular Biology



Based on: Waxman (1999) Arch. Biochem. Biophys. 369, 11-23.

Some Common Nuclear Receptors Controlling CYP Induction

<u>Receptor</u>	<u>Typical Activator</u>
• PPAR α	Fatty acids, Fibrates
• PPAR γ	Rosiglitazone
• CAR	Phenobarbital
• PXR	Steroids, Dexamethasone
• LXR α	Cholesterol
• FXR	Bile acids
• RXR	Retinoic acid
• TR	Triiodothyronine
• Ah ¹	Polycyclic aromatics, Dioxin

¹ PAS transcription family member, not a nuclear receptor

Experimental Approaches

- Engineered nuclear receptor domains
- Primary cell culture
- In-life exposure followed by biochemical and molecular biological methods
- Transgenic mouse studies
 - Remove or repress receptor
 - Insert human form of receptor

Species Differences in PPAR α

- Humans less responsive than rodents
 - Lower human levels of PPAR α
 - Human PPAR α not associated with hyperplasia
- Use of genetically-modified mice^{1,2,3,4}
 - Using specific activators of PPAR α
 - mPPAR α (natural) – hypertrophy and hyperplasia
 - hPPAR α – hypertrophy but NO hyperplasia
 - No PPAR α – NO hypertrophy and NO hyperplasia

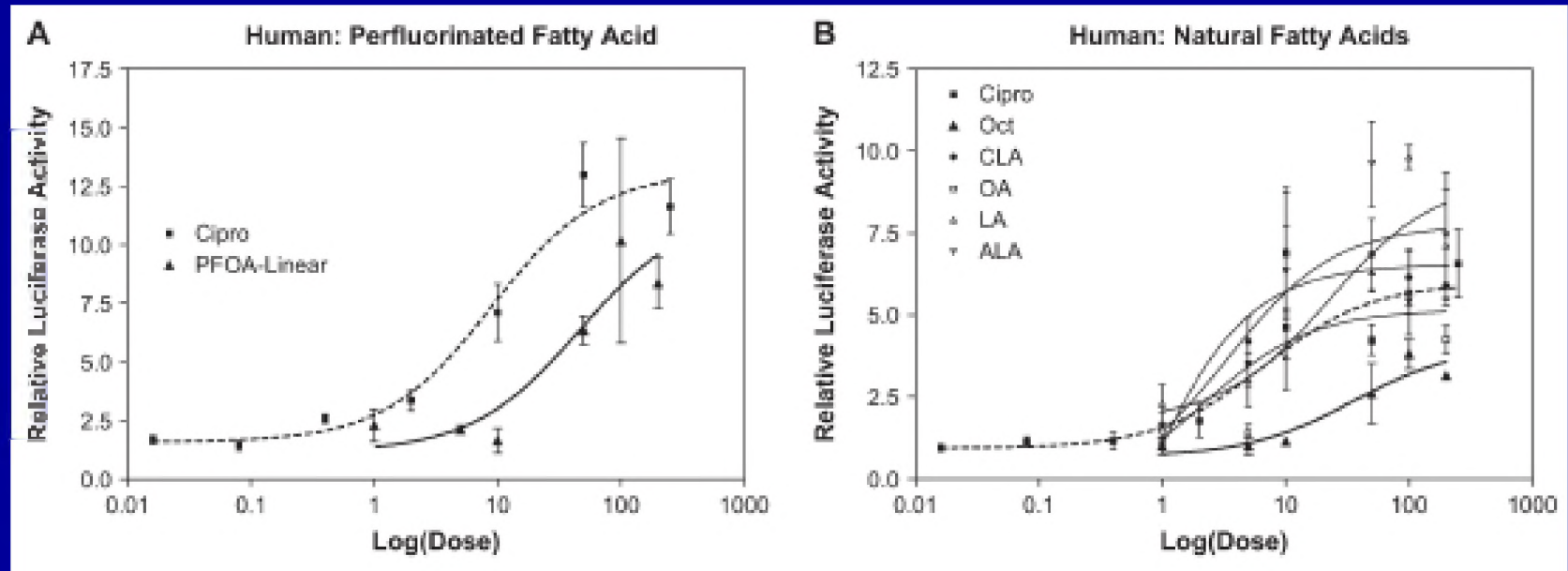
¹Cheung et al. (2004) Cancer Res 64, 3849-3854.

²Morimura et al. (2006) Carcinogenesis 27, 1074-1080.

³Shah et al. (2007) Mol Cell Biol 27, 4238-4247.

⁴Yang et al. (2008) Toxicol Sci 101, 132-139.

Differential Activation of PPAR α in an Engineered System



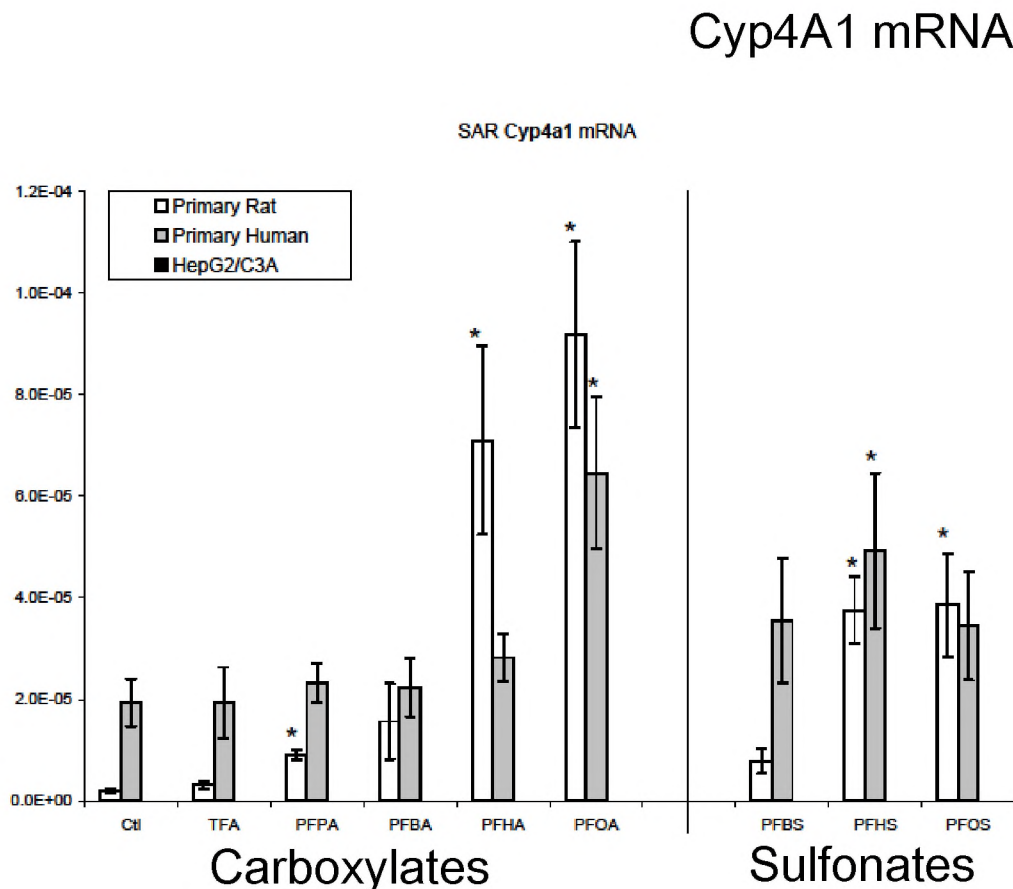
PFOA is a weak activator of PPAR α compared to ciprofibrate and natural fatty acids.

