NTP
National Toxicology Program

Tox21 Targeted Testing

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Division of the National Toxicology Program
December 15, 2011

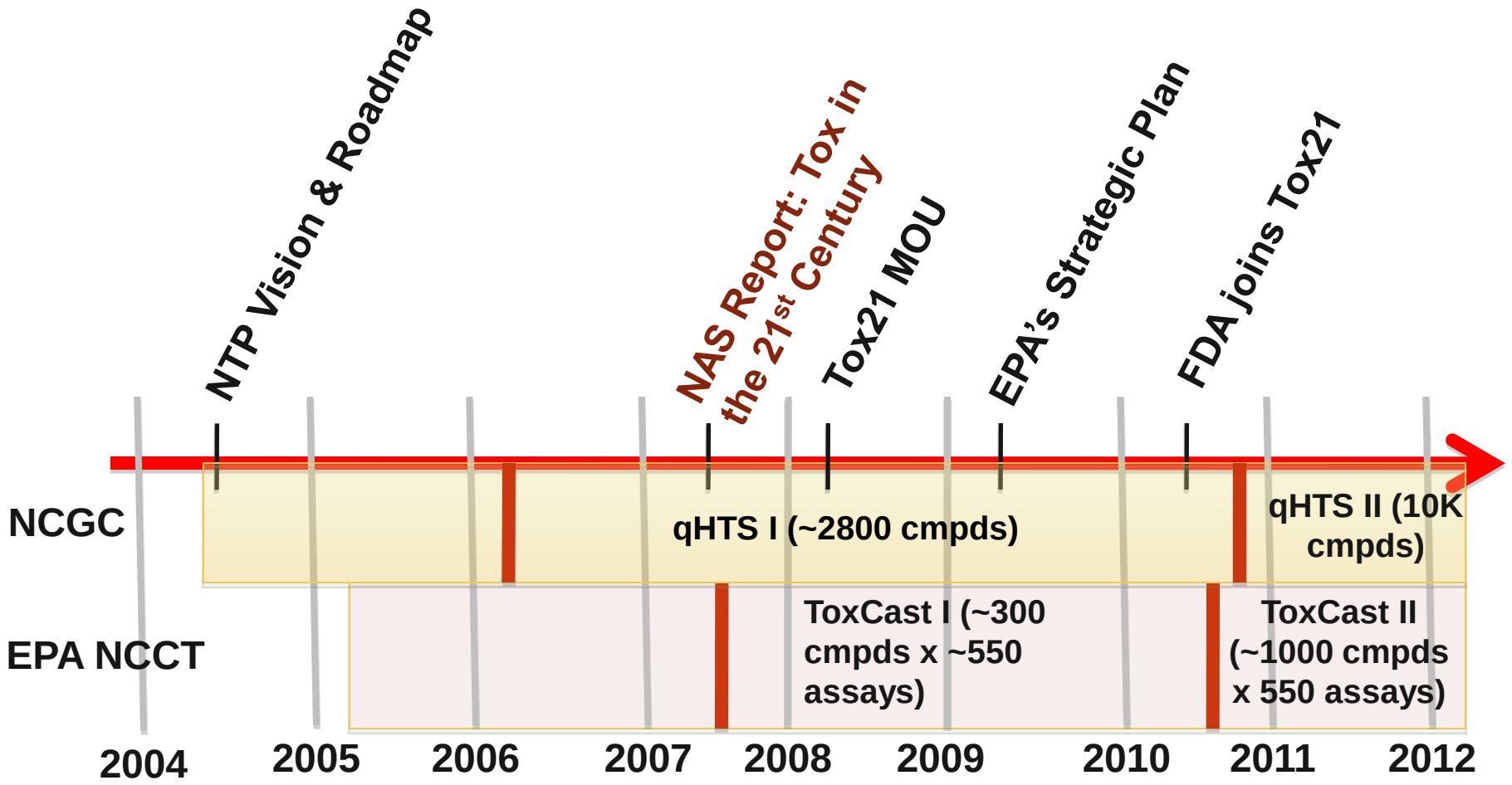
Tox21

DEPARTMENT OF HEALTH & HUMAN SERVICES • USA

Goals of Targeted Testing Work Group

- Evaluate the qualitative and quantitative relationships between *in vitro* HTS assays and predictive models to *in vivo* biological activity and toxicity.
  - *Building bridges between HTS and risk management decisions*
  - *Presently, the only predictive models built on Tox21 data come from ToxCast.*

The Tox21 Screening Timeline



Tox21 - a "Community Resource" Project

Tox21

Agency Points of Contact

Christopher Austin, M.D. (NCGC)
Thomas Colatsky, Ph.D. (FDA)
Robert Kavlock, Ph.D. (EPA)
Raymond Tice, Ph.D. (NTP)

Assays & Pathways Working Group

Co-Chairs
Kevin Gaido, Ph.D. (FDA)
Keith Houck, Ph.D. (EPA)
Kristine Witt, M.S. (NTP)
Menghang Xia, Ph.D. (NCGC)

- Identify toxicity pathways & corresponding assays
- Review nominated assays
- Prioritize assays for qHTS

Chemical Selection Working Group

Co-Chairs
William Leister, Ph.D. (NCGC)
Donna Mendrick, Ph.D. (FDA)
Ann Richard, Ph.D. (EPA)
Cynthia Smith, Ph.D. (NTP)

- Establish a 10K DMSO soluble compound library for qHTS
- Establish QC procedures
- Establish libraries of mixtures and aqueous soluble compounds for qHTS

Informatics Working Group

Co-Chairs
Ruili Huang, Ph.D. (NCGC)
Richard Judson, Ph.D. (EPA)
Jennifer Fostel, Ph.D. (NIEHS)
Weida Tong, Ph.D. (FDA)

- Characterize assay output and evaluate assay performance
- Develop prioritization schemes and prediction models
- Make all data publicly accessible via CEBS, PubChem, ACToR

Targeted Testing Working Group

Co-Chairs
Kevin Crofton, Ph.D. (EPA)
Michael DeVito, Ph.D. (NTP)
David Gerhold, Ph.D. (NCGC)
James Weaver, Ph.D. (FDA)

- Evaluate the relevance of prioritization schemes and prediction models
- Prioritize substances for more complex testing
- Extrapolate *in vitro* conc to *in vivo* dose

Projects ongoing or planned

- Liver Cancer Model
- Reproductive Toxicity model
- Obesity/Diabetes

Liver Targeted Testing Study

- NCCT has developed a statistical model that predicts rodent liver proliferative lesions and rat liver tumors based on the ToxCast Phase I Screening data (Judson et al 2010).
  - Model developed using multivariate analysis based on a subset of 21 ToxCast chemicals with positive rat liver tumor findings.
  - Model was applied to the ToxCast data set

Goals of Liver Targeted Testing Project

- Test for *in vivo* presence of activity seen *in vitro*
  - Sensitivity, specificity
  - Dose-response
- Confirm that previously untested compounds show predicted *in vivo* activity
- See if Reverse Toxicokinetics (RTK) approach gives reasonable estimate of dose for *in vitro* to *in vivo* extrapolation

Hypothesis

- **Hypothesis 1:**
  - **—*In vitro* activation of PPARγ along with one or more of the following pathways CCL2 / AR / OS/PPARα is highly predictive of the corresponding activation *in vivo*, at some dose level**
- **Hypothesis 2:**
  - **—Only at doses for which at least 2 of these pathways or processes are activated will we see liver tumors in the 2-year rat study.**

Assays Associated with Rat Liver Tumors

Assay Name	Partner or Contractor	Assay type	Cell type	Pathway
ATA_PPARγ_TRANS	Attagene	Transactivation Reporter gene	HepG2	PPARγ
ATA_PPARα_TRANS	Attagene	Transactivation Reporter gene	HepG2	PPARα
NCGC_AR-Antagonist	NCGC	Reporter gene	HEK293H	Androgen Receptor
CLZD_HMGCS2_48	CellzDirect/ Invitrogen	RNAse Protection Assay	Primary Human Hepatocytes	PPARα
BSK_SM3C_MCP1_up	BioSeek	Elisa	HUVEC, Primary Human vascular smooth muscle	Cytokine
CLM_Oxidative Stress_24hr	Cellumin	Flourescent	HepG2	H2AFX

ToxCast Markers

In vivo Endpoints	In vitro Assay	Justification
Affymetrix GeneChip Rat RAE230 2.0	PPARγ / CCL2 / AR / OS/PPARα	The in vitro assays are thought to be markers for pathway activation and the arrays are the most efficient method to assay all of these pathways
IHC for phosphor-gamma-H2AX	Cellumin Oxidative stress assay	The in vitro assay is an imaging assay which measures the amount of phosphorylated gamma H2AX. Thus a corresponding assay would be to measure phosphorylated H2AX protein. This method has been used in a variety of studies (ref)
Gene Tox Comet Assay on liver tissue (traditional and oxidative damage specific comet assay)	Cellumin Oxidative stress assay	Since the cellumin oxidative stress assay is really a measure of DNA repair, the design team thought it would be of value to reassess the genetic toxicity of these chemicals with newer methods.
RT-PCR for HMG-CoA synthase (HMGCS2)	CellzDirect hepatocyte assay PPARα	The PPARα assay was the induction of HMGCS2 in human hepatocytes after 48 hour exposure to the test chemical. This is a direct correlate for that assay in vivo.
RT-PCR for hepatic MCAD and PEPCK mRNA	PPARγ - transactivation assay	These two genes are directly regulated by PPAR and induction of these genes is a close correspondence to the in vitro assay for PPARγ activation
RT-PCR for CCL2	CCL2 mRNA induction	This is a direct correspondence to the in vitro assay.
Clinical Chemistry on blood for glucose, cholesterol and triglycerides, HDL, LDL, ALT, SDH	PPARγ - transactivation assay	PPARγ agonists alter glucose and lipid concentrations in rodent serum in short-term assays.

