

Dose Response and Thresholds in Endocrine Toxicity

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Research Toxicology

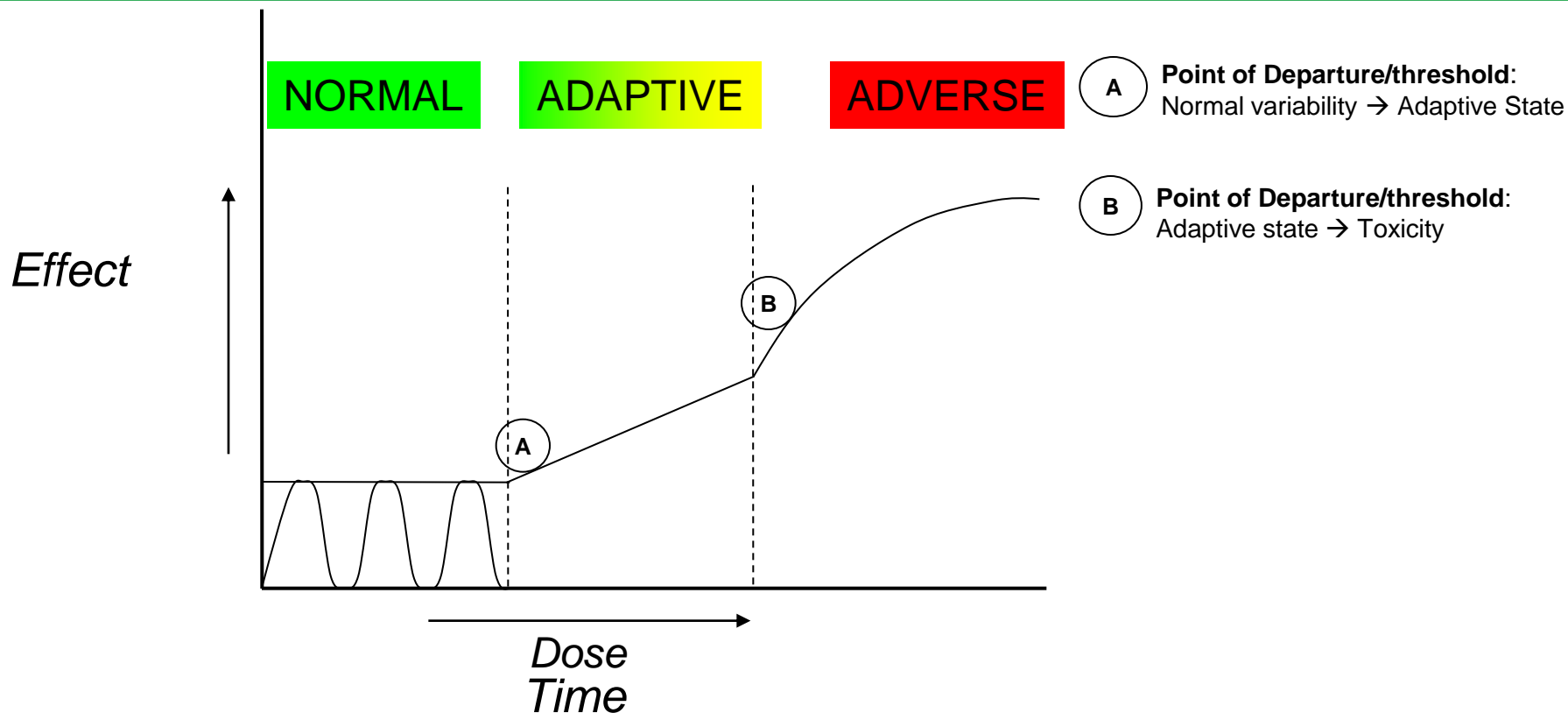


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Outline

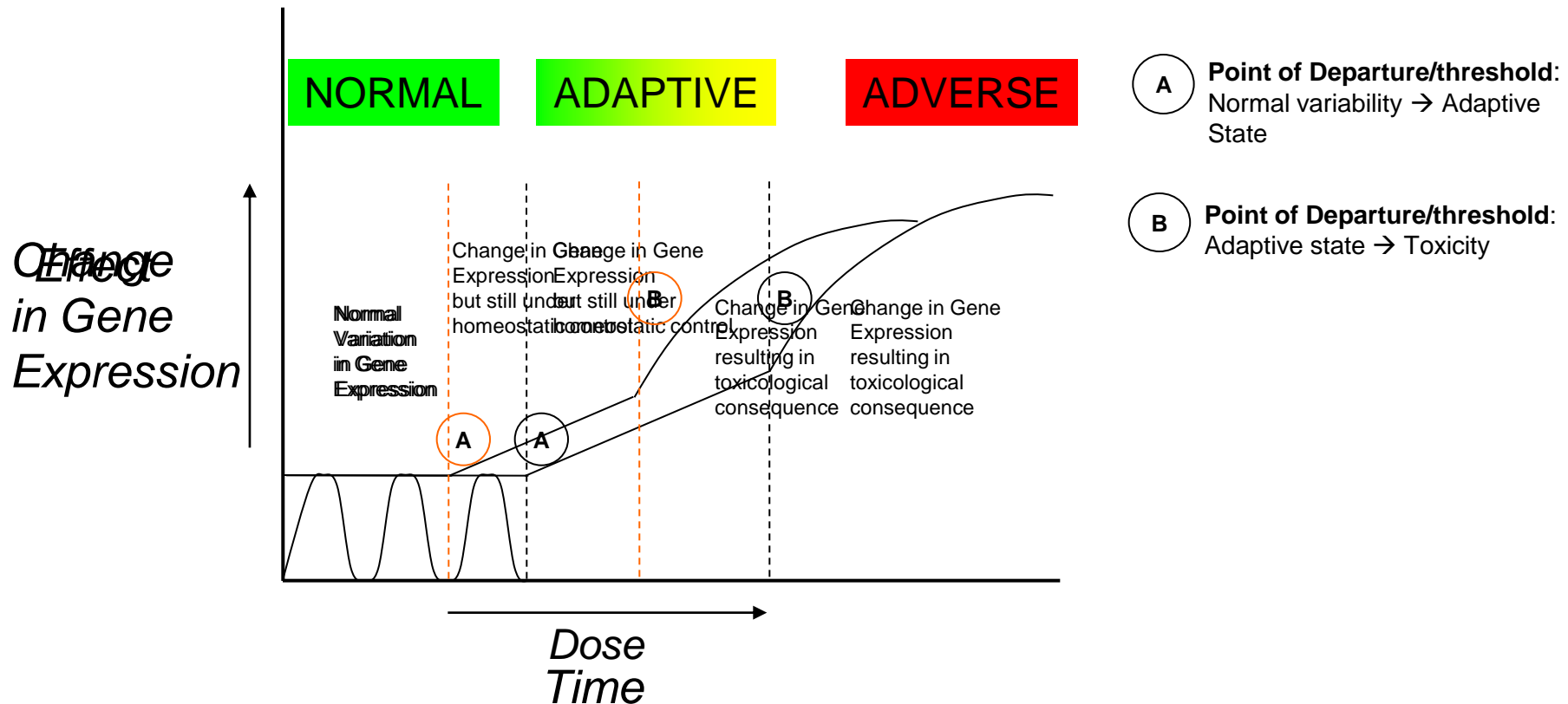
- Introduction
- Cefic EMSG46 research project
- Flutamide data
- DNB data
- Conclusions
- Perspectives

The Challenges to Toxicity Testing (1)



➤ Do such points of departure (POD)/thresholds exist for Endocrine Disrupters?

The Challenges to Toxicity Testing (2)

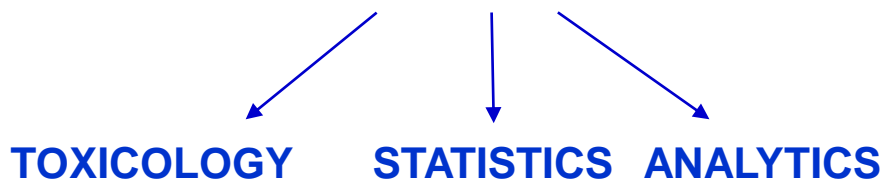


- Do such points of departure (POD)/thresholds exist for Endocrine Disrupters
- What impact will toxicogenomic data have on our PoDs/thresholds?

