From Profile to Mechanistic Understanding

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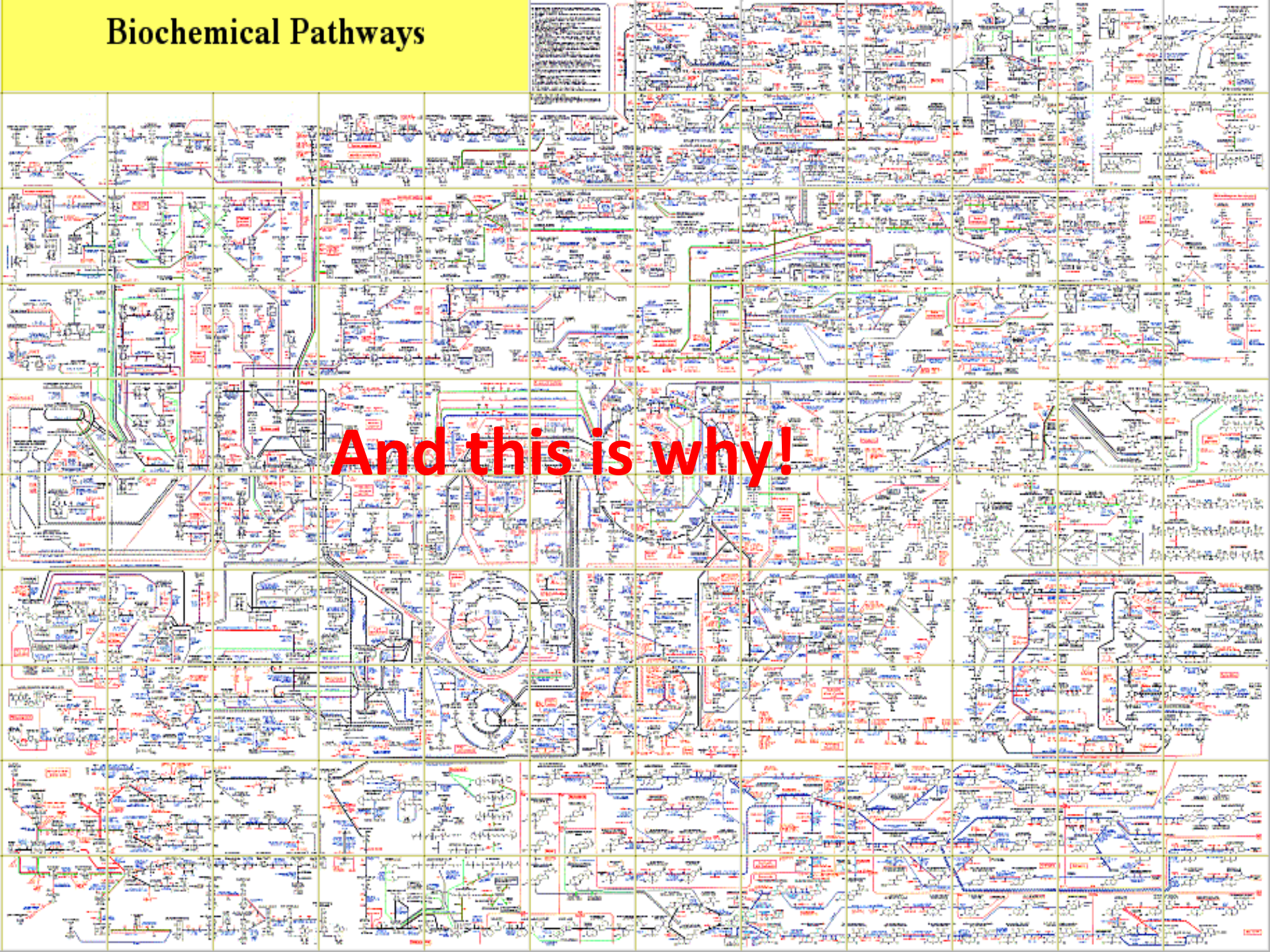
Metabolic Phenotyping

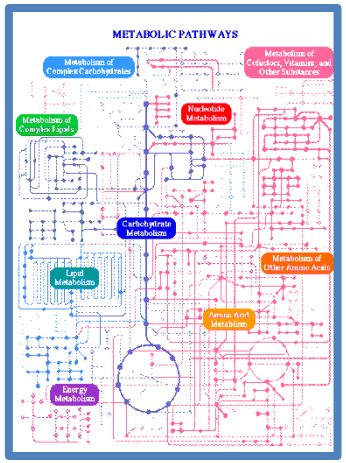
- Metabonomics/metabolomics – untargeted (and intended to be unbiased) metabolic profiling of biological samples
- The aim is to find "biomarkers" in areas such as basic biology, disease, toxicity etc.
- Ideally these should be mechanistic and specific for the condition under investigation
- Often they are not……