Additional Assays

- Serum Markers
  - Cholesterol, triglycerides, HDL, LDL
  - T4 and T3
- miRNA Arrays –
  - Carole Yauk at Health Canada

Tiered Study Design

- **Tier 1 Pilot Study**
  - At the highest dose tested in a bioassay, do we see *in vivo* signatures consistent with *in vitro* results. Evaluate numerous chemicals at one dose and time point.
- **Tier 2 *In vivo* time course and dose response studies**.
  - Dependent upon results of tier 1
  - More limited number of chemicals
  - Tissue dosimetry added as an endpoint Tissue dosimetry
- **Tier 3 Chemicals without 2-year bioassays**
  - Dependent upon results of Tier 2
  - Prior to start of Tier 3, study design presented to PRC

NOTE: Possible that study does not progress beyond Tier 1 based on results

- All negative results from Tier 1 (no change in response to chemical exposures)

Tier 1 Pilot Screening Project

- Iterative process starting with 12 chemicals and maybe as high as 40.
- Single daily exposure by oral gavage to highest dose used in the 2-yr bioassay for 4 days. Sacrifice 4 hrs after last dose.
- Use strain and gender of rat that has positive liver tumor finding
- Measure
  - Body and liver weights at start and T-sac
  - Blood collected for
    - Serum chemistry for liver toxicity markers
    - Serum Thyroid hormones
  - Liver collected for
    - Immunohistochemistry
    - ToxCast markers

Initial Chemicals

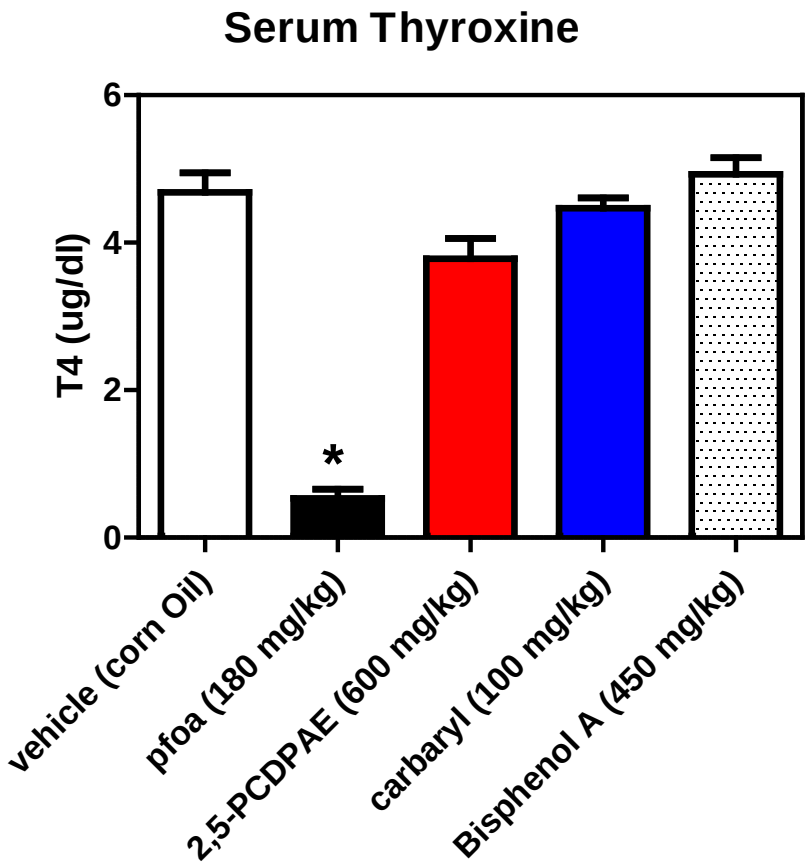
	Tumor Results		ToxCast Results				
CHEMICAL	Rat Liver Tumors (% at high dose)	High Dose (mg/kg)**	PPARγ	Ox Stress	AR	HMGCS2	CCL2
Acetoclor	+ (9)	250	+	+	+	+	-
Dimethenamid	+ (12)	109	+	+	+	-	-
Lactofen	+ (9)	79	+	-	+	+	-
PFOA	+ (13)	300	+	-	-	+	-
Simazine	+ (5)	63	+	-	-	-	+
2,5-Pyridinedicarboxylic acid di-n-propyl ester (2,5-PCADPE)	+ (17)	1000	+	-	-	-	+
PFOS	+ (12)	100	+	-	-	-	-
Carbaryl	+ (2.9)	100	+	+	-	-	-
Triclosan	-	1000	-	-	-	-	-
fludioxonil	-	121	+	+	-	-	-
Bisphenol A	-	1000	+	-	+	-	-
Flusilazole	-	13	+	-	+	-	-

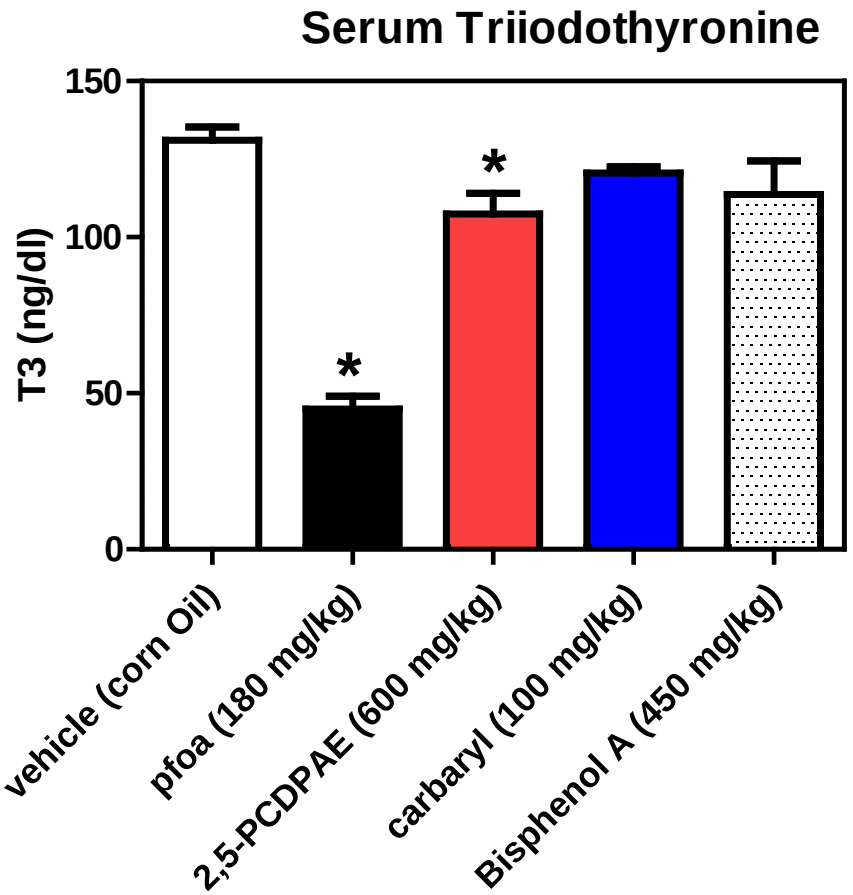
** - The exposures in the bioassay were dietary and exposures are estimated