CEFIC EMSG46: designed to address these challenges using phenotypic anchoring and focussing on the rat testis

EMSG 46: CHARACTERIZATION OF TESTICULAR TOXICITY USING TRADITIONAL AND OMIC TOOLS

3 year project (start: Dec 2007)
4 Partners (Industry and Academia)



Steps necessary to meet the objective

1. Dose-response progression from normal variability → adaptive change → adverse toxicological effects
2. Time dependent progression from normal variability → adaptive change → adverse toxicological effects

Compounds:

Flutamide (FM)	a potent antiandrogen
1,3 dinitrobenzene (DNB)	a direct acting testicular toxicant
Ethinyl estradiol (EE)	a potent estrogen

Dose levels:

Dose levels were selected based on consideration of all available literature.

General Study Design: Dose Response



Oral Gavage
Up to 28 days
Control + 4 dose levels
10 Adult male Wistar rats

Body and
organ
weights

Plasma
testosterone

Testicular
histopathology

Parent/Metabolite
concentrations

Testicular
transcriptome

Standard toxicity parameters

Refinements

Flutamide: Dose Response Investigations



Oral Gavage
28 days

Control + 0.2, 1, 6, 30 mg/kg/d
10 Adult male Wistar rats

Body and
organ
weights

≥1mg/kg/d
SAT wt ↓
ADAPTIVE

Plasma
testosterone

≥6mg/kg/d
Increased
ADVERSE?

Testicular
histopathology

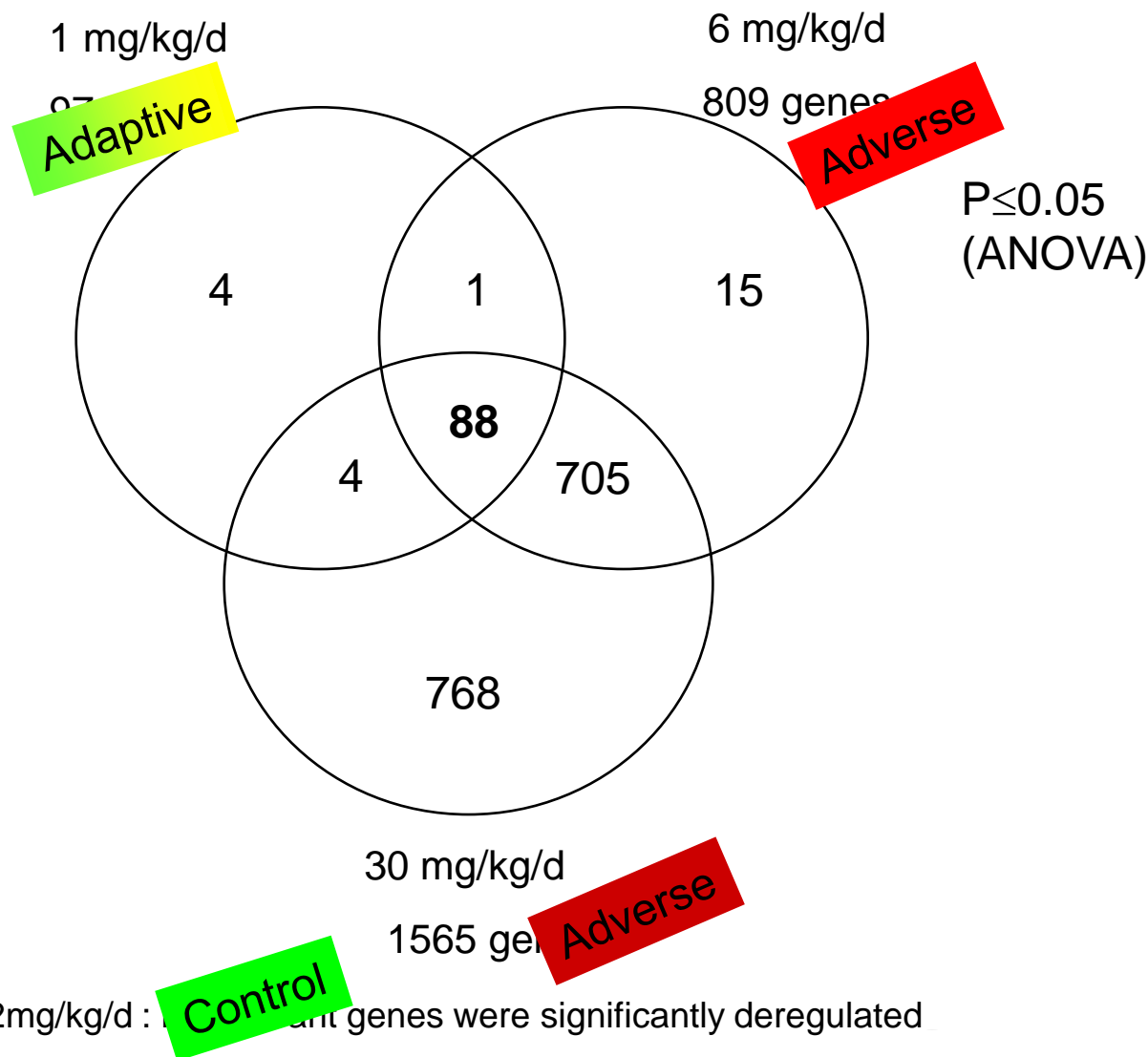
≥6mg/kg/d
Leydig cell
hyperplasia
ADVERSE

Parent/Metabolite
concentrations

≥1mg/kg/d
HydroxyFM
detected in
testes
ADAPTIVE?