Biochemical Pathways

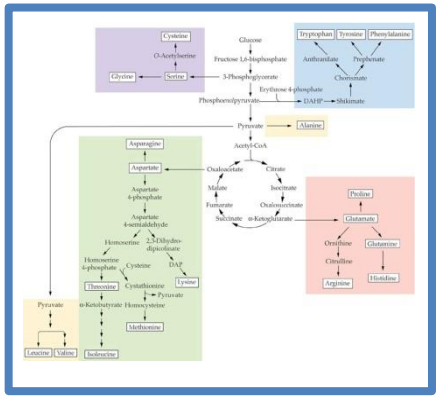


From Metabolic Phenotyping to Biomarkers

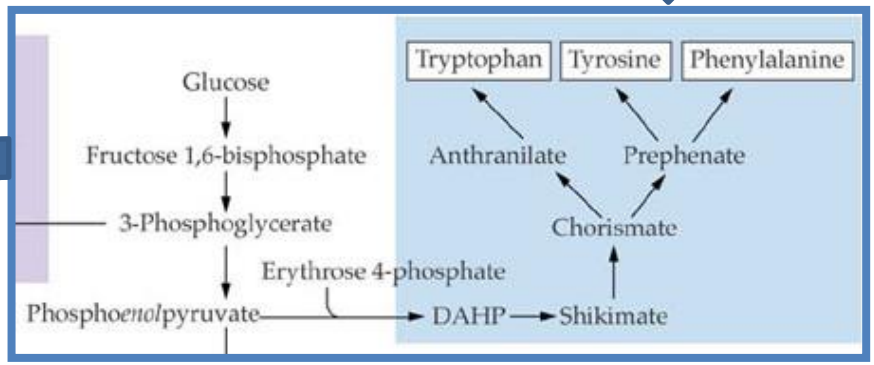
Global profiling – unbiased biomarker **Discovery**