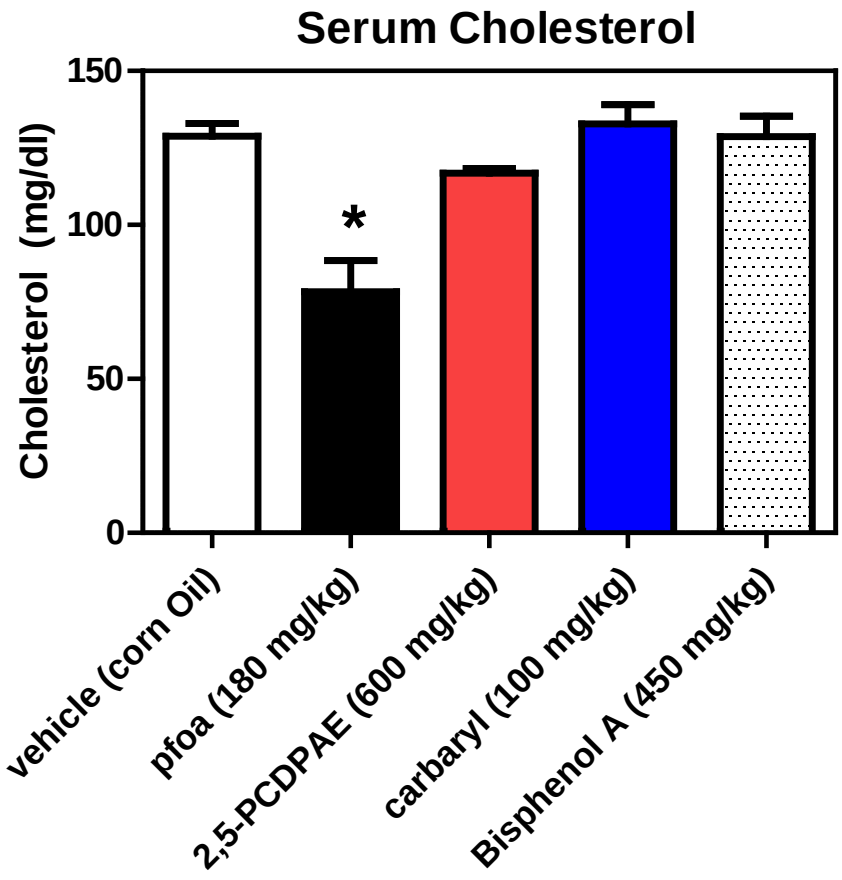
Tox21

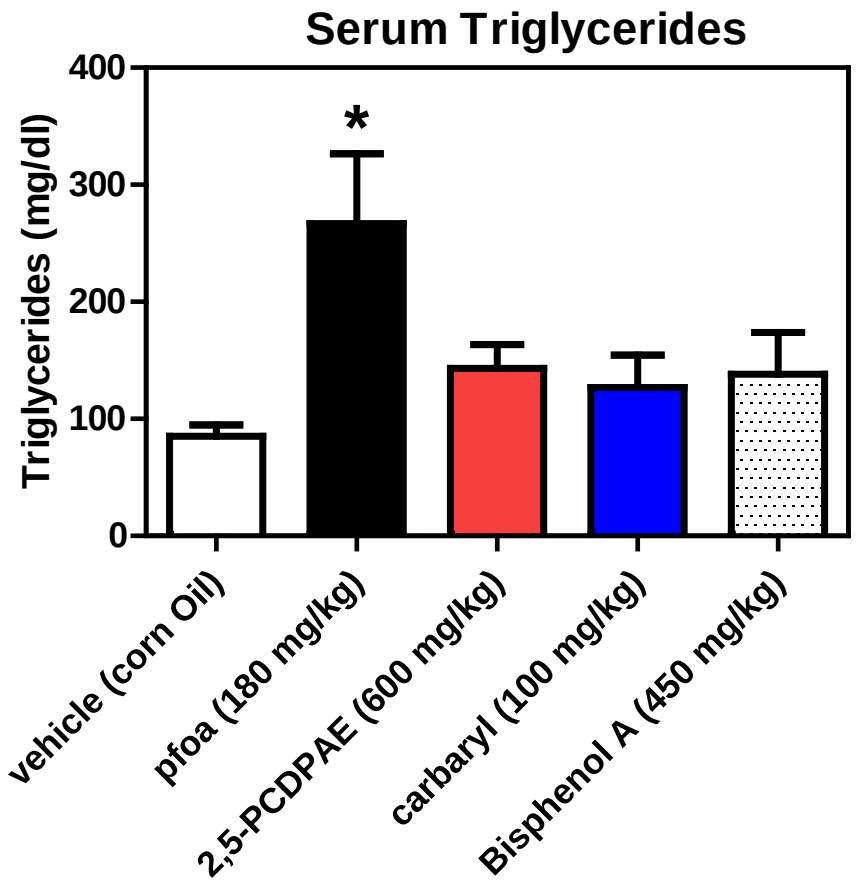
Stage 1 Chemicals

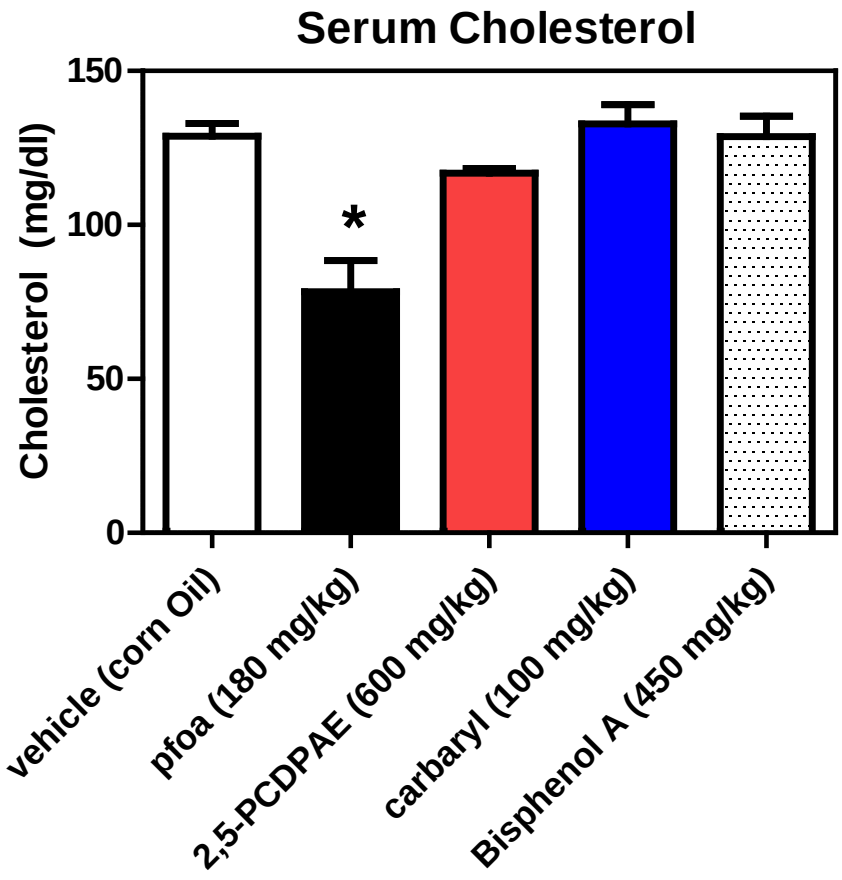
Chemicals	Rat Liver Tumors (% at high dose)	High Dose (mg/kg)	PPARγ	PPARά	Ox Stress	AR	HMGCS2	CCL2
PFOA	+ (13%)	300	+	+	-	-	+	-
2,5-PCADPE	+ (17%)	1000	+	-	-	-	-	+
Carbaryl	+ (3%)	100	+	-	+	-	-	-
Bisphenol A	-	1000	+	-	-	+	-	-

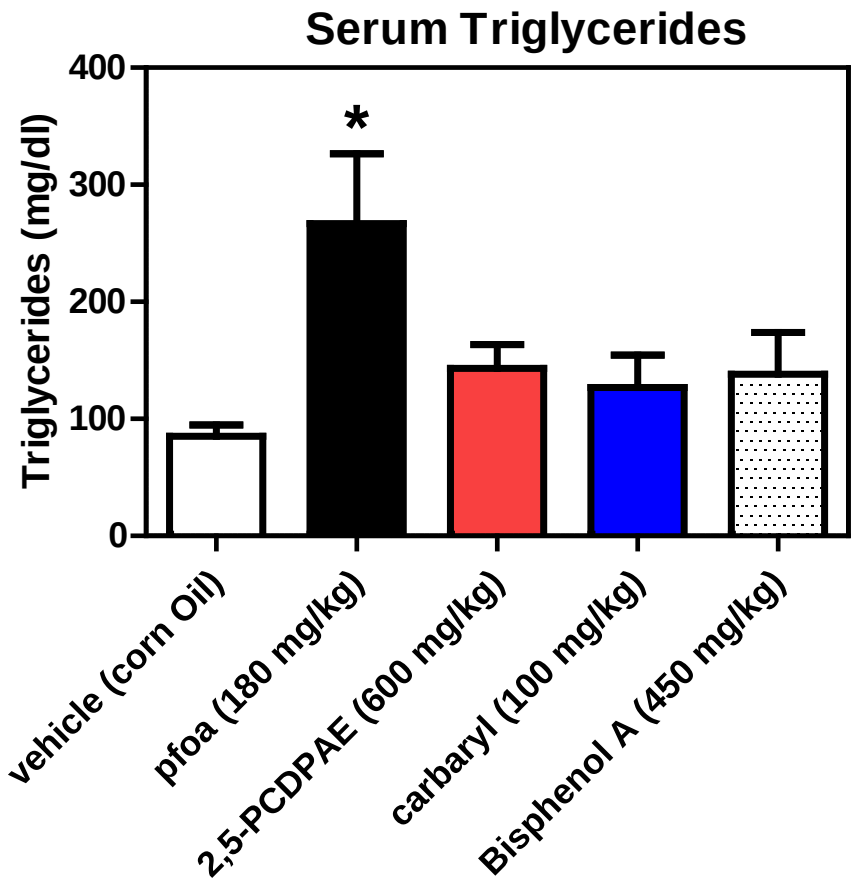

Serum Thyroxine
T4 (ug/dl)
6
4
2
0
*
vehicle (corn Oil)
pfoa (180 mg/kg)
2,5-PCDPAE (600 mg/kg)
carbaryl (100 mg/kg)
Bisphenol A (450 mg/kg)