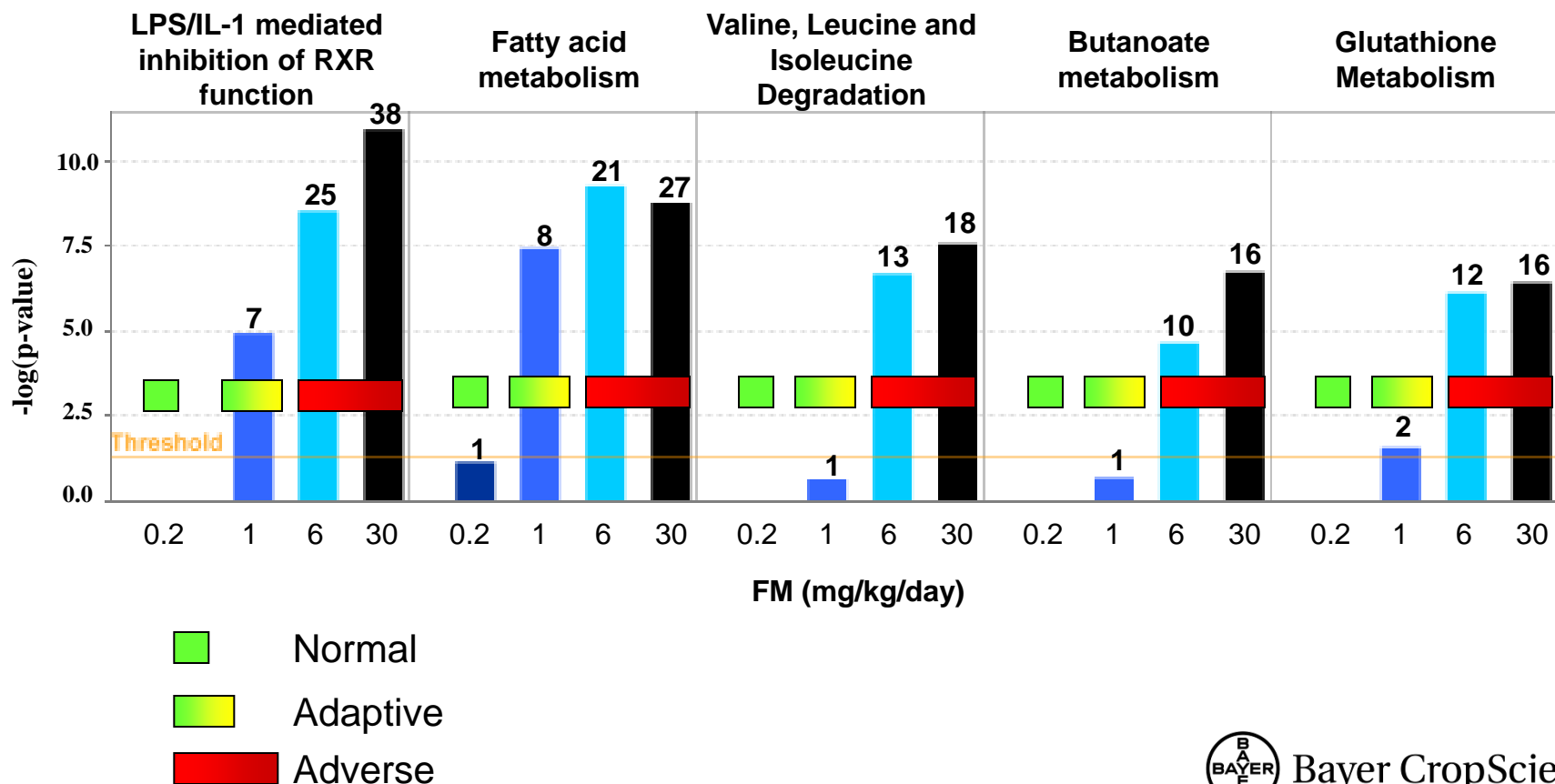
Testicular
transcriptome

Flutamide: Transcriptomics - Microarray Data

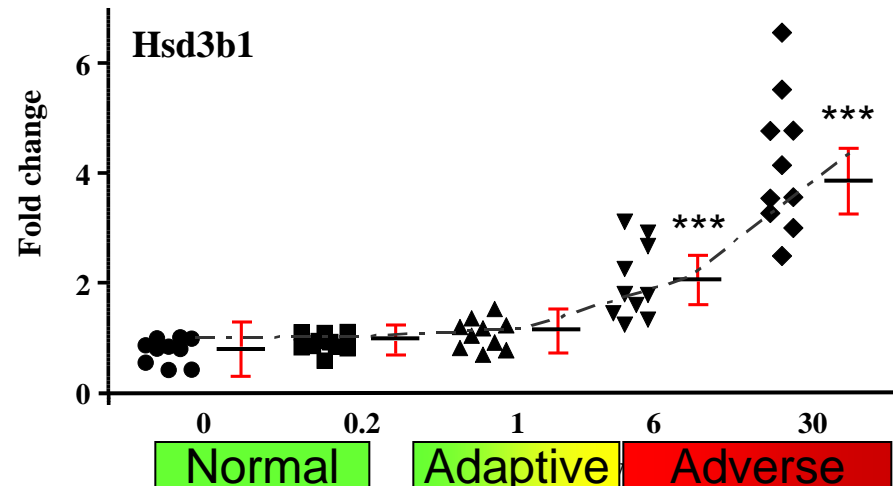
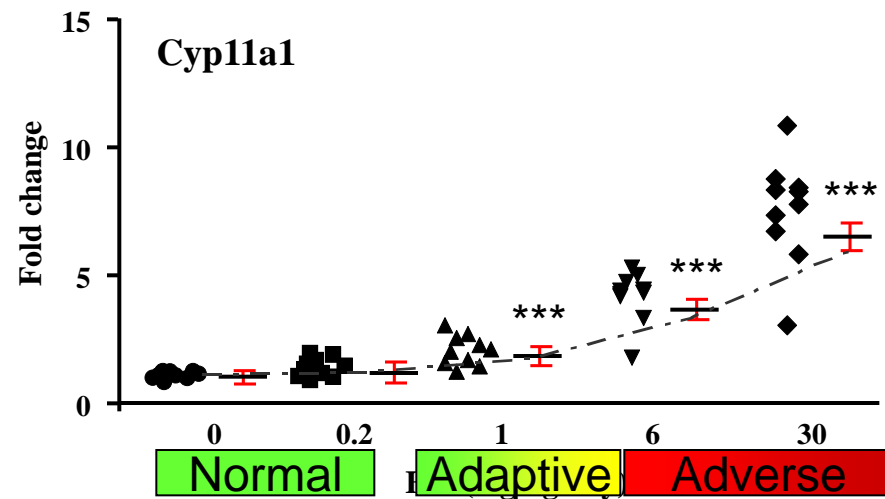
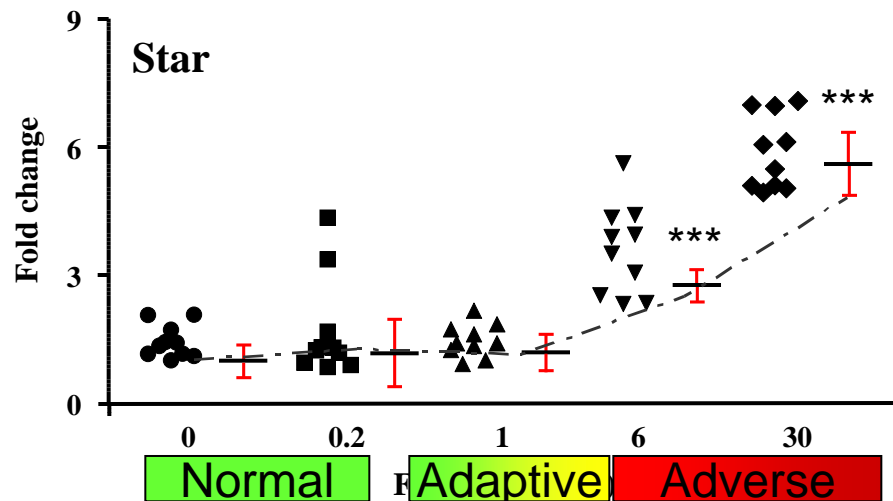


Flutamide: Transcriptomics - Functional Analyses

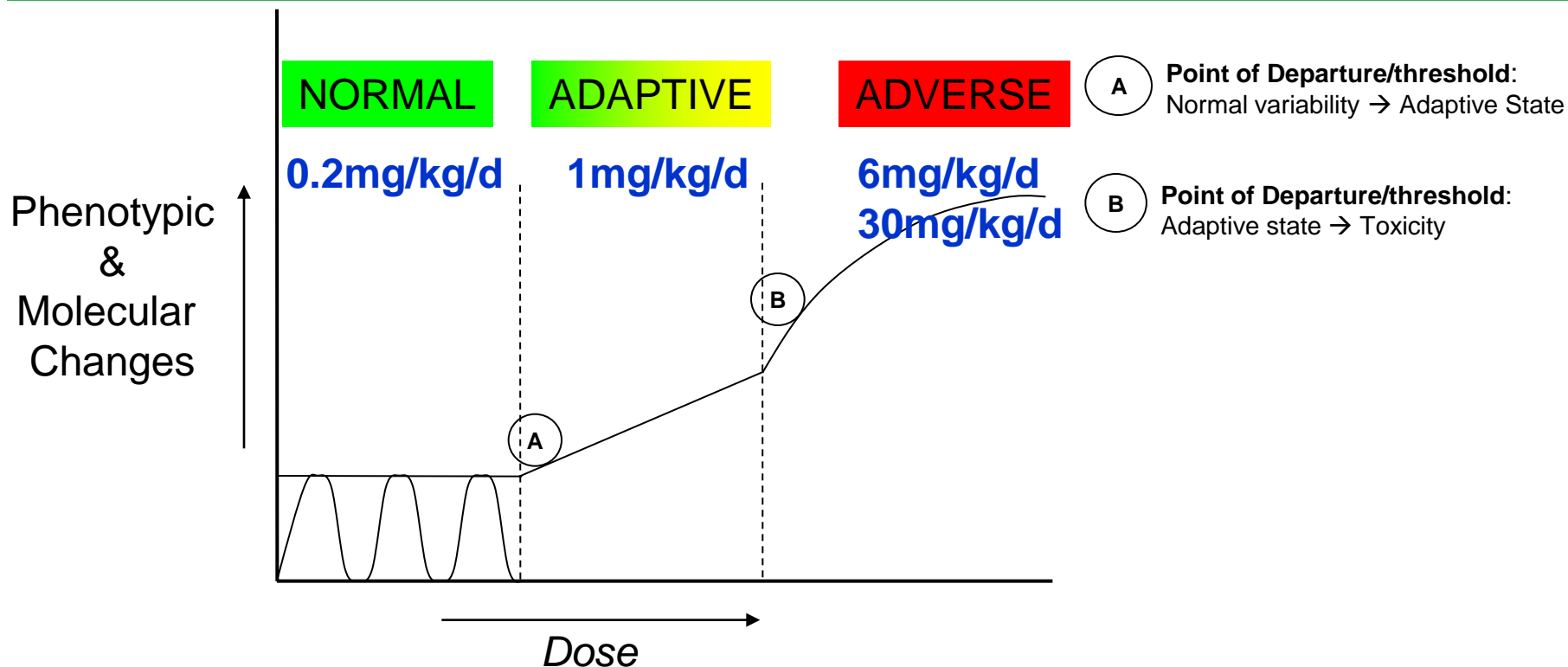
Top 5 Biological Processes Affected by 30mg/kg/day Flutamide



Flutamide: Transcriptomics - Lipid Metabolism



Flutamide: Dose Response Investigations



- Do such points of departure (POD)/thresholds exist for Endocrine Disruptors?
 - **This appears to be the case for testicular toxicity induced by flutamide**

1,3-Dinitrobenzene: Dose Response Investigations



Oral Gavage
4 days

Control + 0.1, 1, 4, 8 mg/kg/d
10 Adult male Wistar rats

Body and
organ
weights

Plasma
testosterone

Testicular
histopathology

8mg/kg/d
Testicular wt ↓
ADAPTIVE?