Targeted **classes** of compounds - **Quantification**



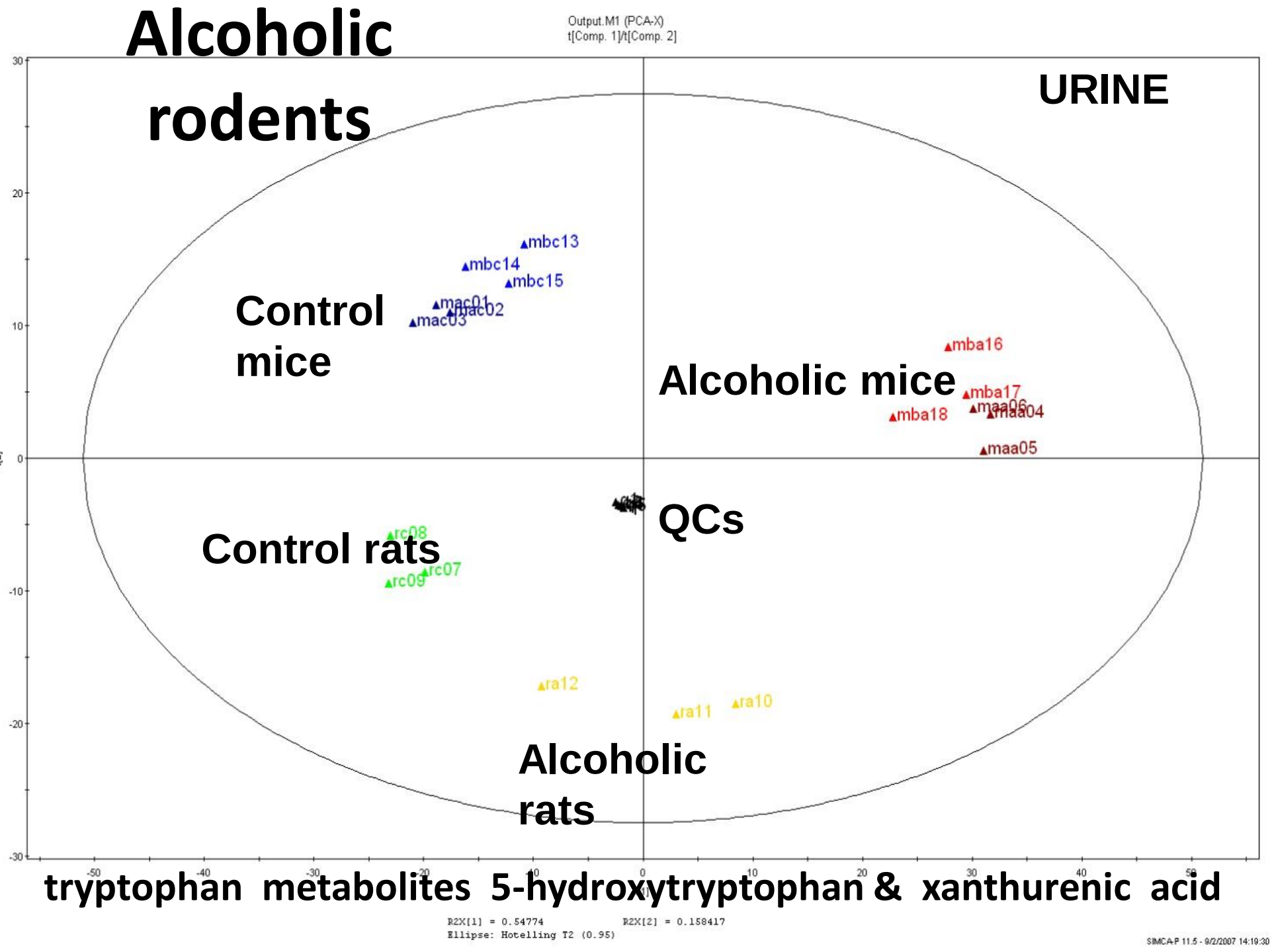
Targeted pathways - **Confirmation**

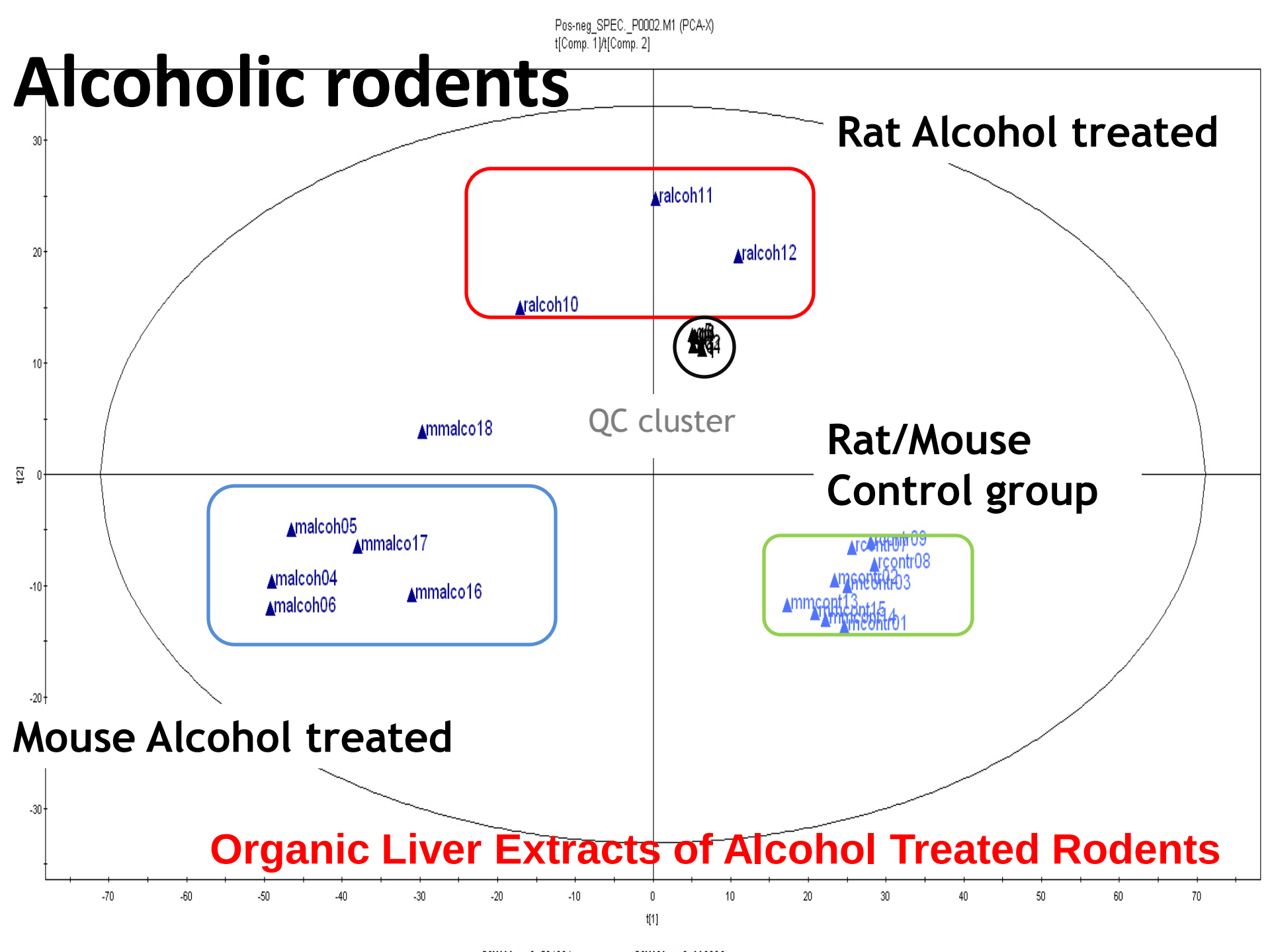