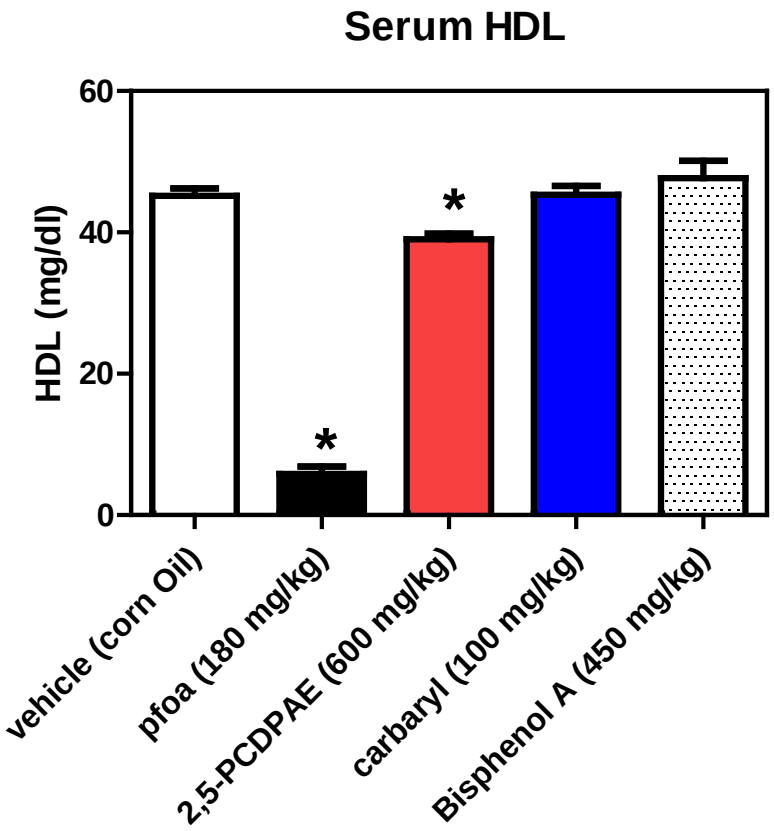

Serum Triiodothyronine
T3 (ng/dl)
150
100
50
0
*
*
vehicle (corn Oil)
pfoa (180 mg/kg)
2,5-PCDPAE (600 mg/kg)
carbaryl (100 mg/kg)
Bisphenol A (450 mg/kg)

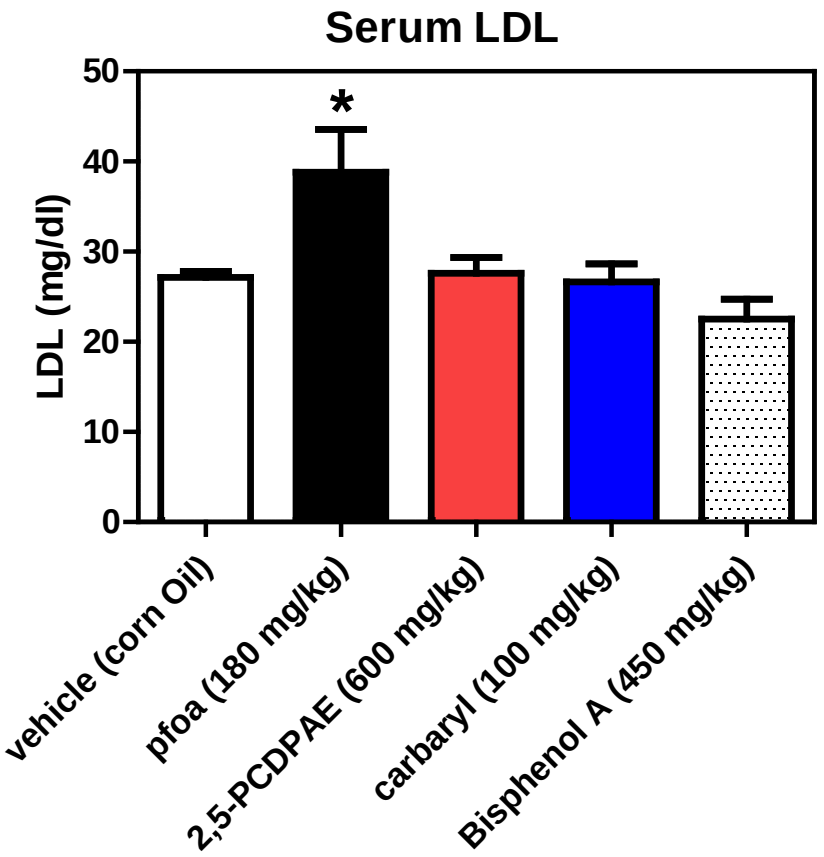

Serum Cholesterol
Cholesterol (mg/dl)
150
100
50
0
*
vehicle (corn Oil)
pfoa (180 mg/kg)
2,5-PCDPAE (600 mg/kg)
carbaryl (100 mg/kg)
Bisphenol A (450 mg/kg)


Serum Triglycerides
Triglycerides (mg/dl)
400
300
200
100
0
*
vehicle (corn Oil)
pfoa (180 mg/kg)
2,5-PCDPAE (600 mg/kg)
carbaryl (100 mg/kg)
Bisphenol A (450 mg/kg)

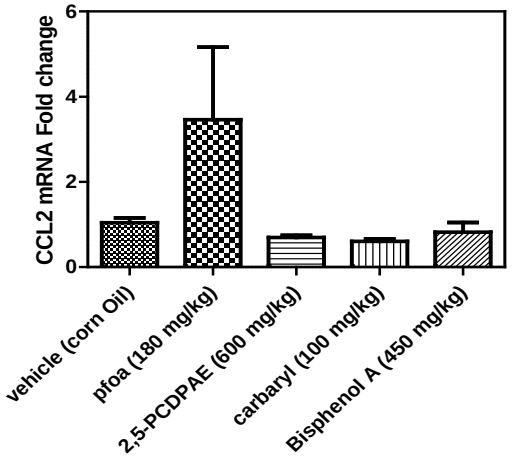

Serum Cholesterol
Cholesterol (mg/dl)
150
100
50
0
*
vehicle (corn Oil)
pfoa (180 mg/kg)
2,5-PCDPAE (600 mg/kg)
carbaryl (100 mg/kg)
Bisphenol A (450 mg/kg)

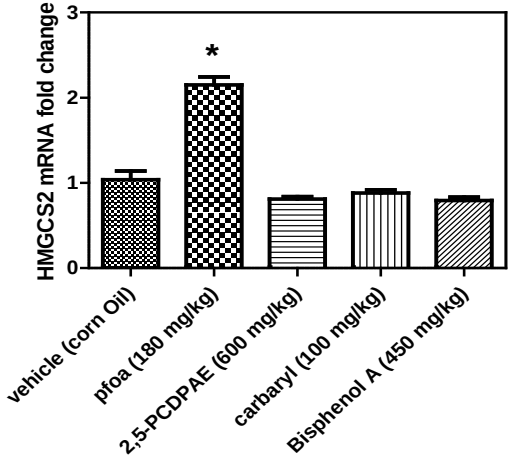

Serum Triglycerides
Triglycerides (mg/dl)
400
300
200
100
0
*
vehicle (corn Oil)
pfoa (180 mg/kg)
2,5-PCDPAE (600 mg/kg)
carbaryl (100 mg/kg)
Bisphenol A (450 mg/kg)

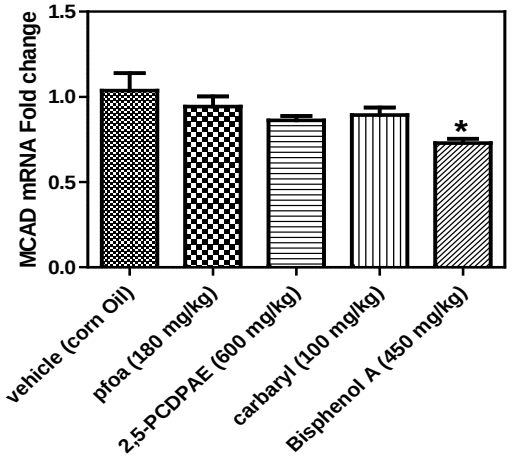

Serum HDL
HDL (mg/dl)
60
40
20
0
*
*
vehicle (corn Oil)
pfoa (180 mg/kg)
2,5-PCDPAE (600 mg/kg)
carbaryl (100 mg/kg)
Bisphenol A (450 mg/kg)