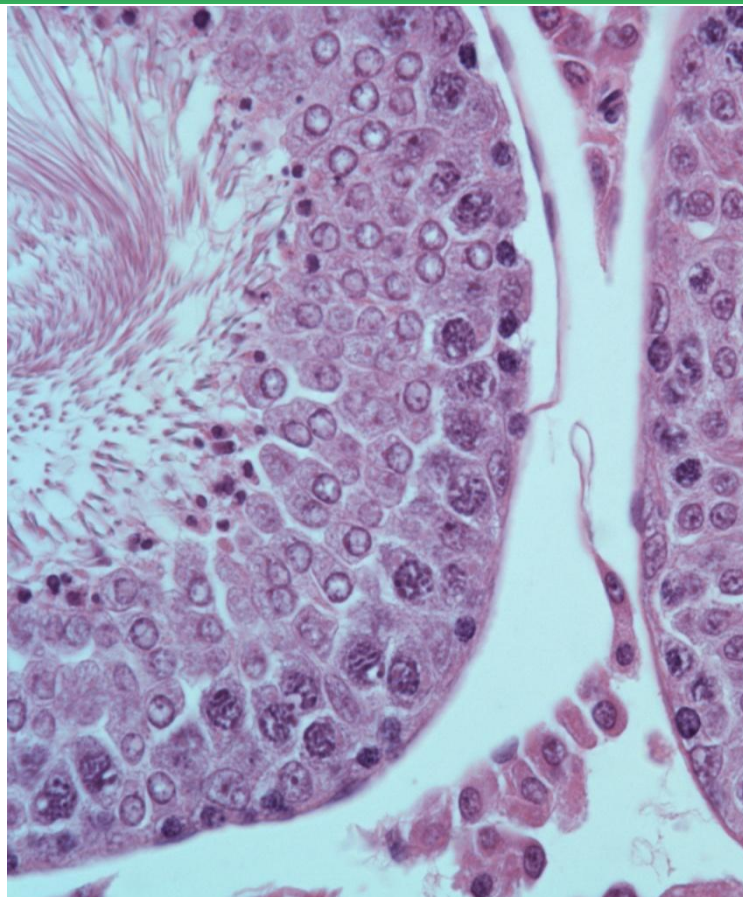
No effects

≥4mg/kg/d
Multiple lesions
ADVERSE

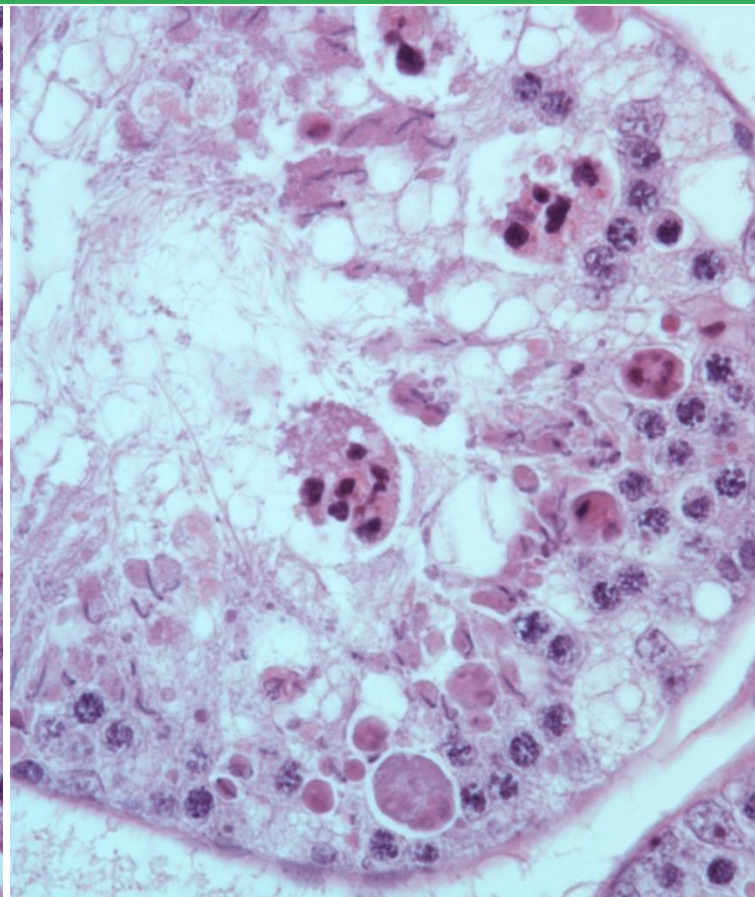
1,3-Dinitrobenzene: Testicular Lesions

Incidence and severity of microscopic changes in the testis					
Dose group (mg/kg/day)	0	0.1	1	4	8
Number examined	10	10	10	10	10
NORMAL			ADAPTIVE?		ADVERSE
Germ cell degeneration/depletion					
Slight → Marked	0	0	0	9	10
Multinucleated giant spermatids					
Minimal → Moderate	0	0	0	7	10
Sertoli cell vacuolation					
Minimal → Marked	0	0	0	9	10

1,3-Dinitrobenzene: Testicular Lesions



Control



8 mg/kg/day

TESTIS (HE, x 400)



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1,3-Dinitrobenzene: Dose Response Investigations



Oral Gavage
4 days

Control + 0.1, 1, 4, 8 mg/kg/d
10 Adult male Wistar rats

Body and
organ
weights

Plasma
testosterone

Testicular
histopathology

Parent/Metabolite
concentrations

Testicular
transcriptome

8mg/kg/d
Testicular wt ↓
ADVERSE

No effects

≥4mg/kg/d
Multiple lesions
ADVERSE

Parent not detected
Metabolite
investigations on-going

1,3-Dinitrobenzene : Transcriptomics - Microarray data

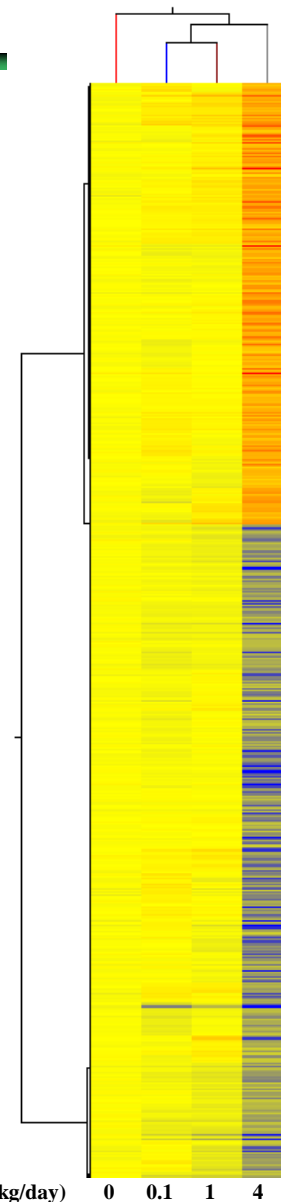
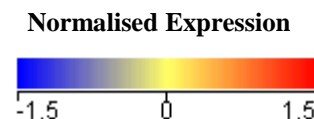
When applying ANOVA p-value of 0.0002:

Adverse

0.1 mg/day: 3918 genes significantly deregulated

Control

0.1 mg/day: No significant changes in gene expression



1,3-Dinitrobenzene : Transcriptomics - Functional Analyses

Molecular and Cellular Functions

Name	p-value	# Molecules
Cell Cycle	3,69E-12 - 3,08E-02	238
Cell Death	2,06E-06 - 3,08E-02	367
DNA Replication, Recombination, and Repair	1,60E-05 - 2,81E-02	142
Gene Expression	4,23E-05 - 2,73E-02	277
Cellular Assembly and Organization	2,04E-04 - 2,85E-02	153