Nuclear Receptor Activation by PFOA and PFOS in an Engineered System

- Mouse, rat, human receptor forms
- PFOA and PFOS activate PPAR α
 - Less potent than clofibrate and endogenous long chain FFA
- PFOS and PFOA are weak agonists for PPAR γ
 - Much less potent than rosiglitazone
- No activation of RXR α or LXR β
- PFOA and PFOS more specific and less potent than endogenous long-chain FFAs.

¹ Vanden Heuvel et al. (2006) Toxicol Sci 92, 476-489.

Human vs. Rat Liver Cells in Primary Culture and PPAR α Activation by PFAs



➤ All PFAs at 25 μ M in cell culture media.

➤ C $\leq$ 4 PFAs have little or no effect.

Courtesy of Dr. Kendall Wallace, U of MN.

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- Nervous system
- Endocrine system (hormones)

Serum Lipids

- Hypolipidemia
 - Reduced serum total cholesterol with
 - PFOS; PFHxS; PFBA; PFOA (not consistently)
 - Early onset clinical observation in lab animals
 - Apparent reduction in HDL (female monkeys)
 - PFOS
 - A basis for MDH HRL for PFOS
 - Mode(s) of action
 - PPAR α activation (evidence strong)
 - HMG CoA reductase inhibition (evidence weak)

Serum Lipids

- Hyperlipidemia
 - Inconsistent epidemiological association of serum PFOS and PFOA with increased serum cholesterol in humans
- C8 Science Panel Report
 - “In multivariate models adjusting for other factors ... all lipid outcomes except HDL were higher when serum PFOA and PFOS levels were higher. The positive trends were statistically significant in all cases, again with the exception of HDL.”

A Case of Reverse Causation?

- Do higher serum lipids increase serum binding capacities for PFOA and PFOS?
- Is there an experimental basis for causation?
- Continuing areas of research
 - Serum lipid biochemical studies
 - Binding of PFOS and PFOA to serum lipoproteins
 - Pharmacokinetic distribution studies

Serum Lipids

Experimental model:

- “Humanized” lipoprotein-profile transgenic mice
- Developed by TNO in The Netherlands
- Studying PFBS, PFHxS, PFOS
- Western-style diet (high fat)
- PFOS, PFHxS, PFBS at ~ 3, 6 and 30 mg/kg body weight/d in diet, respectively.