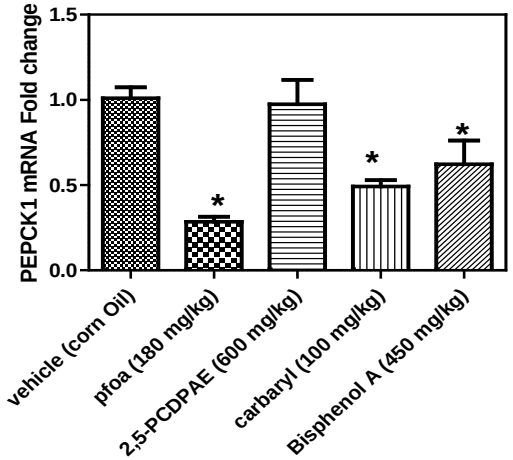

Serum LDL
LDL (mg/dl)
50
40
30
20
10
0
*
vehicle (corn Oil)
pfoa (180 mg/kg)
2,5-PCDPAE (600 mg/kg)
carbaryl (100 mg/kg)
Bisphenol A (450 mg/kg)

mRNA changes using PCR assays

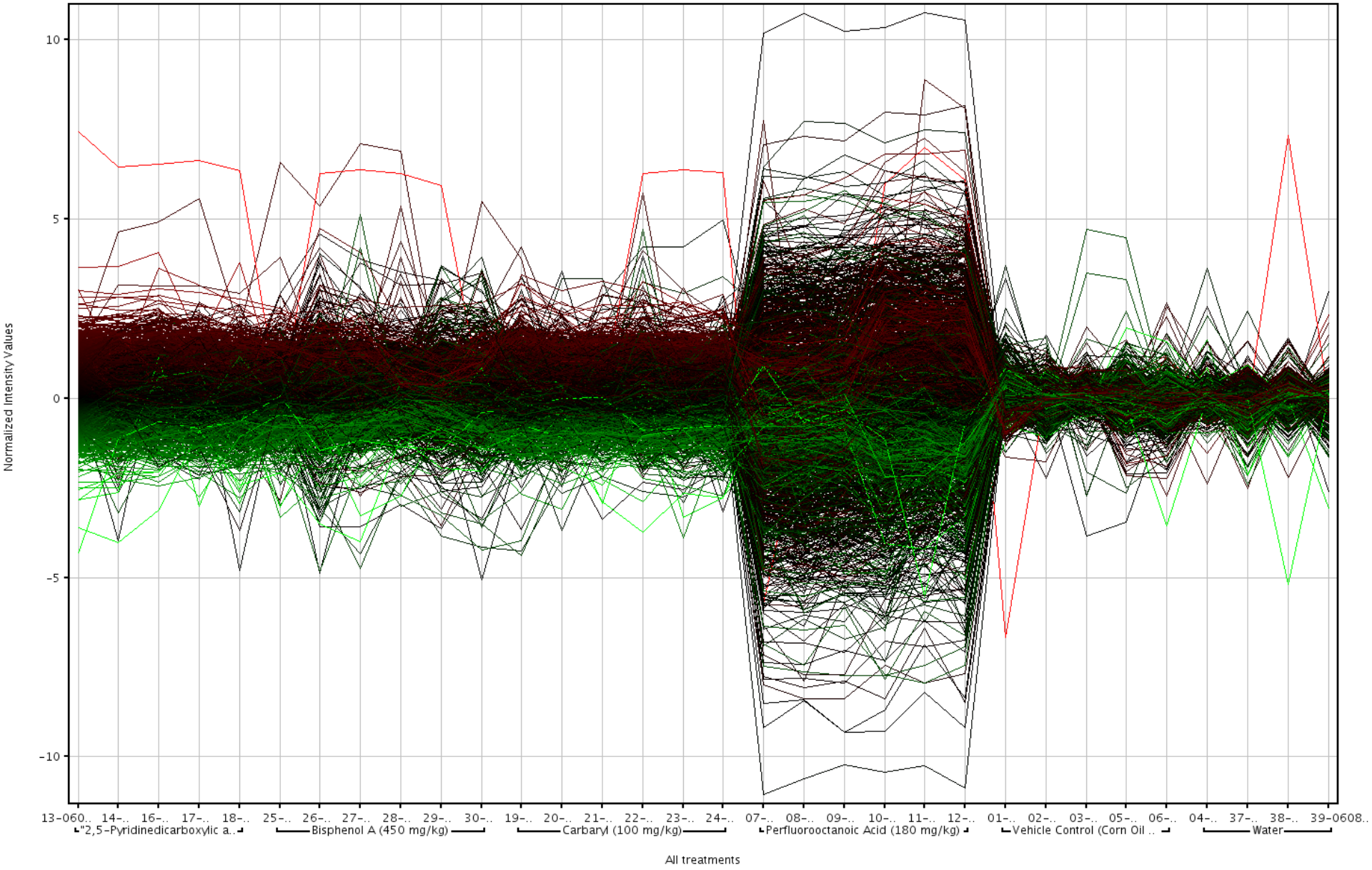




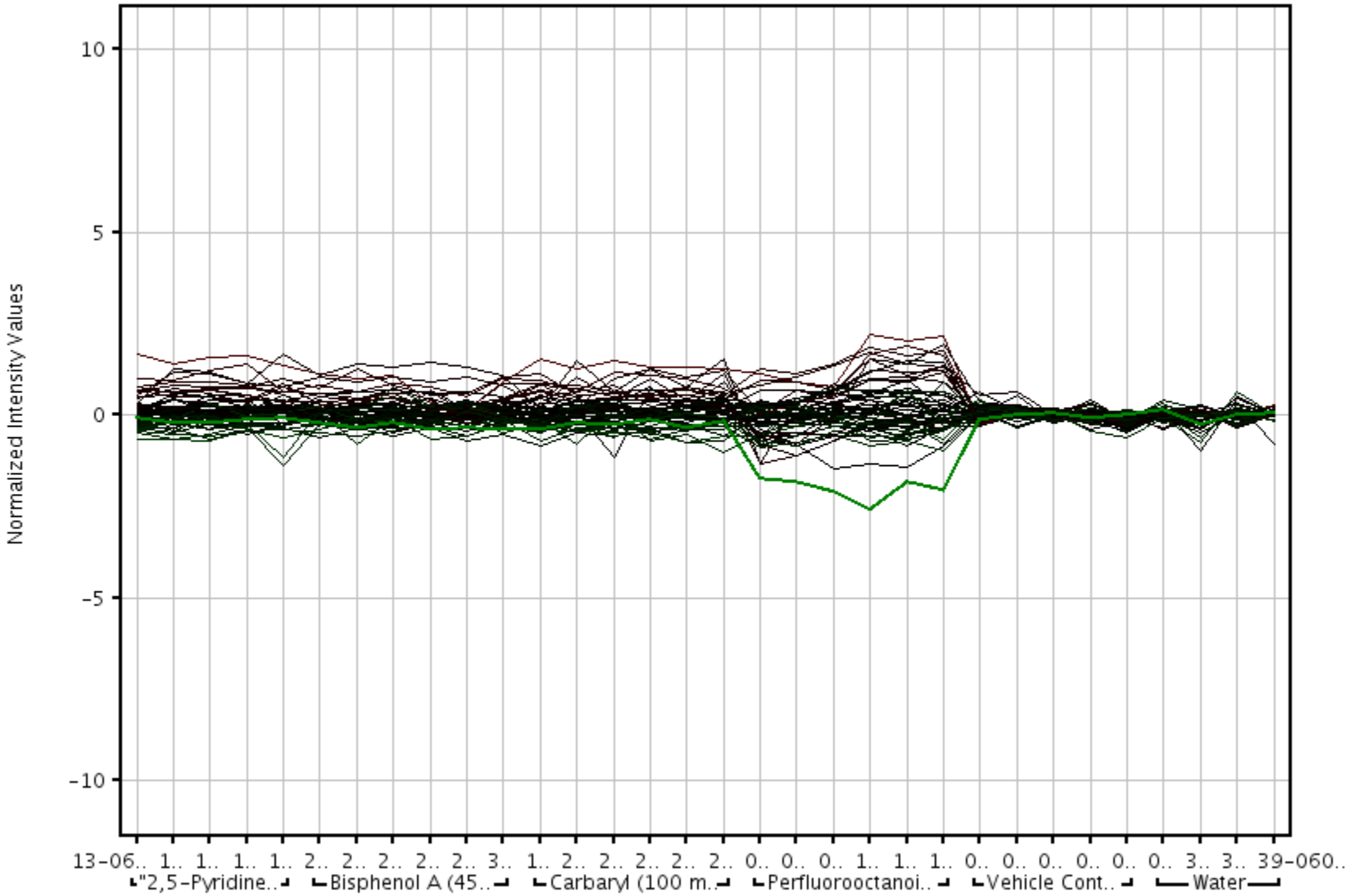