1,3-Dinitrobenzene: Transcriptomic Data – Cell Cycle Arrest

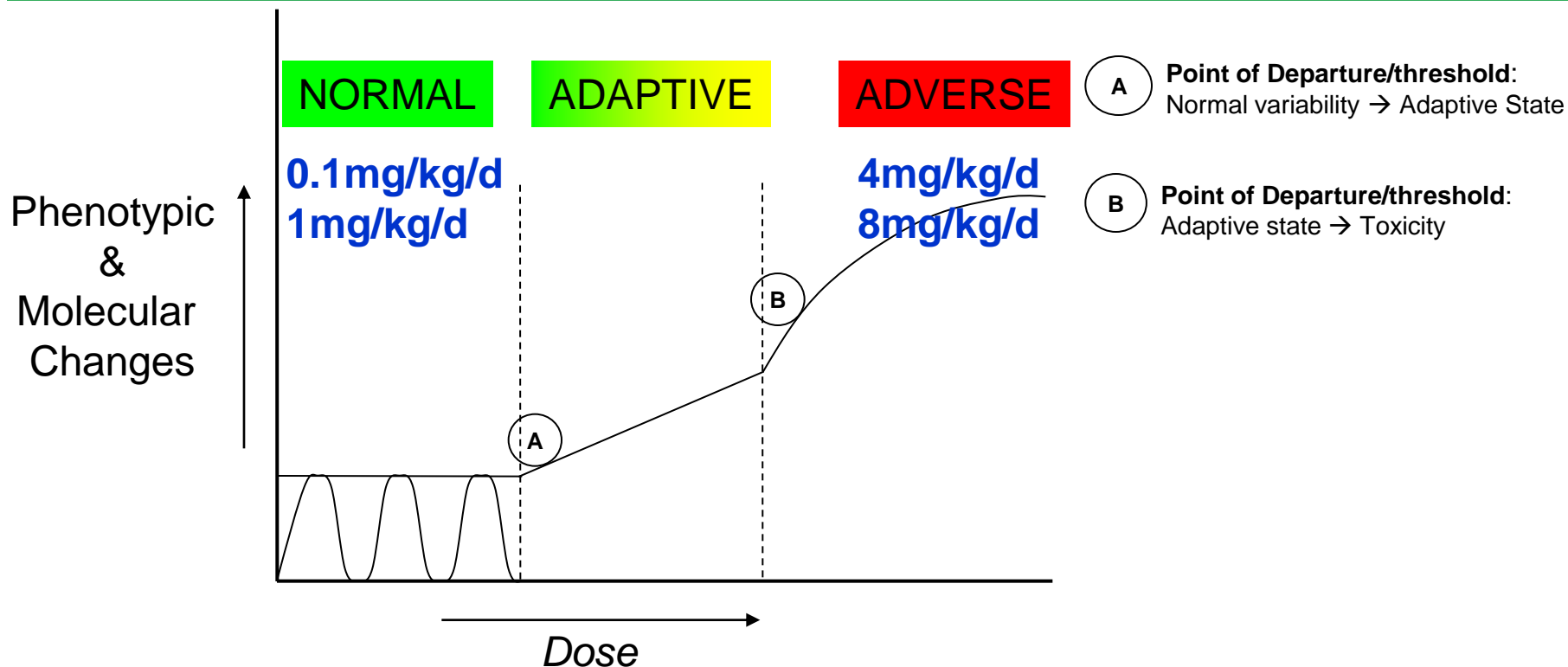
Mitotic Role of Polo-Like Kinase: Effects at 4mg/kg/day

■ Down-regulation
■ Up-regulation

1,3-Dinitrobenzene: Transcriptomic Data – Cell Cycle Arrest

GenBank	Gene Description (gene symbol)		NORMAL/ ADAPTIVE?		ADVERSE
			0.1	1	4
NM_019296	cell division cyle 2 homolog A (<i>Cdc2a</i>)	Array	0.97	0.96	0.63***
		qPCR	0.94	0.97	0.48***
NM_00101274	wee 1 homolog (<i>Wee1</i>)	Array	1.02	1.02	1.23***
		qPCR	0.99	1.15**	1.14**
XM_229115	heat shock protein 1, alpha (<i>Hsp90aa</i>)	Array	0.96	0.91	0.72***
		qPCR	1.01	1.02	0.66***
NM_019349	serine/threonine kinase 2 (<i>Slk</i>)	Array	0.99	1.03	1.38***
		qPCR	1.00	1.02	1.19***
NM_00102541	protein phosphatase 2 (formerly 2A), regulatory subunit A, beta isoform (<i>Ppp2r1b</i>)	Array	1.01	0.97	0.71***
		qPCR	1.02	0.96	0.65***
NM_00102474	cell division cycle 16 homolog (<i>Cdc16</i>)	Array	0.95	0.95	0.79***
		qPCR	0.95	1.00	0.69***
NM_031683	structural maintenance of chromosomes 1A (<i>Smc1l1</i>)	Array	0.99	1.00	1.21***
		qPCR	1.09	1.19	1.36*
NM_171993	cell division cycle 20 homolog (<i>Cdc20</i>)	Array	1.01	1.02	0.78***
		qPCR	0.96	0.99	0.61***
NM_017100	polo-like kinase 1 (<i>Plk1</i>)	Array	0.99	0.98	0.77***
		qPCR	1.02	0.96	0.65***

1,3-Dinitrobenzene: Dose Response Investigations



- What impact will toxicogenomic data have on our PoDs/thresholds?
 - **Our testicular data for 1,3-DNB that the PoDs are similar between the adverse phenotypic effects and the molecular changes**

1,3-Dinitrobenzene: Time Response Investigations



**Single Oral Gavage
Control + 4 mg/kg/d
Sacrifice: 8, 24, 48, 72h after dosing
10 Adult male Wistar rats**

Body and
organ
weights

Plasma
testosterone

Testicular
histopathology

Parent/Metabolite
concentrations

Testicular
transcriptome

Effects

No effects

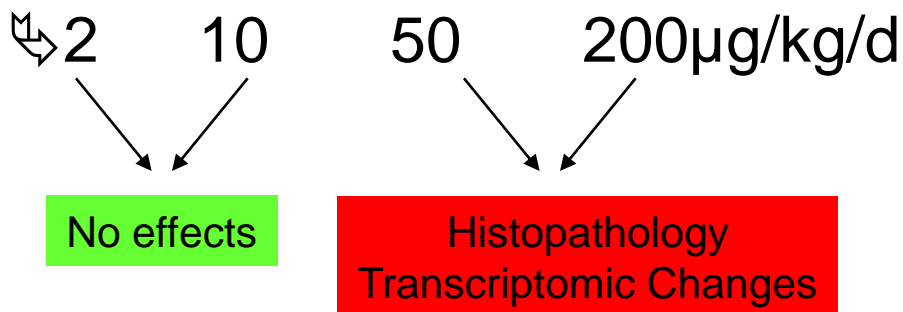
No effects

Conclusions (1)

Do points of departure (POD)/thresholds exist for Endocrine Disrupters?

↪ Our Flutamide data indicate that thresholds can be identified, at least for testicular toxicity.

↪ A similar profile was observed for Ethinyl Estradiol:



Conclusions (2)

What impact will toxicogenomic data have on our PoDs/thresholds?

- ✚ Transcriptomic data provide evidence that can support the identification of NOAELs
- ✚ However, transcriptomic data appear to be more suited to:
 - ✚ Providing information concerning the mode of action of a compound
 - ✚ Providing alerts in short-term studies as to what could be expected in the long-term (eg cancer bioassays) studies.

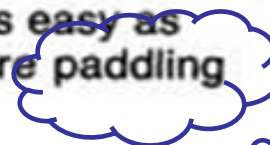
Perspectives

- Completion of the time response studies
- Publications
 - Flutamide dose response (submission end of Nov 2010)
 - DNB testicular effects (in preparation)
 - Statistical approaches (in preparation)

Acknowledgements



"The trick is to make it look as easy as possible while, underneath, you're paddling like hell."