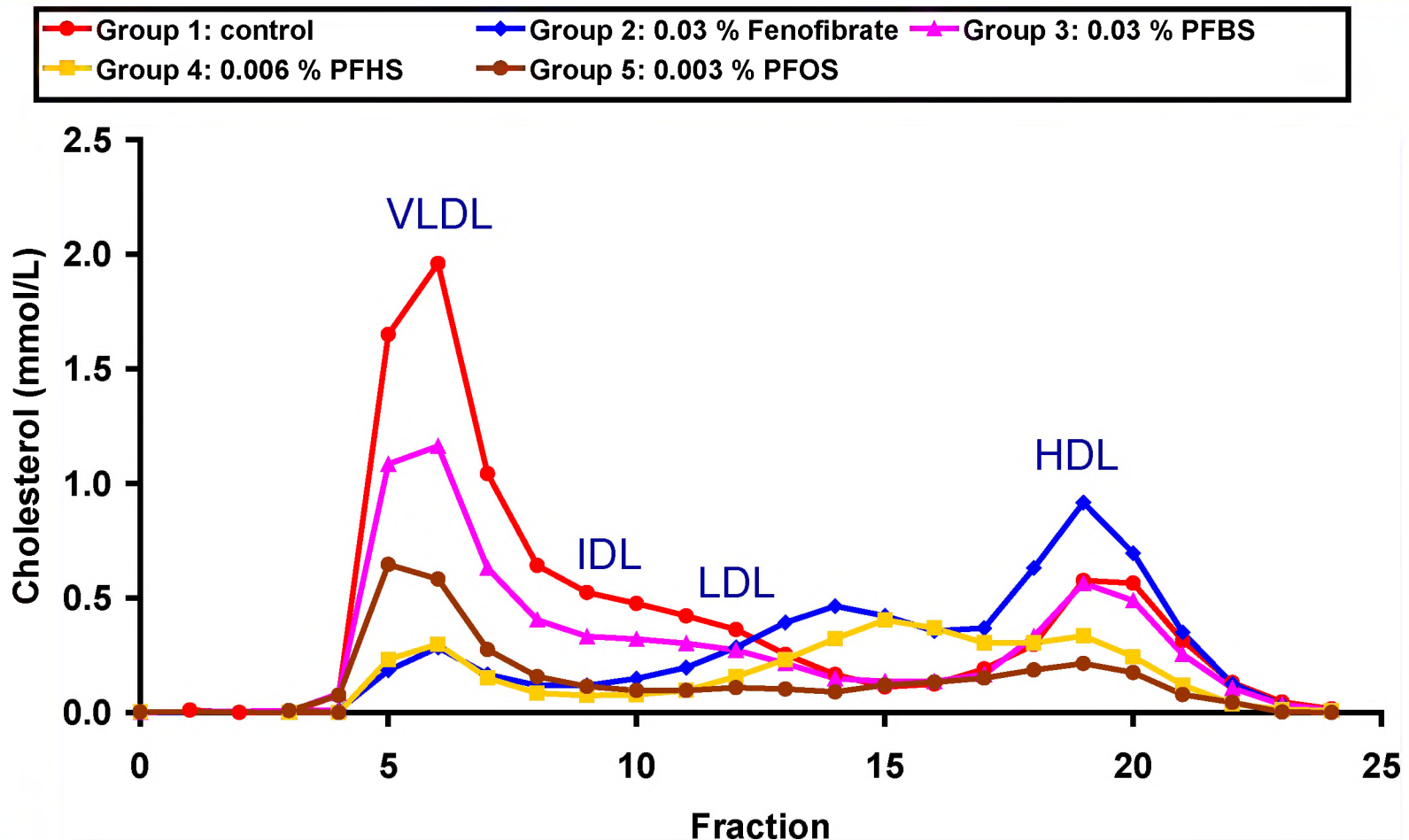
APOE*3Leiden Mouse Study

- PFOS and PFHxS
 - Reduced total cholesterol and triglycerides
 - Decreased cholesterol 7- α -hydroxylase
 - Increased liver size
 - Increased fatty acid oxidation
 - Suggests a PPAR α agonist mode of action
- PFBS had no effect.

APOE*3Leiden.CETP Mouse Studies

- Incorporate cholesterol ester transfer protein
- PFOS and PFHxS
 - reduced total cholesterol and triglycerides via
 - decreased VLDL production
 - increased VLDL lipolysis and clearance
 - increased HDL clearance
- PFBS
 - reduced total cholesterol and triglycerides
 - to a lesser extent and via
 - reduced VLDL production and
 - increased VLDL clearance
 - no effect on HDL

PFBS, PFHS, PFOS & Hypolipidemia APOE*3Leiden.CETP Mouse



Association of PFOS and PFOA with Hyperlipidemia in Epi Studies

- APOE*3Leiden mouse model argues against causation.
- Serum binding studies show affinity of PFOS and PFOA for lipoproteins.
- Additional serum binding work may help prove reverse causation.

Percent Binding to Isolated Human Serum Protein Fractions at 10 µg/mL

	PFBS	PFHS	PFOS	PFOA
Albumin	93.5	> 99.9	99.8	99.7
γ-Globulin	< 0.1	26.1	24.1	3.0
α-Globulin	< 0.1	13.7	59.4	11.0
Fibrinogen	< 0.1	< 0.1	< 0.1	< 0.1
α-2-Macro- globulin	< 0.1	< 0.1	< 0.1	< 0.1
Transferrin	< 0.1	6.4	<0.1	2.1
β-Lipo-protein	< 0.1	64.1	95.6	39.6

3M Company and Southern Research Institute, unpublished data

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Additional Experimental Approaches

- Binding interaction studies
 - Exploit
 - ^{35}S -PFOS made at Stockholm University in Åke Bergman's lab.
 - Biochemical expertise of Joe DePierre's research group.
- In-life experiments under consideration
 - Exploit APOE*3Leiden.CETP mice
 - Dietary manipulation of lipoprotein profile

Responses of Laboratory Animals To Perfluorinated Alkyls (PFAs)

- Liver function and health
- Serum lipid chemistry
- **Body-weight change**
- Tumorigenesis
- Reproduction/Development
- Immune system
- Nervous system
- Endocrine system (hormones)

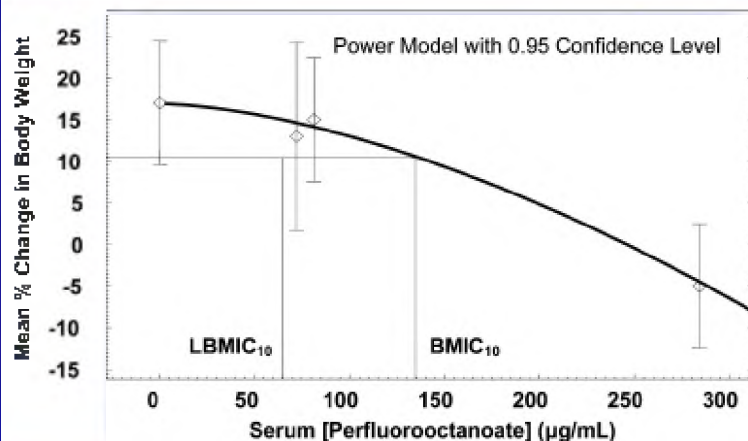
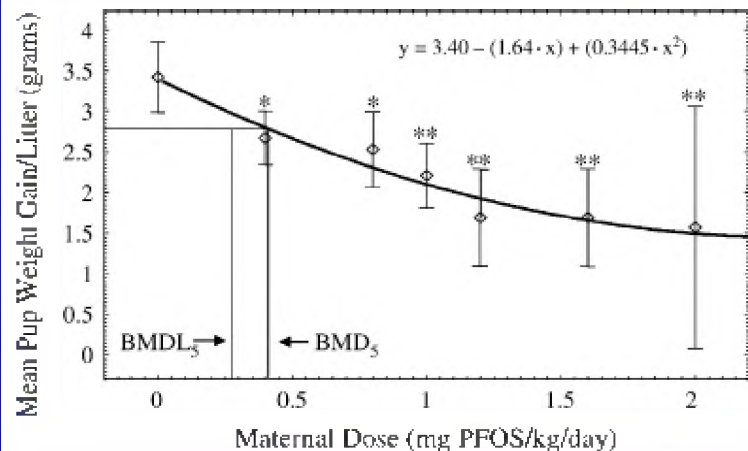
Body Weight

- Decreased weight gain in growing animals

Rat pups

- Weight loss at sufficient dose

Male monkeys



Body Weight

- Hypotheses
 - Increased burning of fat
 - Uncoupling of oxidative phosphorylation (mitochondria)
 - Only with certain sulfonamides (NOT PFOS or PFOA)
 - Increased mitochondrial bodies (PFOA)
 - Evidence from rat and monkey studies
 - PPAR α activation
 - Strong evidence from mouse studies
 - Decreased appetite
 - Some evidence
 - Malabsorption of nutrients
 - Not fully investigated

Biological Interactions - Mitochondria

- 3M sponsored
 - Starkov and Wallace (2002) Toxicol Sci 66, 244-252.
 - O'Brien et al. (2008) Toxicol Appl Pharmacol 227, 184-195.
 - Berthiaume and Wallace (2002) Toxicology Lett 129, 23-32.
 - Butenhoff et al. (2002) Toxicol Sci 69, 244-257.
 - Mitochondrial proliferation mode of action (current)
- NTP sponsored (i.e., they think its important)
 - Mitochondrial interactions of PFCs *in vitro* (Wallace)