Total transcriptional changes

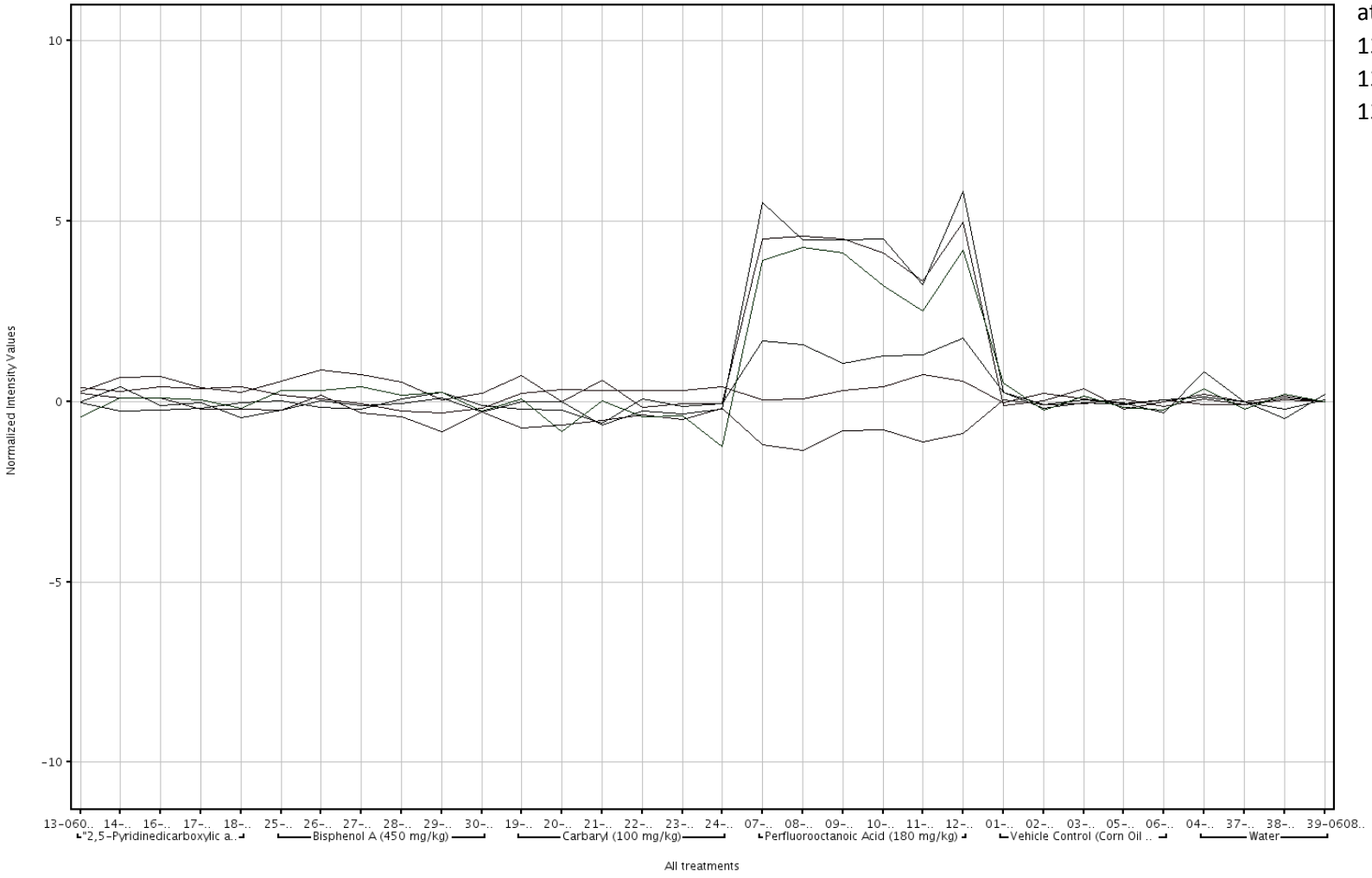


Androgen receptor signaling

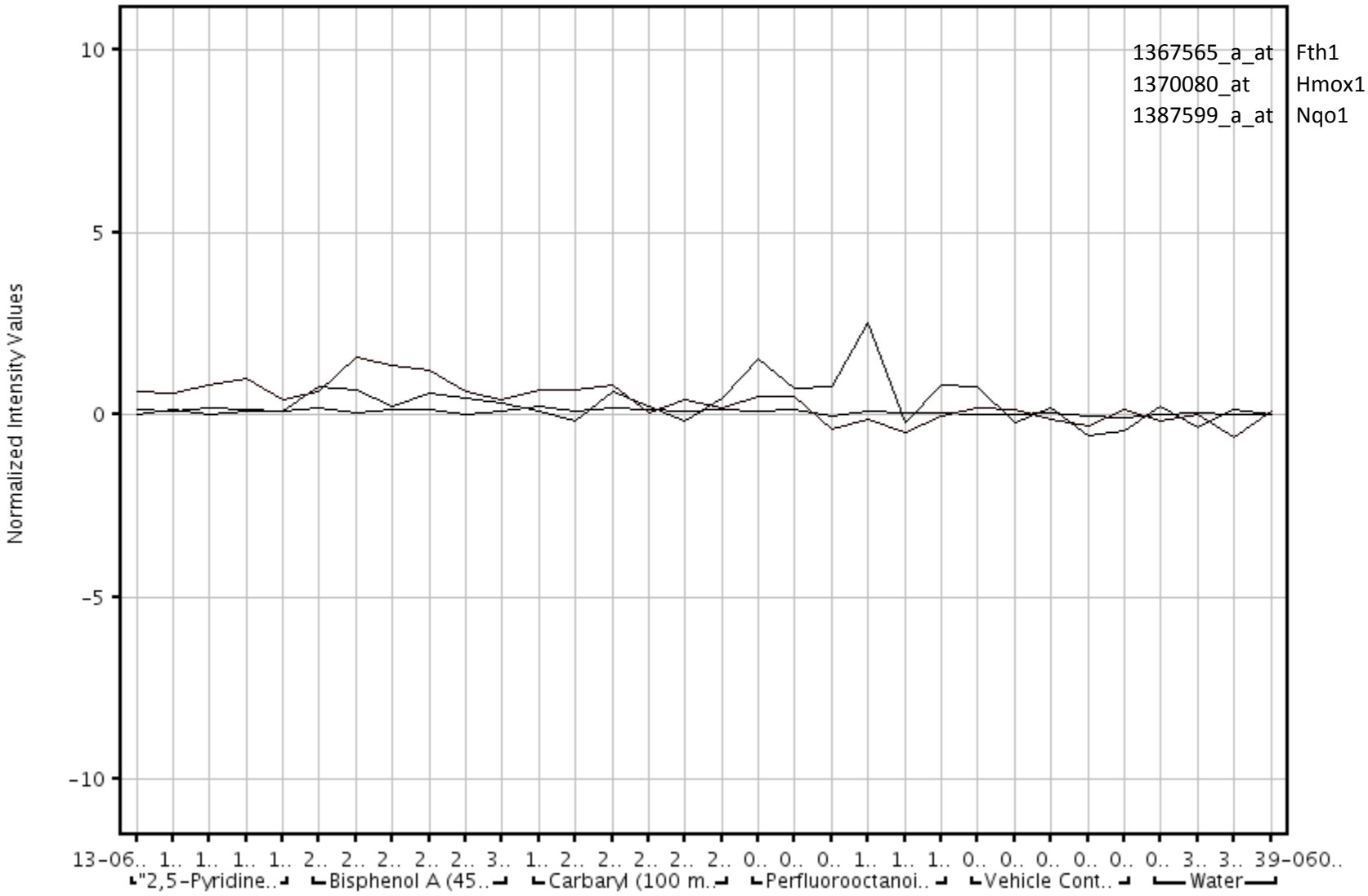


PPAR Gamma genes

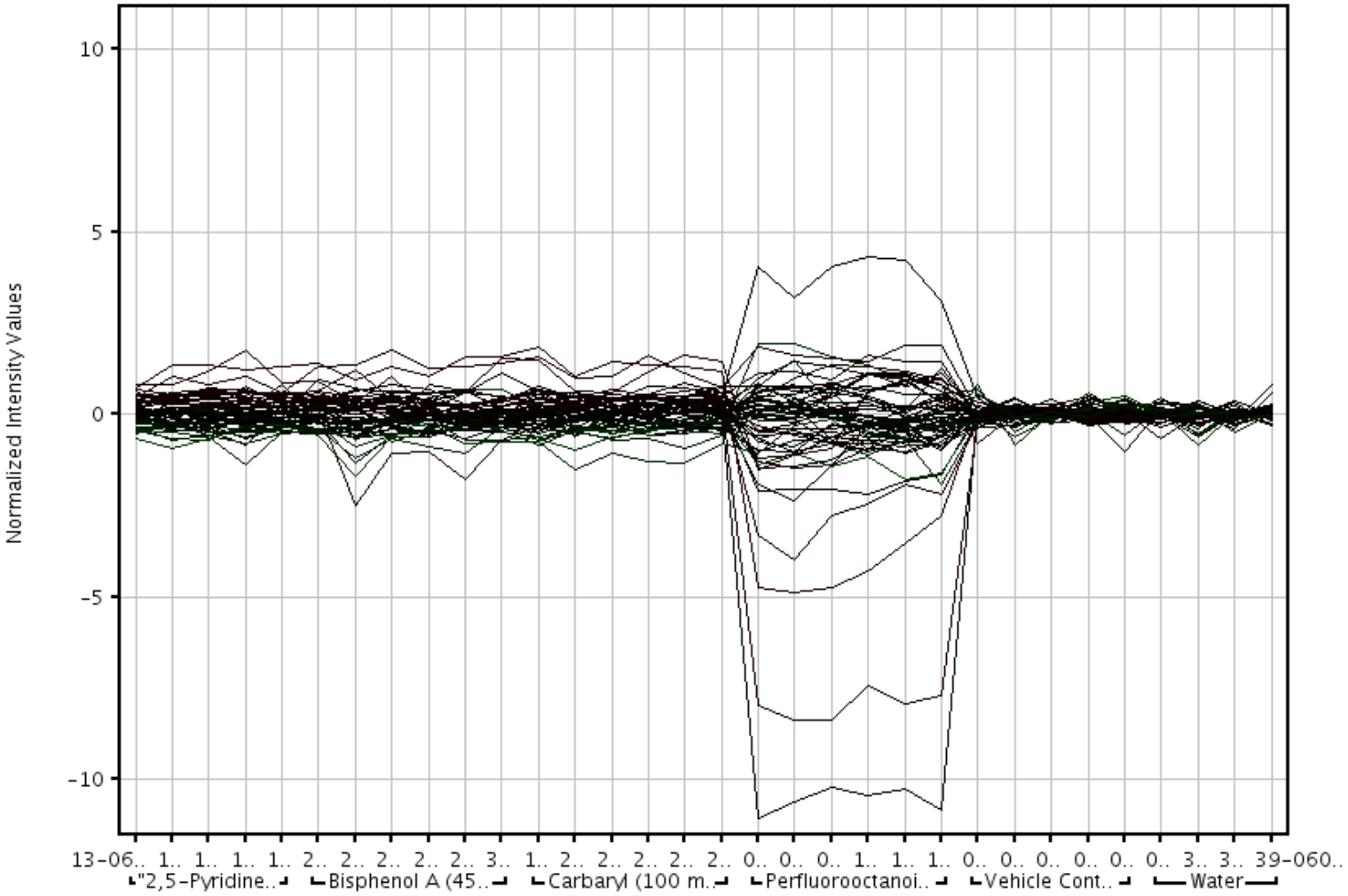
Probe Set ID	Gene Symbol
1367689_a_at	Cd36
1367702_at	Acadm
1368271_a_at	Fabp4
1368669_at	Ucp2
1372264_at	Pck1
1386901_at	Cd36