Specific metabolites - **Application**

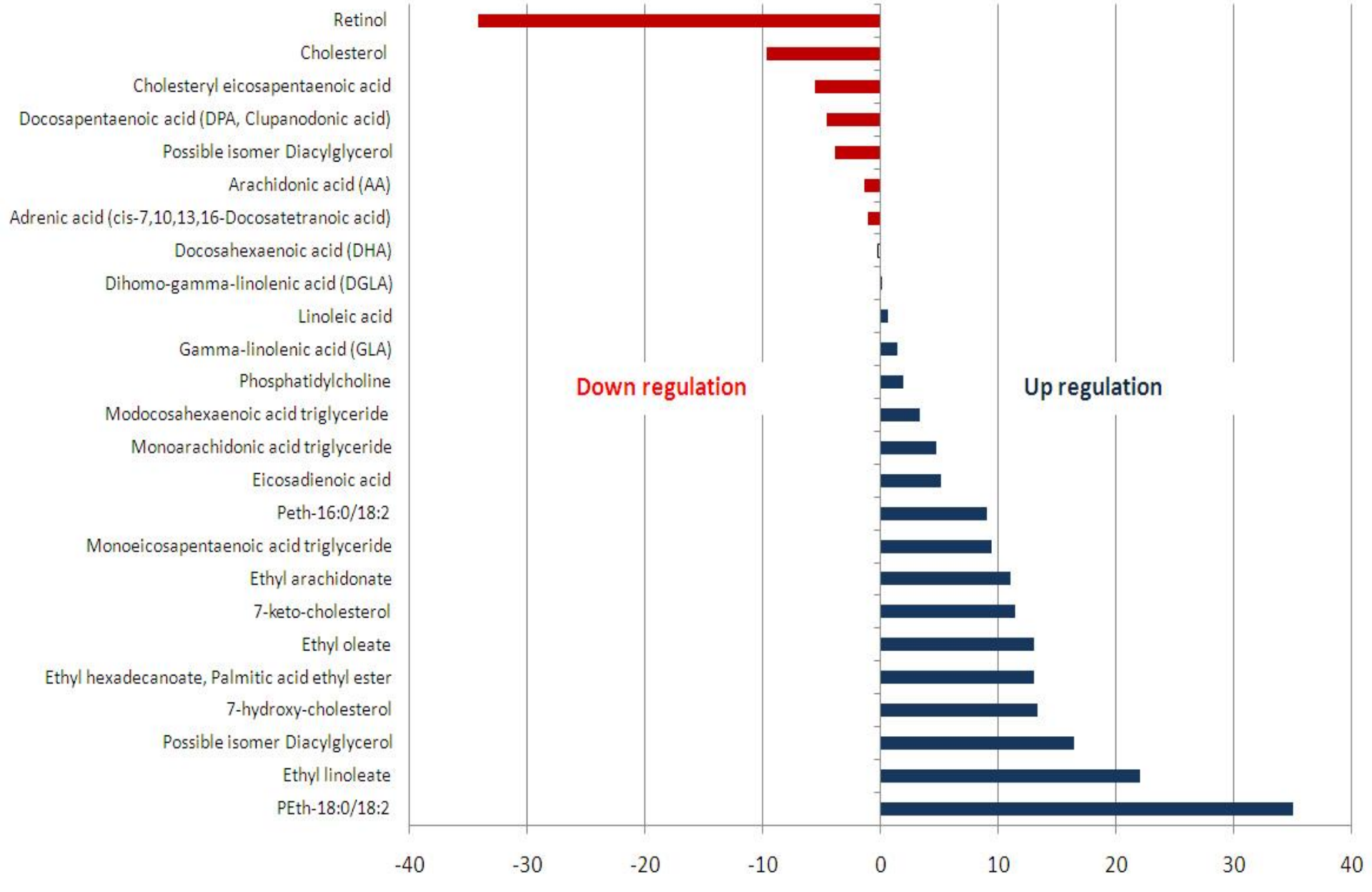
A Rodent Model of Alcoholism

- A rodent model of alcoholism, mice/rats fed intra-gastrically with ethanol.
- Urine and liver extracts analysed by UPLC-MS with PCA for “biomarker” detection.
- What do we see? And what does it tell us?