Responses of Laboratory Animals To Perfluorinated Alkyls (PFAs)

- Liver function and health
- Serum lipid chemistry
- Body-weight change
- **Tumorigenesis**
- Reproduction/Development
- Immune system
- Nervous system
- Endocrine system (hormones)

Tumorigenicity in SD Rats

- PFOA

- At 300 ppm in diet (~15 mg/kg body weight)
 - Hepatocellular adenoma (males)
 - Pancreatic acinar-cell adenoma (males)
 - Testicular Leydig-cell adenoma(“Tumor triad” pattern seen with other PPAR α agonists)
 - No increased tumor incidence in females

- PFOS

- At 20 ppm in diet (~1 mg/kg body weight)
 - Hepatocellular adenoma (males and females)
 - Thyroid follicular cell adenoma (20 ppm stop-dose males)

Tumorigenesis - PFOA

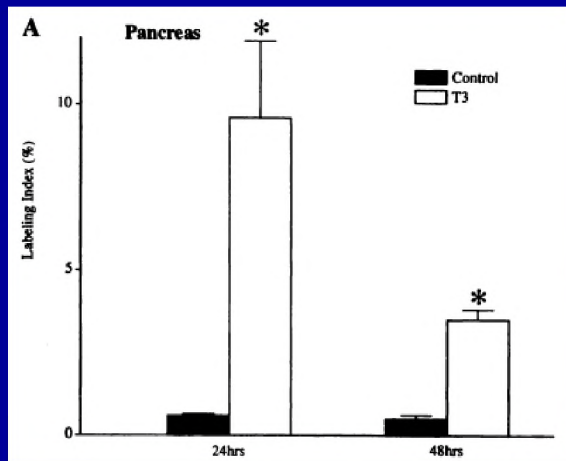
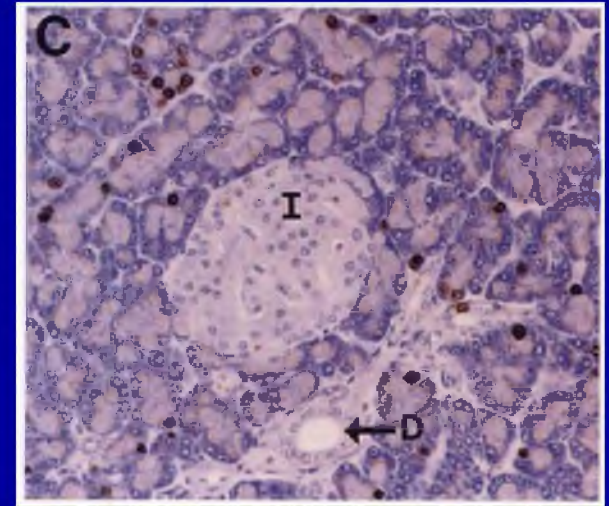
- Hepatocellular
 - Consequences of PPAR α activation
 - Oxidative stress
 - Potential for contribution of CAR activation
- Testicular Leydig cell adenoma
 - Consequences of PPAR α activation
 - Induction of aromatase enzyme leading to increased estrogen
- Pancreatic acinar cell adenoma
 - Consequences of PPAR α activation
 - Increased cholecystokinin hormone (evidence weak)
 - Mitogenic activity of thyroid hormone, retinoids (not tested).

Pancreatic acinar cell proliferation

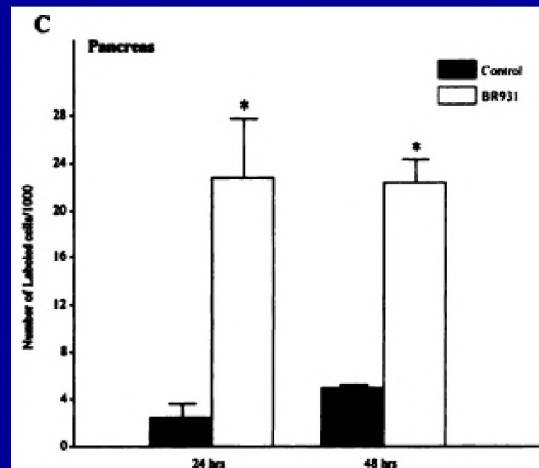
From: Ohmura et al. (1997) Can Res 57, 795-798.

Thyroid hormone (T3) is a strong mitogen for rat pancreatic acinar cells, as are BR931 and 9-cisRA.

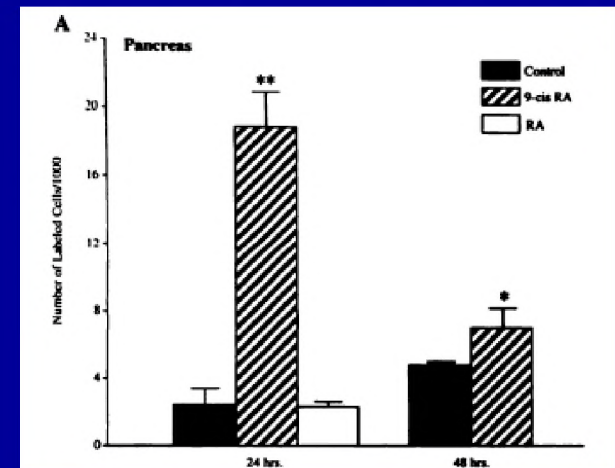
BrDU staining showing proliferation of acinar cells and not ductal or islet cells in rat pancreas stimulated with T3.