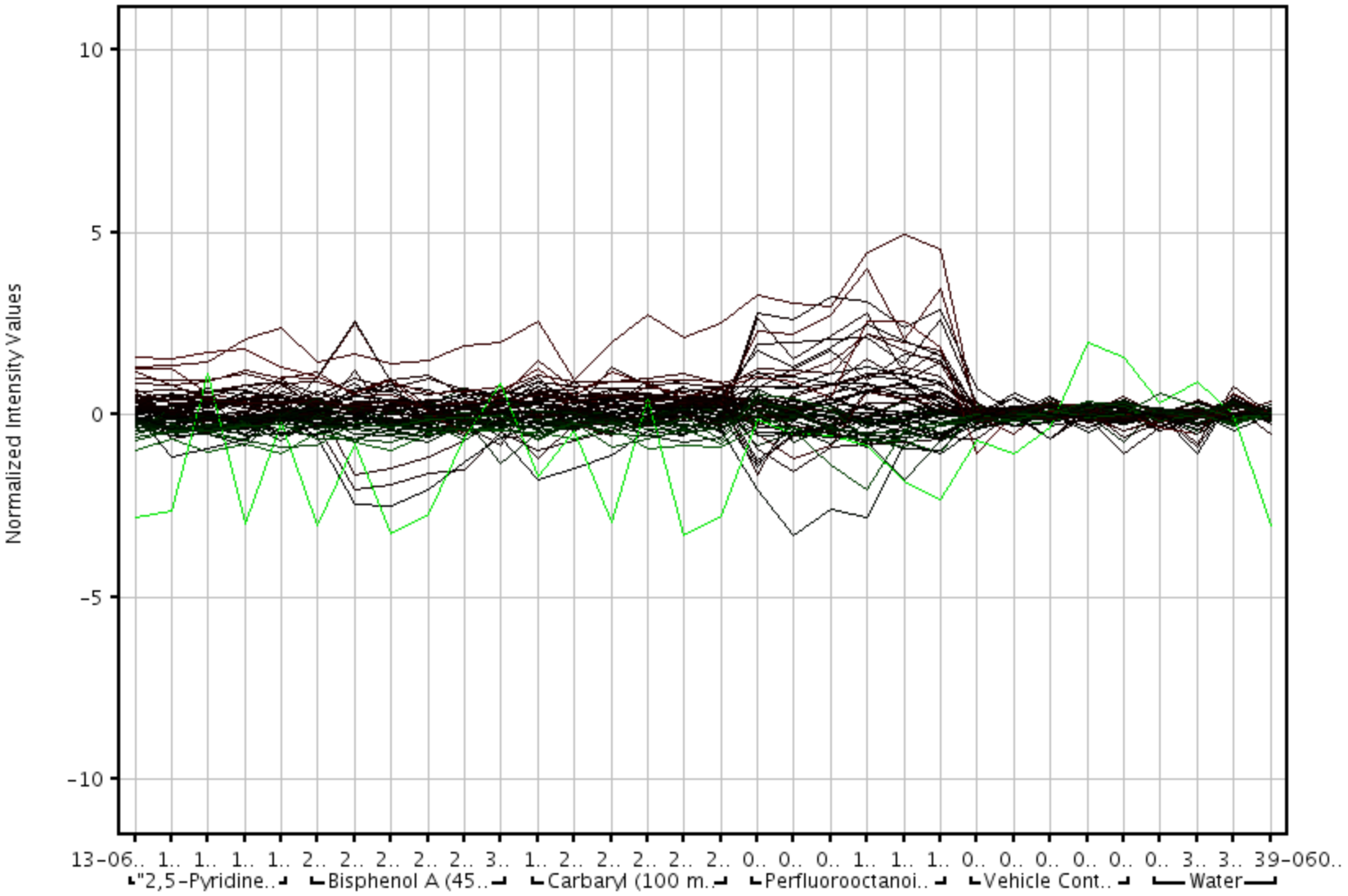
Nrf2 responsive genes



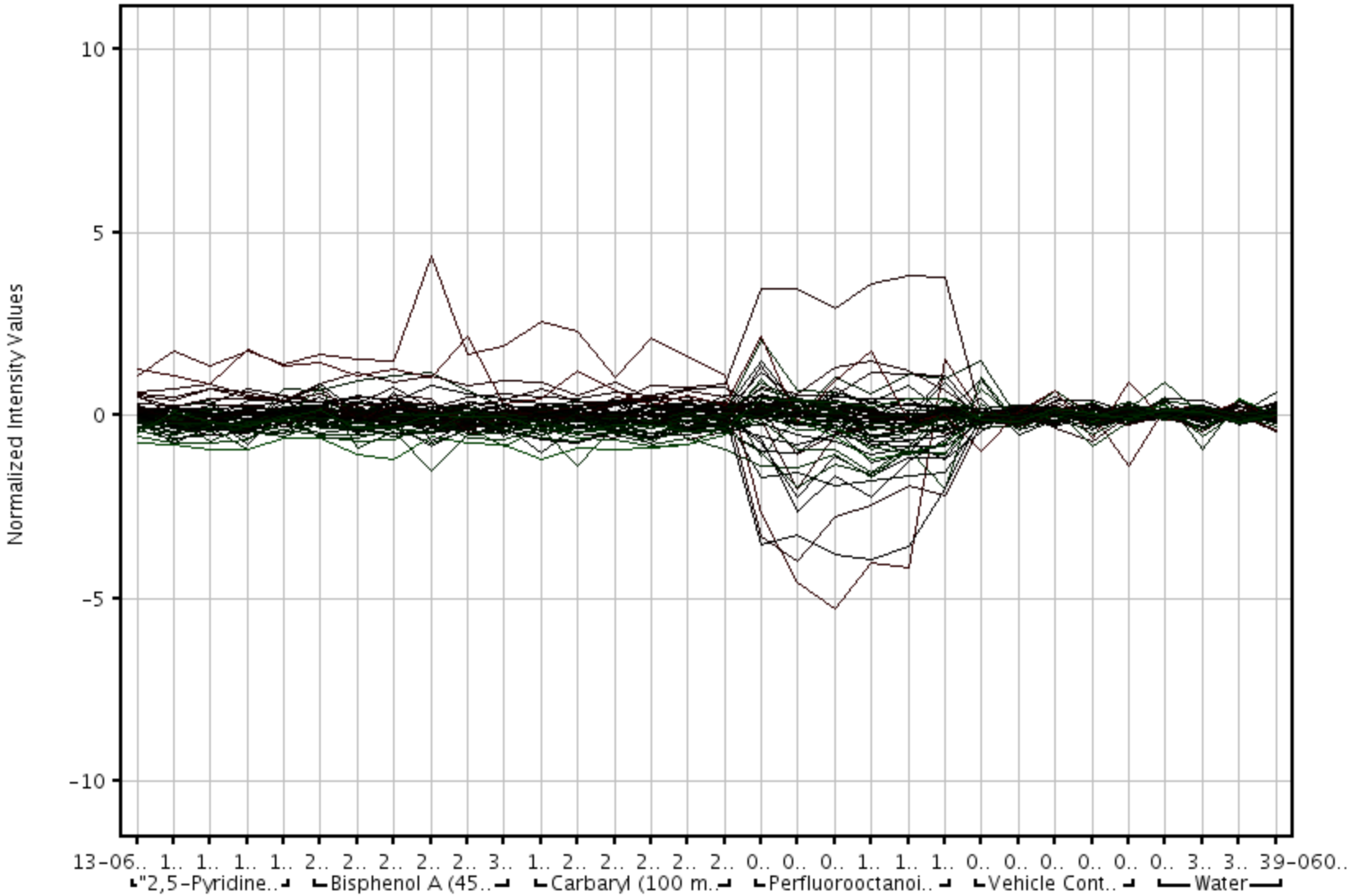
Response to Oxidative stress



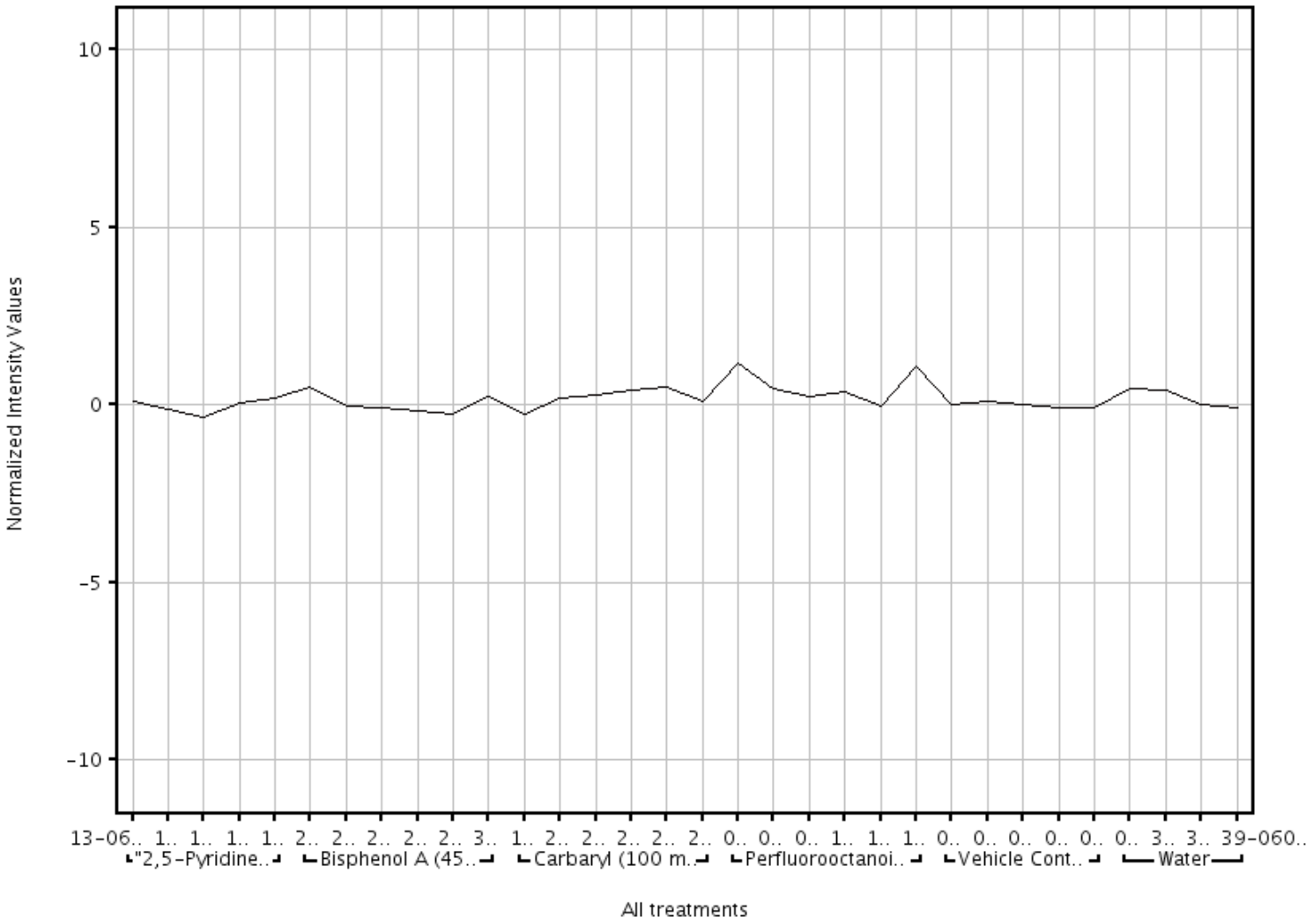
Response to DNA Damage



Inflammatory response



Ccl2


Normalized Intensity Values
10
5
0
-5
-10
13-06.. 1.. 1.. 1.. 1.. 2.. 2.. 2.. 2.. 2.. 3.. 1.. 2.. 2.. 2.. 2.. 2.. 0.. 0.. 0.. 1.. 1.. 1.. 0.. 0.. 0.. 0.. 0.. 0.. 3.. 3.. 39-060..
"2,5-Pyridine..
Bisphenol A (45..
Carbaryl (100 m..
Perfluorooctanoi..
Vehicle Cont..
Water
All treatments

miRNA

- No effect for 2, 5-Pyridinedicarboxylic acid or carbaryl vs Control
- BPA - 1.26 fold increase in miR-193
- PFOA – 26 miRNA changed by 1.3 – 2.9 fold.

Assays still ongoing

- Gene Arrays
- IHC for H2AX
- Elisa for serum CCL2

Initial Chemicals Tested in Male SD rats

	Tumor Results			ToxCast Results				
CHEMICAL	Rat Liver Tumors (% at high dose)	High Dose (mg/kg)**	PPARγ	PPARa	Ox Stress	AR	HMGCS2	CCL2
PFOA	+ (13)	300	+ +	+ +	-	-	+ +	- +
2,5-Pyridinedicarboxylic acid di-n-propyl ester (2,5-PCADPE)	+ (17)	1000	+ -	- -	-	-	- -	+ ?
Carbaryl	+ (2.9)	100	+ -	- -	+ -	-	- -	- -
Bisphenol A	-	1000	+	- -	-	+	- -	- -

Stage 2 Chemicals

Chemicals	Rat Liver Tumors (% at high dose)	High Dose (mg/kg)	PPARγ	PPARά	Ox Stress	AR	HMGCS2	CCL2