Alcoholic rodents
Output.M1 (PCA-X)
t[Comp. 1]/t[Comp. 2]
URINE
Control mice
mbc13
mbc14
mbc15
mac01
mac02
mac03
Alcoholic mice
mba16
mba17
mba18
maa06
maa04
maa05
QCs
Control rats
rc08
rc07
rc09
Alcoholic rats
ra12
ra11
ra10
tryptophan metabolites 5-hydroxytryptophan & xanthurenic acid
R2X[1] = 0.54774
R2X[2] = 0.158417
Ellipse: Hotelling T2 (0.95)
SIMCA-P 11.5 - 9/2/2007 14:19:30


Pos-neg_SPEC._P0002.M1 (PCA-X)
t[Comp. 1]/t[Comp. 2]
Alcoholic rodents
Rat Alcohol treated
ralcoh11
ralcoh12
ralcoh10
QC cluster
mmalco18
Rat/Mouse
Control group
malcoh05
mmalco17
malcoh04
malcoh06
mmalco16
rcontr08
rcontr03
mmcont13
Mouse Alcohol treated
Organic Liver Extracts of Alcohol Treated Rodents
t[1]
t[2]

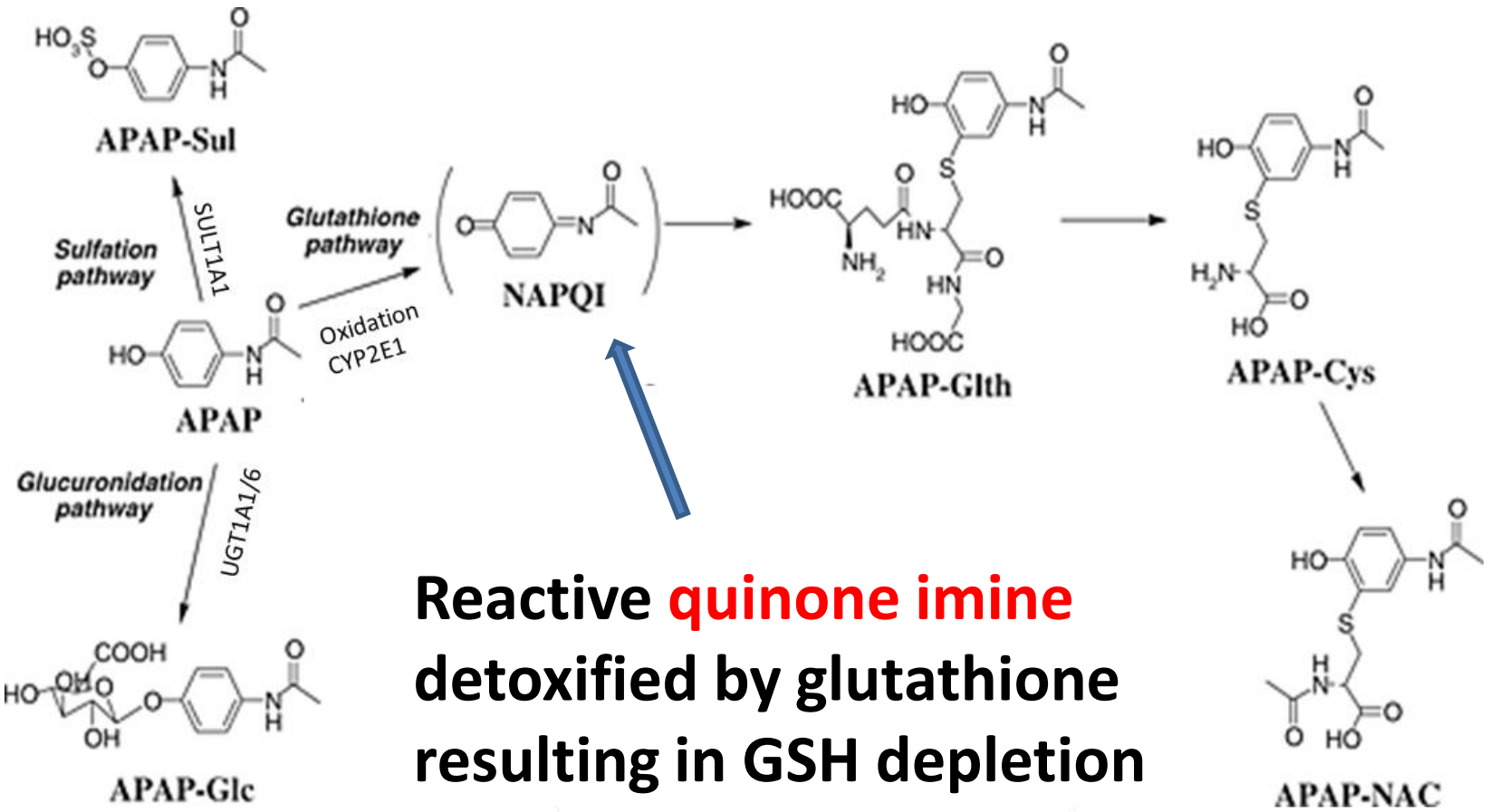
Liver “biomarkers” of alcohol treatment



Paracetamol Toxicity

- Paracetamol (acetaminophen) is a safe drug when taken at therapeutic doses but a major human hepatotoxin in overdose
- Results in a very large number of liver transplants
- Toxicity is the result of the formation of reactive metabolites