Thyroid hormone (T3)



Peroxisome proiferator



Retinoid

Tumorigenesis - PFOS

- Was PPAR α activation responsible?
- PFOS - CXR investigation results
 - Liver
 - PFOS is a mixed agonist in the rat
 - PPAR α , CAR, PXR
 - Thyroid
 - No effect of PFOS

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PFAs Studied for Reproduction and Developmental Effects

- PFBS
- PFHxS
- PFOS
- PFBA
- PFOA

Results of Major Laboratory Studies

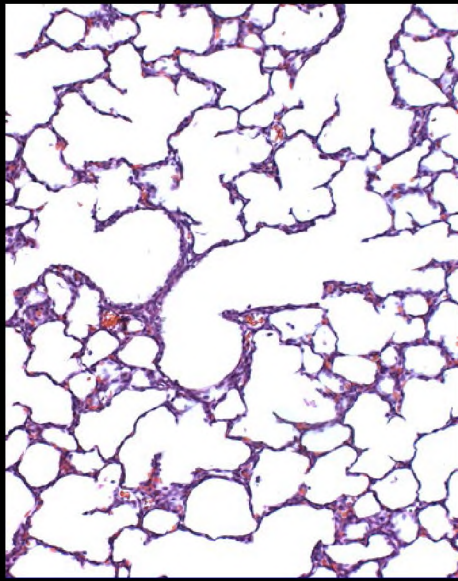
- No effect on functional aspects of reproduction
- Structural anomalies associated with dosing causing maternal stress
- Developmental delays noted in some cases
- Birth weight and weight gain affected in some cases
- Neonatal mortality with PFOS and PFOA

Modes of Action - Current Thoughts

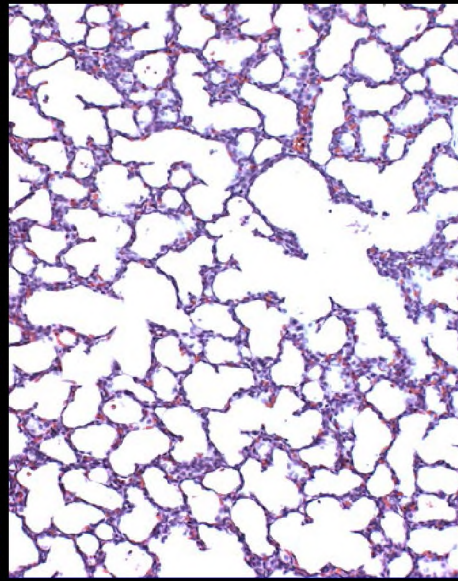
- Although *in utero* exposure of both PFOS and PFOA caused neonatal mortality, the adverse effects may be mediated by separate mechanisms
- PFOA likely acts through the PPAR α signaling pathway that regulates intermediary metabolism
- PFOS likely interacts with phospholipids of lung surfactant and interferes with lung inflation and pulmonary function

Lung Histology and Morphometry

Control



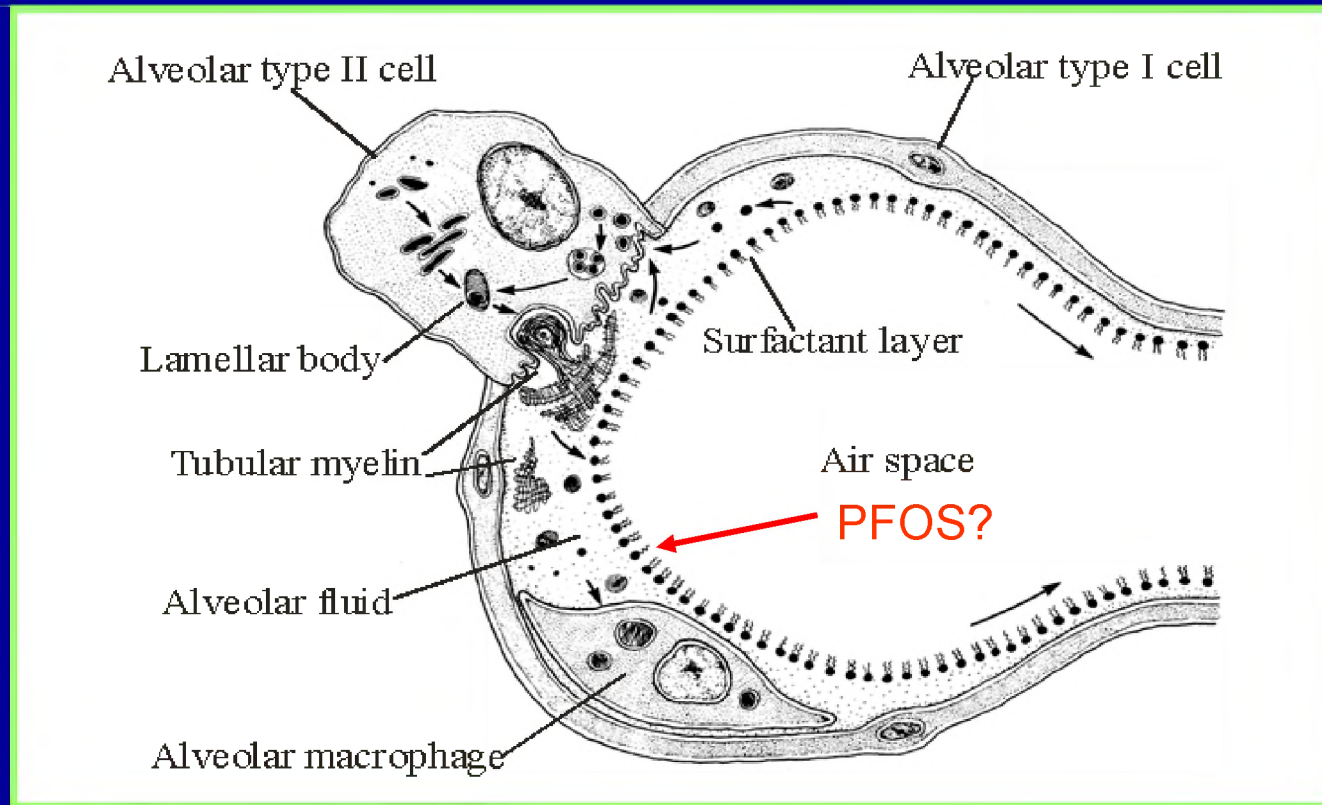
PFOS



Dose (mg/kg)	Air Space (%)	Septal Space (%)
0	63.9 ± 1.5	31.6 ± 1.3
5	56.7 ± 2.1	41.2 ± 2.0 *
10	55.2 ± 2.2*	43.6 ± 1.9 *

Alveolar Structure

Surfactant prevents lungs from collapsing during end-expiration by reducing the surface tension at the air-liquid interface



Modified from Hawgood & Clements, 1990.