• Sophie Ludwig
PhD Student



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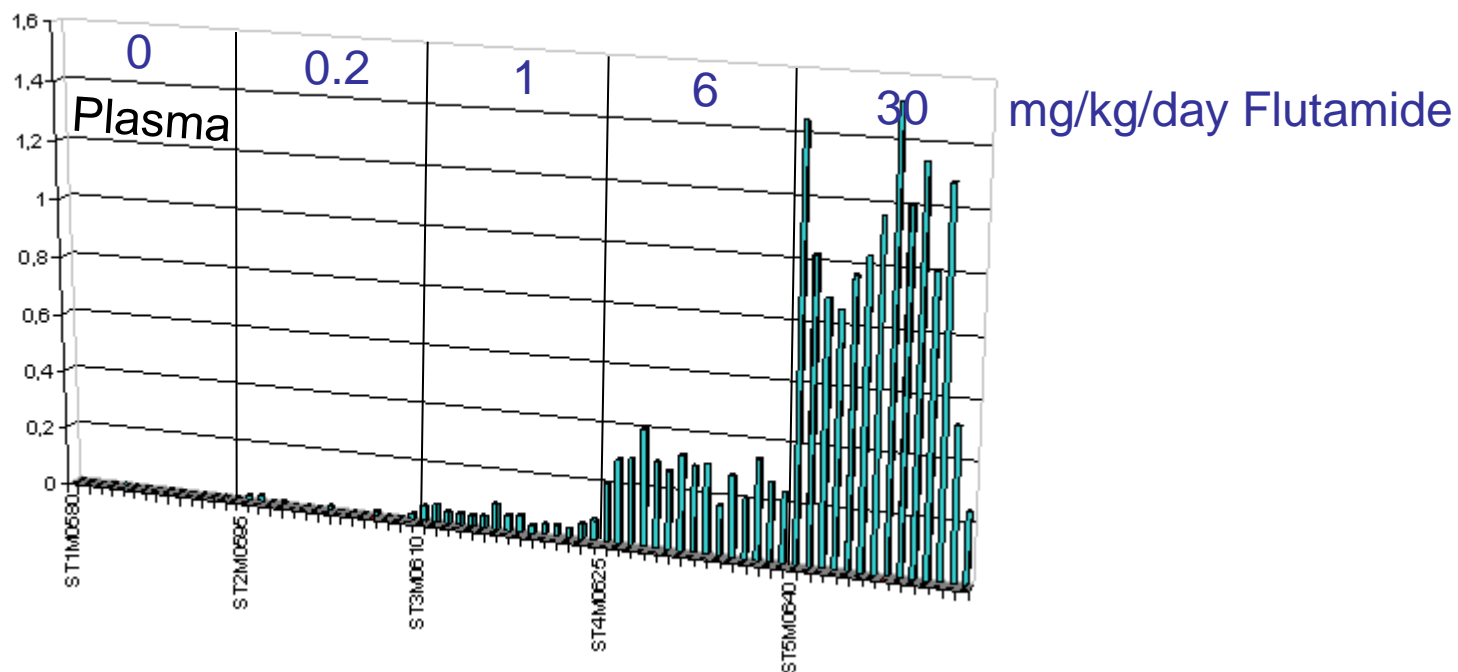
Thank you for your kind attention😊

Spare/back-up slides

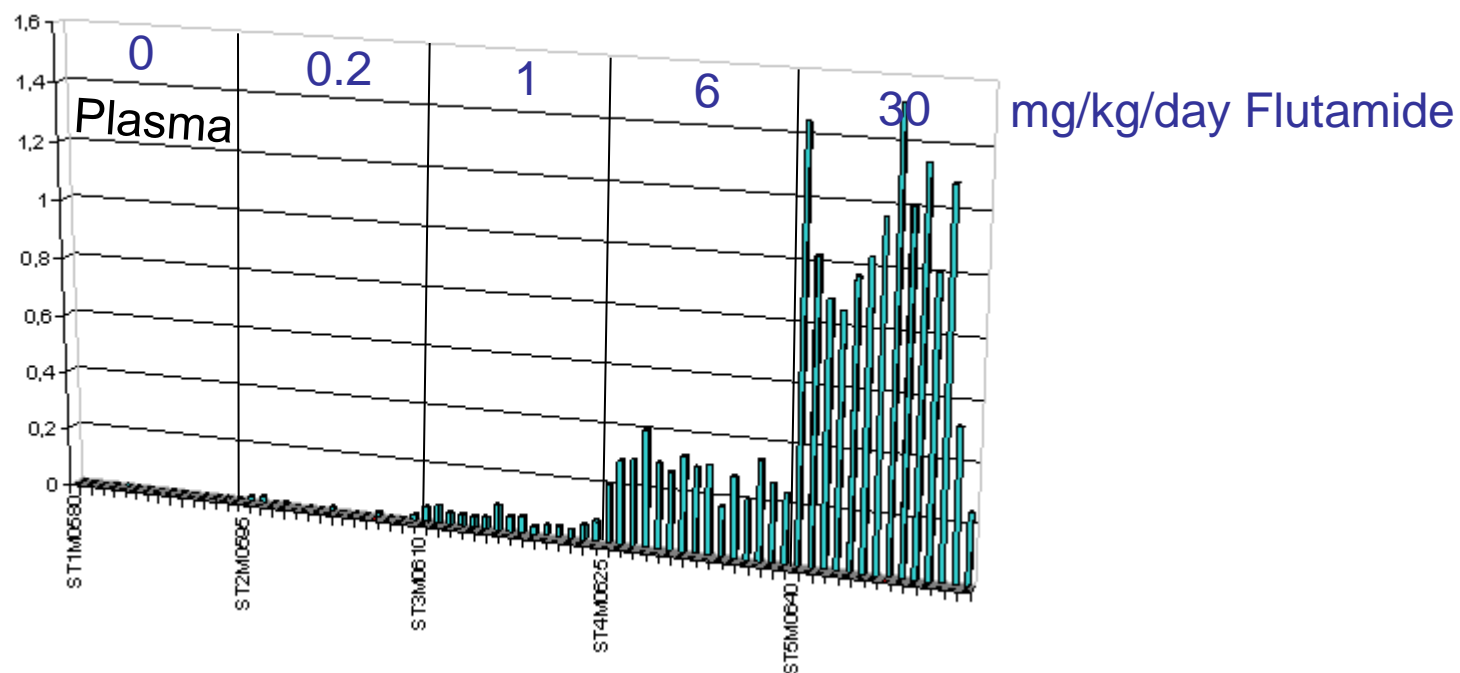
Flutamide : Testicular Lesions

Microscopic changes in the testes: Diffuse Leydig cell HYPERPLASIA					
Dose group (mg/kg/day)	0	0.2	1	6	30
	NORMAL	ADAPTIVE		ADVERSE	
Minimal	0	0	0	10	2
Slight	0	0	0	0	6
Moderate	0	0	0	0	2
Total	0/10	0/10	0/10	10/10	10/10

Plasma



µg/ml



Ethinyl Oestradiol : Histopathologie

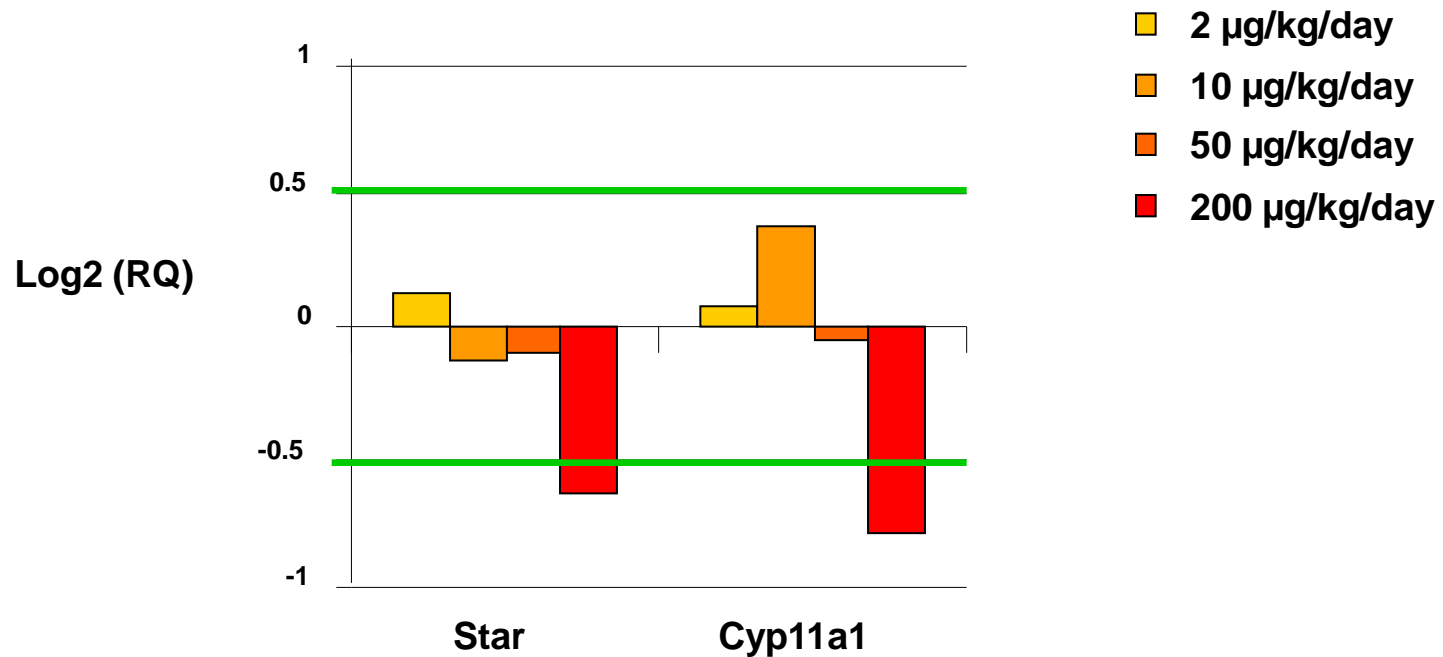
Microscopic changes: TESTIS					
Dose group (µg/kg/day)	0	2	10	50	200
Number examined	10	10	10	10	10
Leydig cell atrophy: diffuse					
Minimal	0	0	0	3	2
Slight	0	0	0	0	3
Moderate	0	0	0	0	2
Marked	0	0	0	0	2
Total	0	0	0	3	9