Paracetamol Metabolism

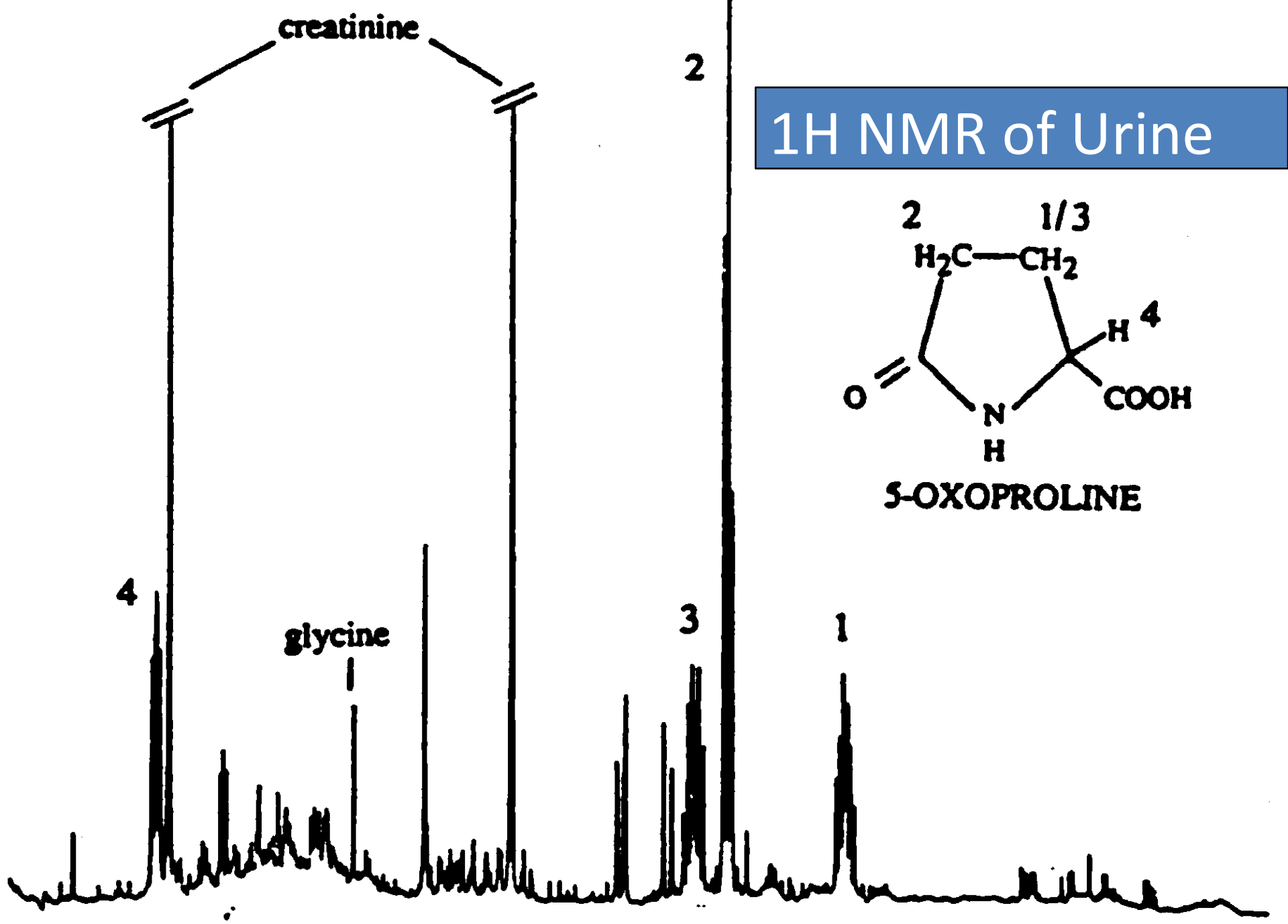


Reactive quinone imine detoxified by glutathione resulting in GSH depletion

Matabonomics &Paracetamol Toxicity

- **F.Y. Ghauri** *et al* Induction of **5-oxoprolinuria** in the rat following chronic feeding with N-acetyl 4 aminophenol (paracetamol), *Biochem. Pharmacol. 46 (1993) 953–957.*
- ***An NMR-based study examining rat urine***
- **T. Soga** *et al* Differential metabolomics reveals **ophthalmic acid** as an oxidative stress biomarker indicating hepatic glutathione consumption, *J. Biol. Chem. 281 (2006) 16768–16776.*
- ***A CE-MS-based study in mice***

1H NMR of Urine



5-OXOPROLINE

Elevated 5-Oxoproline reported in human overdose - translatable to humans…….

JAOA CASE REPORT

5-Oxoproline–Induced Anion Gap Metabolic Acidosis After an Acute Acetaminophen Overdose

David T. Lawrence, DO; Laura K. Bechtel, PhD; Nathan P. Charlton, MD; and Christopher P. Holstege, MD

5-OXOPROLINEMIA CAUSING ELEVATED ANION GAP METABOLIC ACIDOSIS IN THE SETTING OF ACETAMINOPHEN USE

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Alan H. B. Wu, PHD,* and Somnath Mookherjee, MD‡