PFOS and Pulmonary Surfactant

- PFOS was detected in amniotic fluid that bathed the fetal lung
- Oral gavage of newborn rats failed to cause mortality
 - chemical has to reach within the lung
- PFOS interacts with phospholipids (Xie et al., 2007)
 - Dipalmitoylphosphatidylcholine (DPPC) is a major component of lung surfactant
 - *In vitro* study: PFOS had strong tendency to partition into and disrupt DPPC bilayers
 - PFOS > PFOA >> OS
- Definitive evidence is needed

Non-Occupational Human Studies - Summary

Endpoint	PFA	Apelberg	Fei	Monroy
Gestational Age	PFOS	NS	NS	NS
	PFOA	NS	NS	NS
Birth Weight (g)	PFOS	NS (-69 ^a , T ^b)	NS	NS
	PFOA	NS (-104 ^a , T)	-10.6	NS
Birth Length (cm)	PFOS	NS	NS	N/A
	PFOA	NS	-0.69	N/A
Head Circum. (cm)	PFOS	-0.32 (T)	NS	N/A
	PFOA	-0.27 (T)	NS	N/A
Abdominal Circum. (cm)	PFOS	N/A	NS	N/A
	PFOA	N/A	-0.059	N/A
Ponderal Index	PFOS	-0.074 (T)	NS	N/A
	PFOA	-0.074 (T)	NS	N/A
Placental Weight	PFOS	N/A	NS	N/A
	PFOA	N/A	NS	N/A

^a Stat. sign. when adjusted for gest. age but not sign. in fully-adjusted analysis.

^b Log transformed (change for 2.7-fold change in PFA concentration).

Birth Weight - Another Case of Reverse Causation?

- Plasma volume expansion positively associated with increased birth weight.
- Concentrations of plasma constituents may decrease during pregnancy.
- Research approach:
 - Modeling of pharmacokinetics in pregnancy
 - Contract with The Hamner Institutes

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PFOS and PFOA & Immune System

- Suppression of adaptive immunity in mice
 - Thymic and splenic atrophy
- Enhancement of innate immunity in mice
- Attenuated by knocking out PPAR α
- Appears to be a high-dose effect (DePierre)
- However, Peden-Adams report on PFOS effect at 91 ppb PFOS in serum.
- Epi studies?

Immune System and PFOS - Mice

- Dr. DePierre's research group at Stockholm University
 - Carefully repeated Peden-Adams et al. work.
 - Not able to reproduce observed effects.
 - Likely due to methodological issues with Peden-Adams et al. study.
- Human data would be helpful

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Nervous System

- Decreased habituation consistently observed with PFOS in developing male rats and mice (transient)
 - Publishing DNT study
- Delayed pupillary reflex in male rats given PFOA and PFBA
 - Grant to Dr. Donald Fox, U of Houston
- Brain uptake studies
 - Collaborative with USEPA
 - Grant to Dr. Grant Anderson, U of MN

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Endocrine System

- PFAs can interfere with free hormone measurement
- Current focus on thyroid hormones
 - Publication of remaining PFOS work
 - Publication of PFBA work
- Human thyroid hormone displacement studies planned
- Follow-up to PFBA planned using ultrafiltration and LC-MS/MS T4 method

Pharmacokinetics

Key Questions

- What are the mechanisms of PF₆AA transport and elimination?
- What are the determinants of interspecies elimination differences?
- How can interspecies dose-response extrapolations best be accomplished?

3M-Sponsored Research

- Joe DePierre's lab at Stockholm U
 - Distribution and binding
- Hagenbuch's lab at KUMC
 - Renal and liver transport
- Anderson's lab at Univ of MN
 - Thyroid hormone transport interactions
 - Brain uptake
- The Hamner Institute
 - Pharmacokinetic modeling

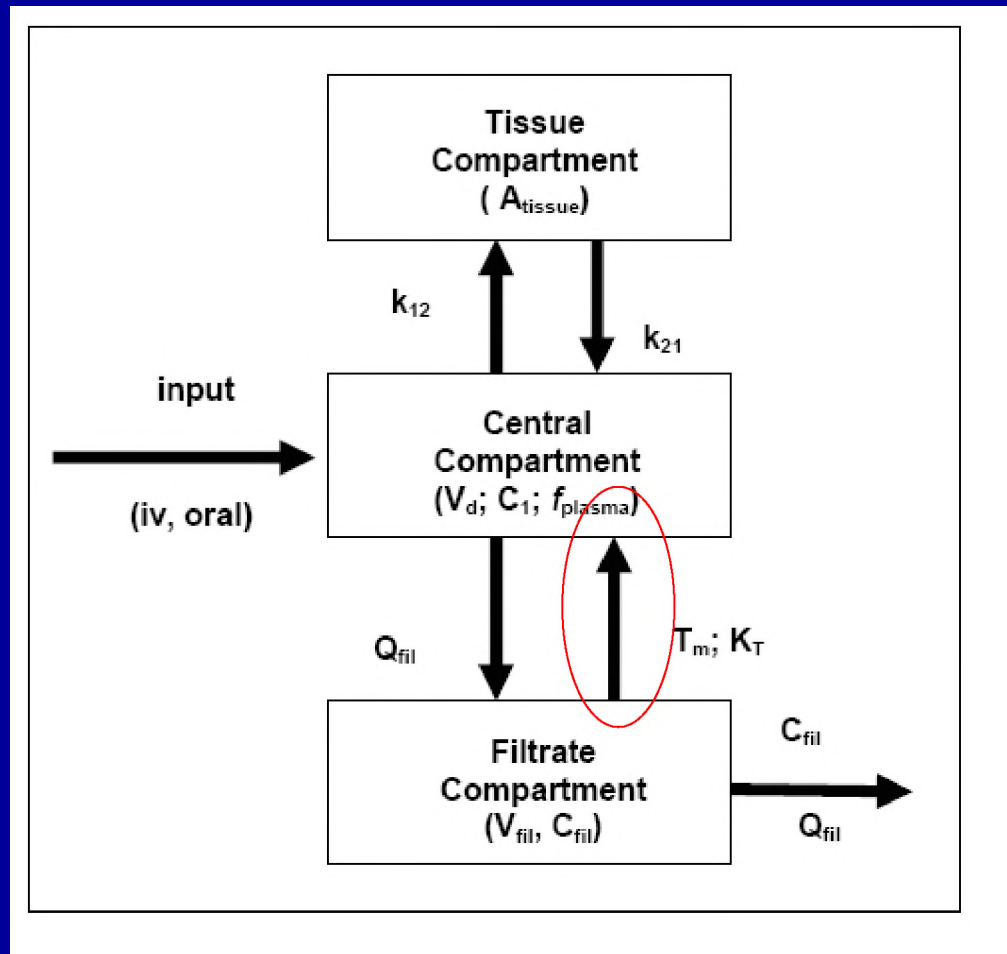
Pharmacokinetics – Tissue Distribution of Radiolabelled PFCs