Ethinyl Oestradiol : Histopathologie

Microscopic changes: TESTIS					
Dose group (µg/kg/day)	0	2	10	50	200
Number examined	10	10	10	10	10
Leydig cell atrophy: diffuse					
Minimal	0	0	0	3	2
Slight	0	0	0	0	3
Moderate	0	0	0	0	2
Marked	0	0	0	0	2
Total	0	0	0	3	9

Ethinyl oestradiol : Transcriptomique

Expression dans les groupes traités à l'EE



1,3-Dinitrobenzene:

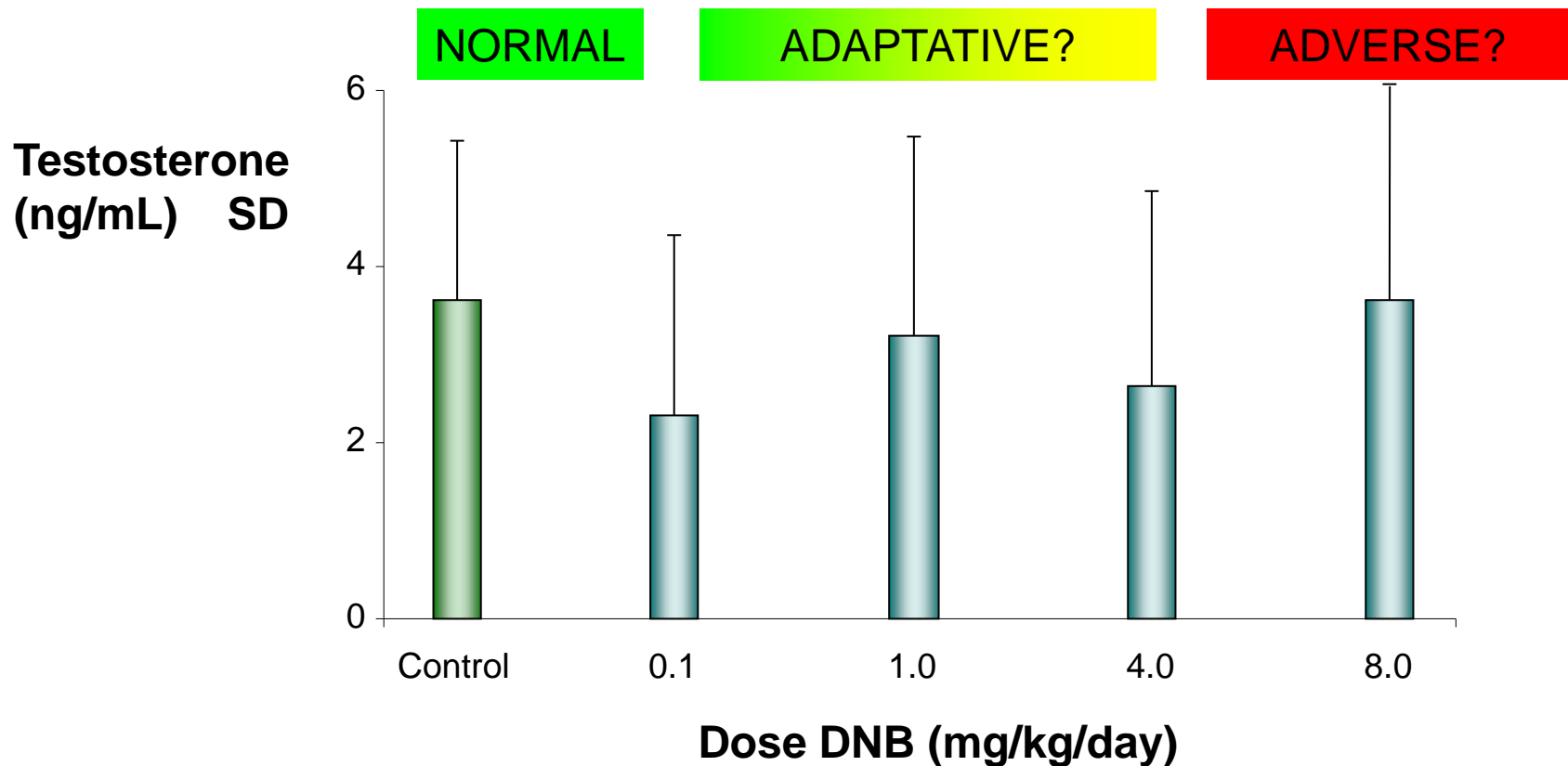
Body and organ weights

	1,3-Dinitrobenzene				
	Control	0.1 mg/kg/d	1 mg/kg/d	4 mg/kg/d	8 mg/kg/d
Body weight (g)					
Initial	409±15	409±16	410±15	411±17	401±19
Terminal	421.7±19.6	419.3±18.6	423.3±18.8	420.3±16.6	407.0±24.4
% organ to body weight					
Liver	3.47±0.25	3.53±0.30	3.58±0.30	3.62±0.37	3.53±0.24
Absolute organ weight (g)	NORMAL	ADAPTATIVE?			ADVERSE?
Testis	1.84±0.53	1.80±0.14	1.73±0.15	1.74±0.28	1.54 0.27*
Epididymis	0.56±0.06	0.56±0.06	0.53±0.05	0.53±0.06	0.52±0.05
Prostate	0.55±0.12	0.48±0.10	0.47±0.09	0.46±0.10	0.49±0.09
Seminal Vesicle	1.7±0.4	1.5±0.3	1.5±0.3	1.4±0.3	1.5±0.3
Adrenal glands	0.0573±0.0088	0.0562±0.0090	0.0581±0.0108	0.0574±0.0101	0.0530±0.0113
Pituitary glands	0.0100±0.0019	0.0094±0.0018	0.0107±0.0027	0.0097±0.0014	0.0105±0.0033

*p<0.05.

1,3-Dinitrobenzene: Plasma Testosterone

No effects on testosterone levels after 4 days dosing with 1,3-DNB



Flutamide – Body and organ weights

	Flutamide				
	Control	0.2 mg/kg/d	1 mg/kg/d	6 mg/kg/d	30 mg/kg/d
Terminal Body weight (g)	416.9±29.4	417.2±29.0	417.9±21.0	412.6±25.9	401.1±26.2
% organ to body weight					
Liver	3.53±0.22	3.57±0.28	3.54±0.26	3.64±0.16	4.19±0.18**
Absolute organ weight (g)	NORMAL	ADAPTATIVE?		ADVERSE?	
Testis	1.83±0.11	1.78±0.14	1.78±0.24	1.85±0.23	1.98±0.52
Right Epididymis	0.53±0.04	0.48±0.03	0.47±0.08	0.40 0.04**	0.38 0.05**
Prostate	0.44±0.12	0.39±0.09	0.38±0.15	0.29 0.07**	0.21 0.05**
Seminal Vesicle	1.39±0.30	1.29±0.32	1.13 0.258*	0.90 0.22**	0.55 0.19**
Adrenal glands	0.051±0.005	0.052±0.009	0.052±0.007	0.055±0.008	0.061 0.011*
Pituitary glands	0.011±0.002	0.011±0.002	0.011±0.001	0.011±0.001	0.012±0.002

*p<0.05, **p<0.01.