Acetoclor	+ (9%)	250	+	-	+	+	+	-
Simazine	+ (12%)	109	+	+	-	-	+	-
Triclosan	-	1000	-	-	-	-	-	-
Flusilazole	-	13	+	-	-	+	-	-
Nn-DMPT	Not run in ToxCast; NTP chemical of interest.							

In life is done, samples are being processed

Liver Targeted Testing Team

- NTP
  - Mike DeVito
  - Scott Auerbach
  - Alex Merrick
  - Kristine Witt
  - Dave Marlarkey
- EPA
  - Richard Judson
  - Imran Shah
  - Chris Corton
- NCGC
  - Dave Gerhold

ReproductiveToxicity Model Assessment

- Had initial meeting
- Goal is to have study design presented to NTP Project Review Committee by Feb 2012.

Reproductive Toxicity Team

- NTP
  - Mike DeVito
  - Paul Foster
  - Chad Blystone
  - Cynthia Rider
  - Barry McIntyre
- EPA
  - Matt Martin
  - Richard Judson
  - Thomas Knudsen
  - David Reif
  - David Dix
  - Kevin Crofton
- NCGC

  Dave Gerhold

NTP Workshop:

Role of Environmental Chemicals in the Development of Diabetes and Obesity

January 11-13, 2011

Michael Gallo, Workshop Chair
Dept. of Environmental & Occupational Health, University of Medicine & Dentistry of New Jersey

Kristina Thayer, Director NTP Office of Health Assessment and Translation

http://cerhr.niehs.nih.gov/evals/diabetesobesity/