- Recent synthesis of ^{35}S -PFOS at Stockholms Universitet:
 - Initial distribution study in mice completed.
 - Whole-body distribution in progress
 - Fetal, age effects, intracellular investigations planned
 - Protein binding studies to be addressed

Role of Organic Anion Transport

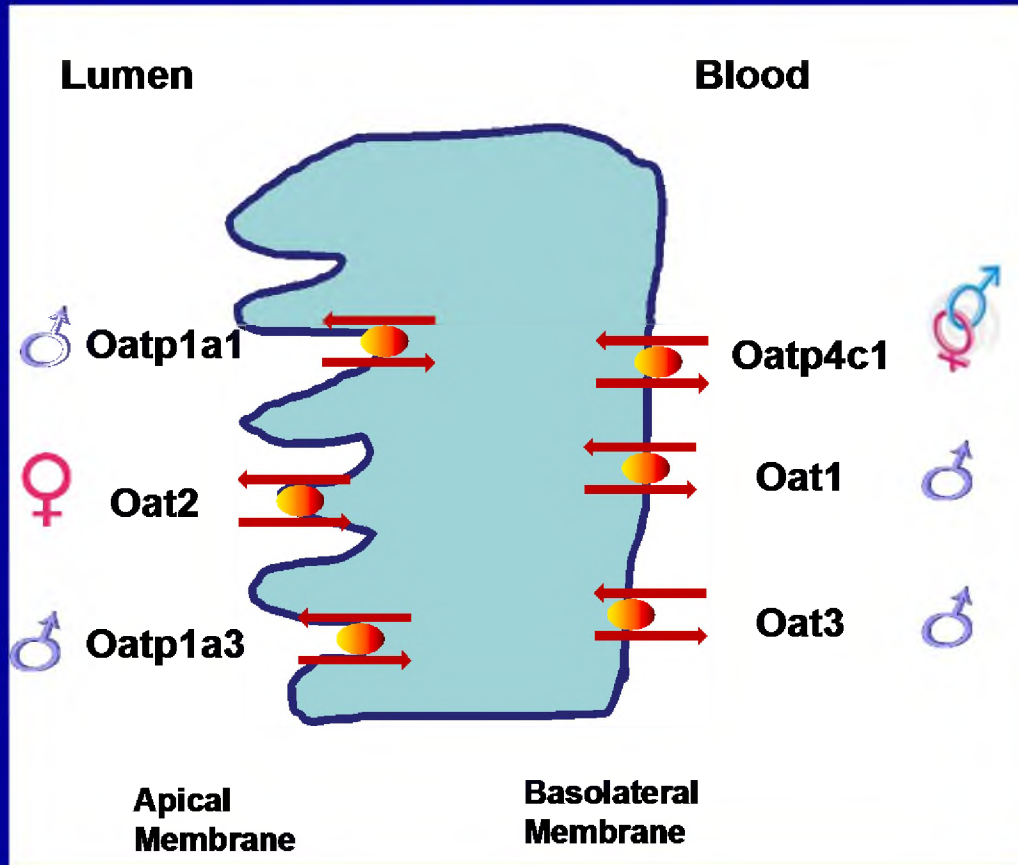
- Active renal proximal tubular reabsorption
- First suggested by Kudo et al. (2002)
 - Based on increased mRNA for Oatp1 in male rats
- First modeled by Andersen et al. (2006)
 - Cynomolgus monkey PK data for PFOA and PFOS fit resorption model
- Evidence in rat by Katakura et al. (2007)
 - Oat3 and Oatp1 may be reabsorption transporters

A Schematic for a Physiologically-Motivated Renal Resorption Pharmacokinetic Model¹



¹ Andersen *et al.* (2006) Toxicology 226, 156-164.

Uptake transporters in renal proximal tubule cells



Based on subcellular localization, Oat1 and Oat3 may be responsible for active renal secretion of PFHA, PFOA and PFNA while Oatp1a1 may be responsible for reabsorption of PFDA, PFNA and PFOA. (From poster by Weaver and Hagenbuch, 2008).

Pharmacokinetics – PBPK Models

- The Hamner Institutes (3M funding)
 - Andersen et al. (2006) Toxicology 227, 156-164.
 - Tan et al. (2008) Toxicol Lett 177, 38-47.
- EPA
 - Wambaugh et al. (2008) J Pharmacokinet Pharmacodyn 35, 683-713.
 - Harris and Barton (2008) Toxicol Lett 181, 148-156.
 - Lou et al. (2009) Toxicol Sci 107, 331-341.

Protein Ionic Binding

- Albumin
 - Major carrier protein in serum^{1,2,3}
 - Saturable¹
 - Competition with endogenous substrates
 - Steroid hormones¹
 - Thyroid hormones⁴
 - Carbon number (size) and solubility

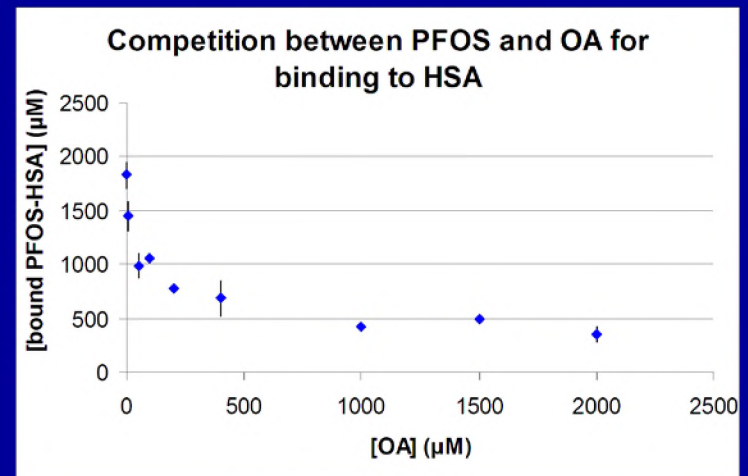
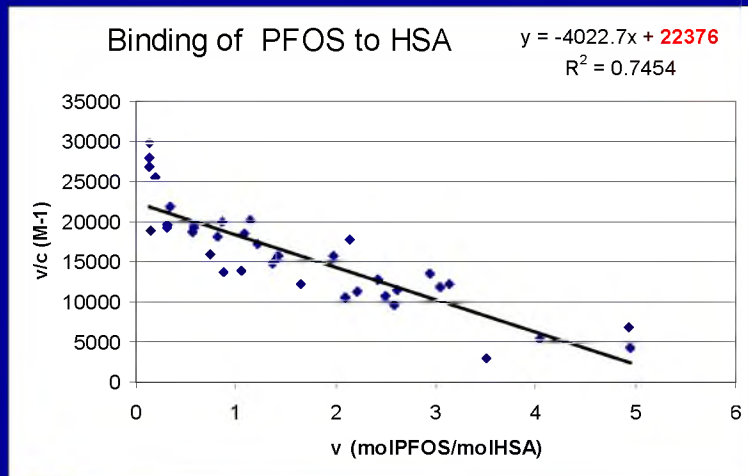
¹Jones et al. (2003) Environ Toxicol Chem 22, 2639-2649.