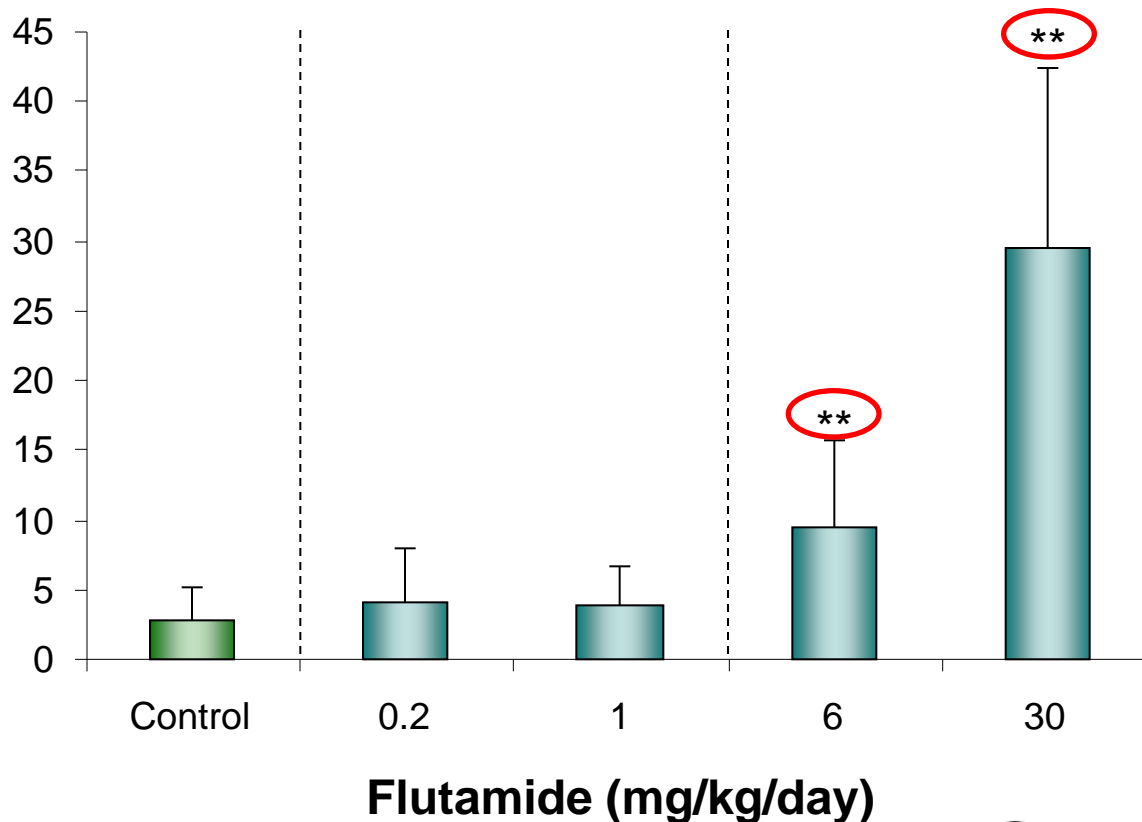
Flutamide: Plasma Testosterone

NORMAL

ADAPTATIVE?

ADVERSE

Plasma
testosterone
(ng/mL) SD



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Transcriptome principal component analysis

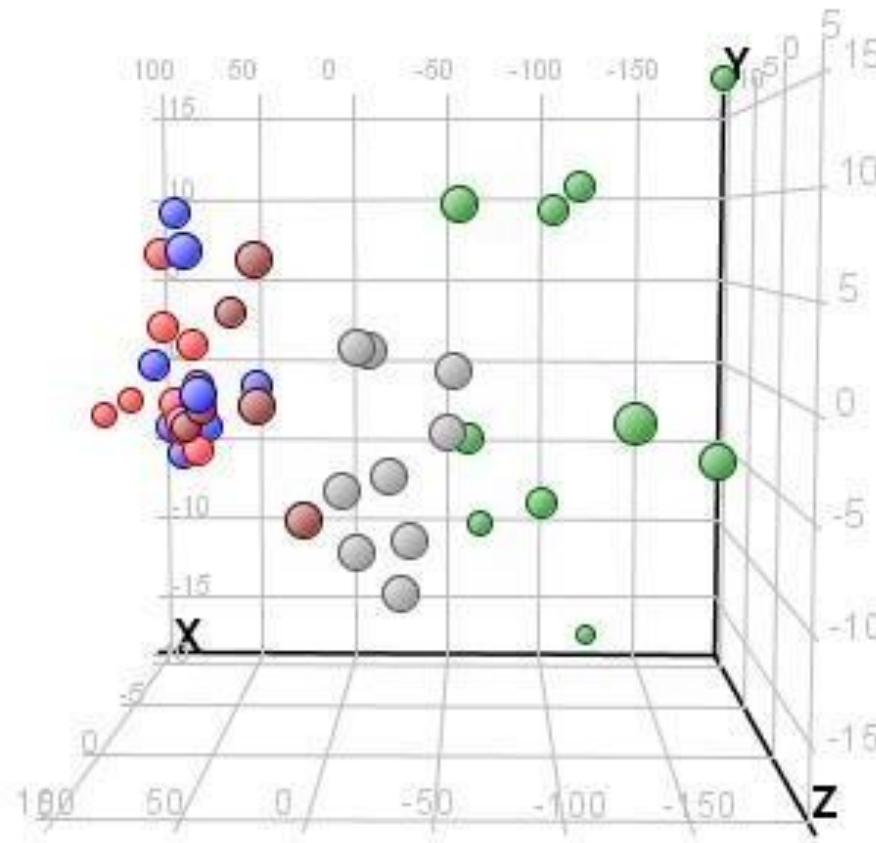
NORMAL

ADAPTATIVE

ADVERSE

Color by treatment

- 0 mg/kg/day
- 0.2 mg/kg/day
- 1mg/kg/day
- 6mg/kg/day
- 30mg/kg/day

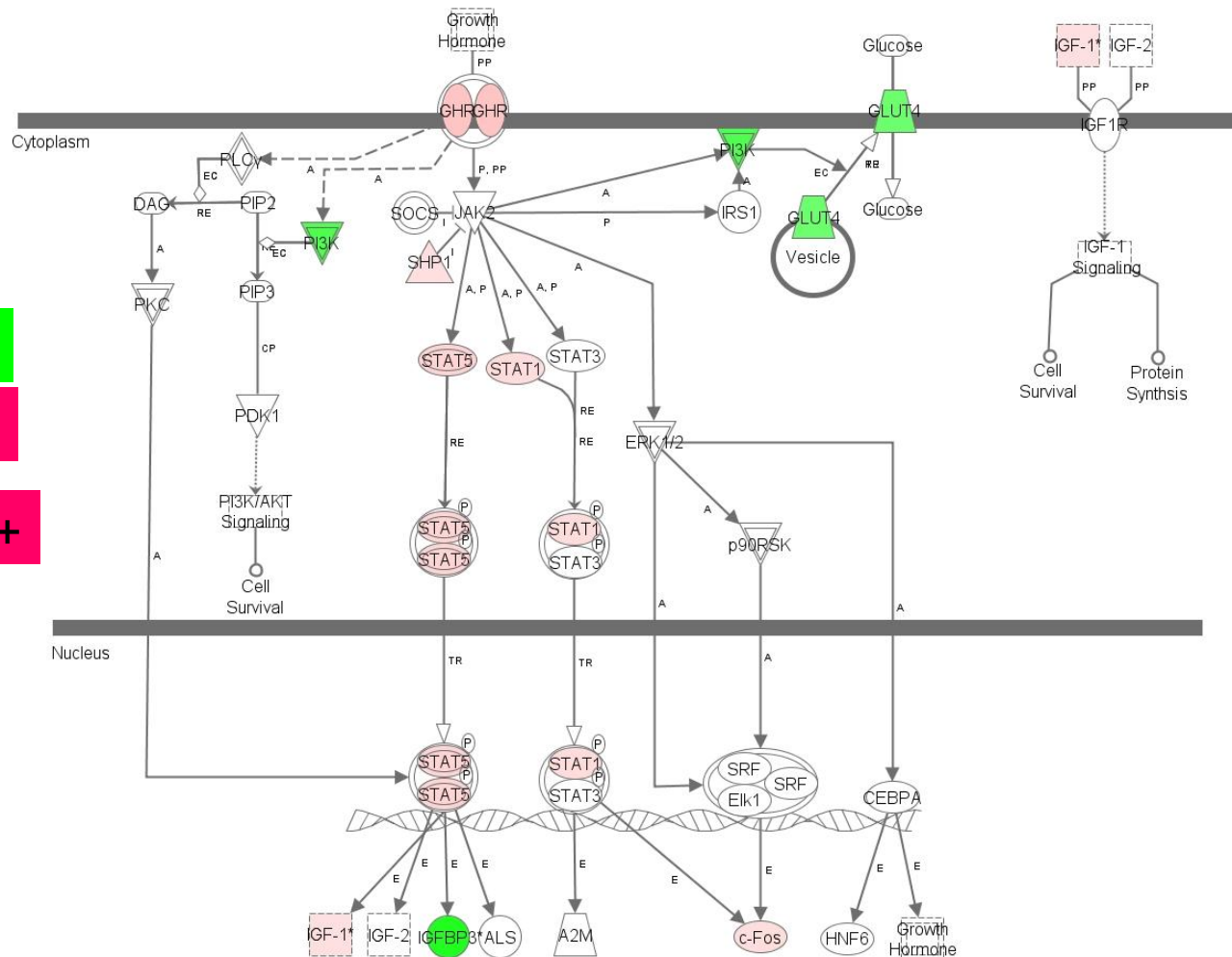


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Toxicity pathway analysis

Growth Hormone Signaling

30 mg/kg/day FLUTAMIDE



ADAPTATIVE

ADVERSE +

ADVERSE ++