²Han et al. (2003) Chem Res Toxicol 16, 775-781.

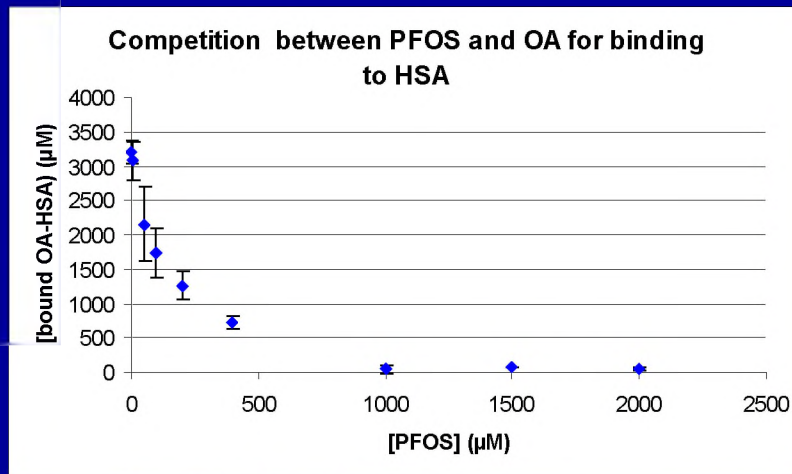
³3M and Southern Research Institute, unpublished report, USEPA Docket AR-226.

⁴Chang et al (2008) Toxicology 243, 330-339.

Binding of PFOS to HSA

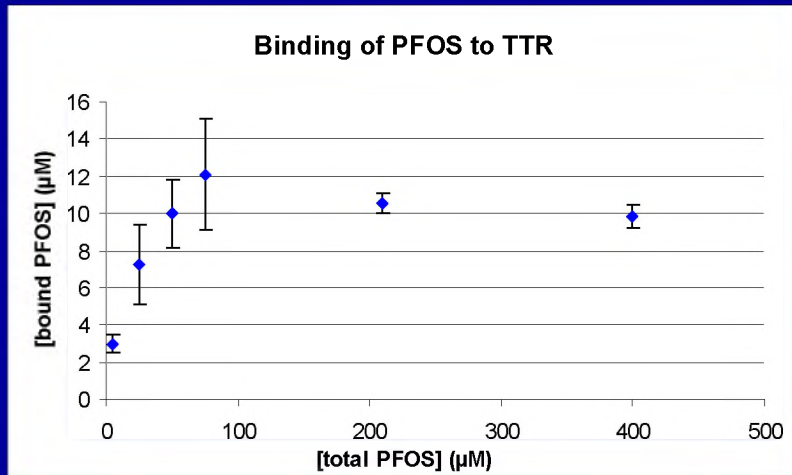


50 μM PFOS fixed concentration

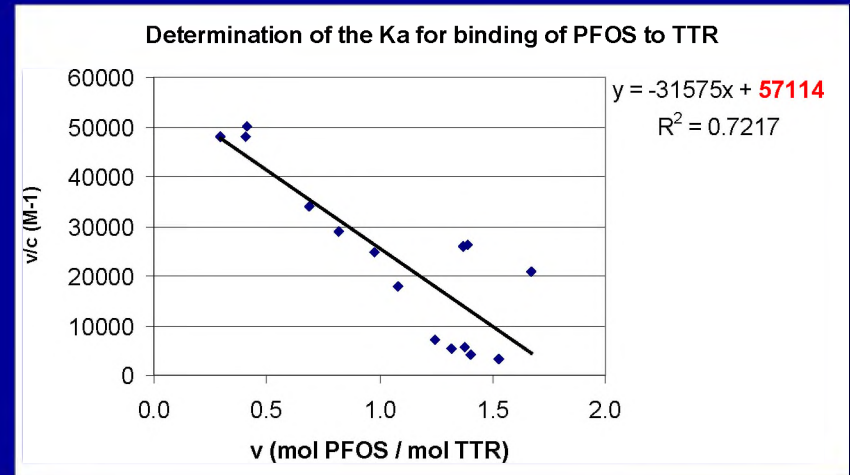


10 μM OA fixed concentration

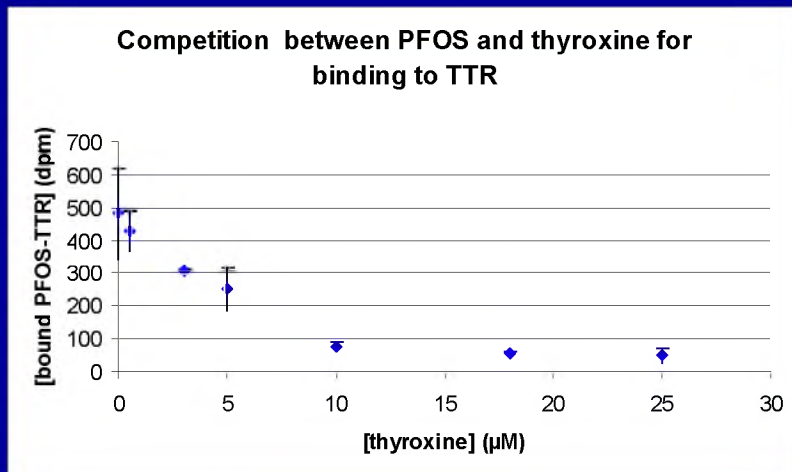
Binding of PFOS to TTR



Saturable



1 -2 binding sites



5 μM PFOS fixed concentration

Summary – Key Research Areas

- Differential effects: human vs. lab animals
- Mechanism of effects on serum lipids
- Immune effects – human relevance
- Transporters – species differences
- Pharmacokinetic models; e.g, pregnancy
- Distribution studies
- Binding studies