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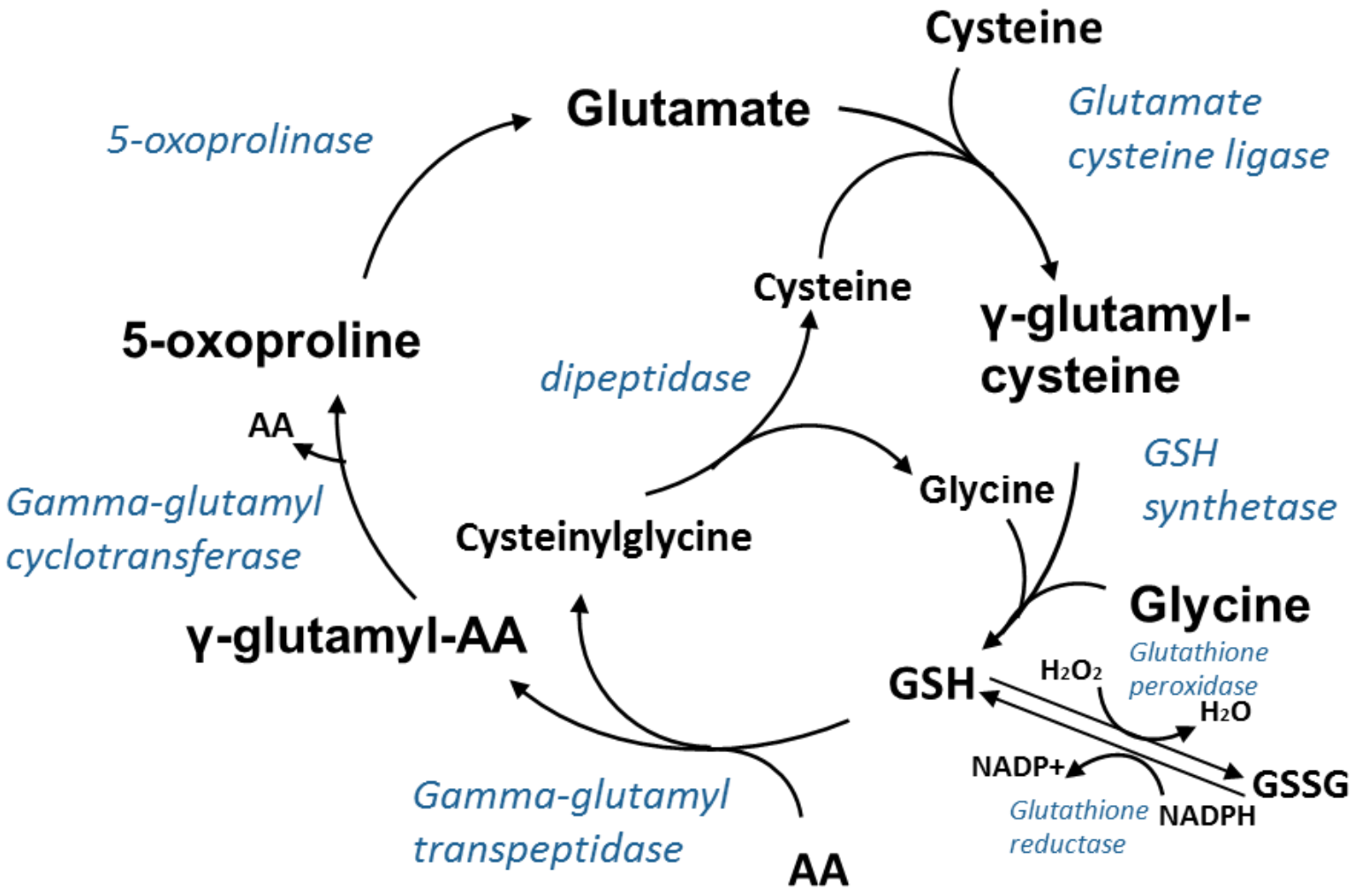
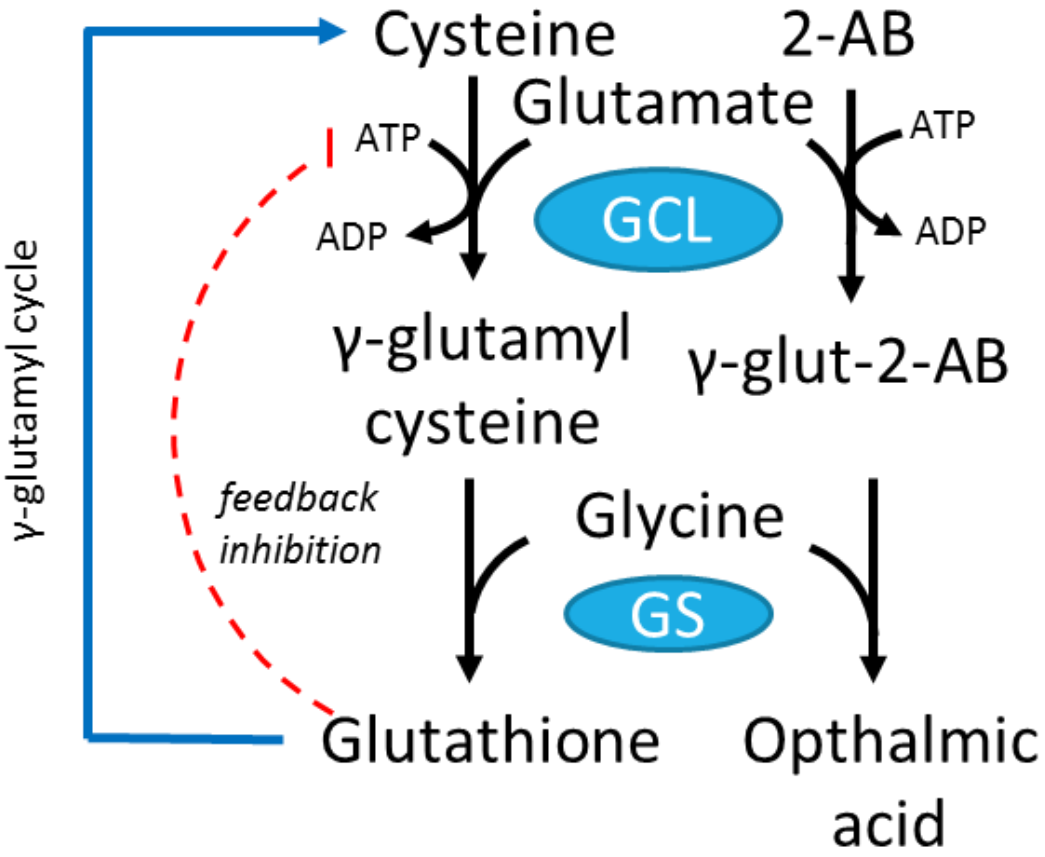
So what is the role of 5-oxoproline?

5-Oxoproline & Glutathione Biosynthesis

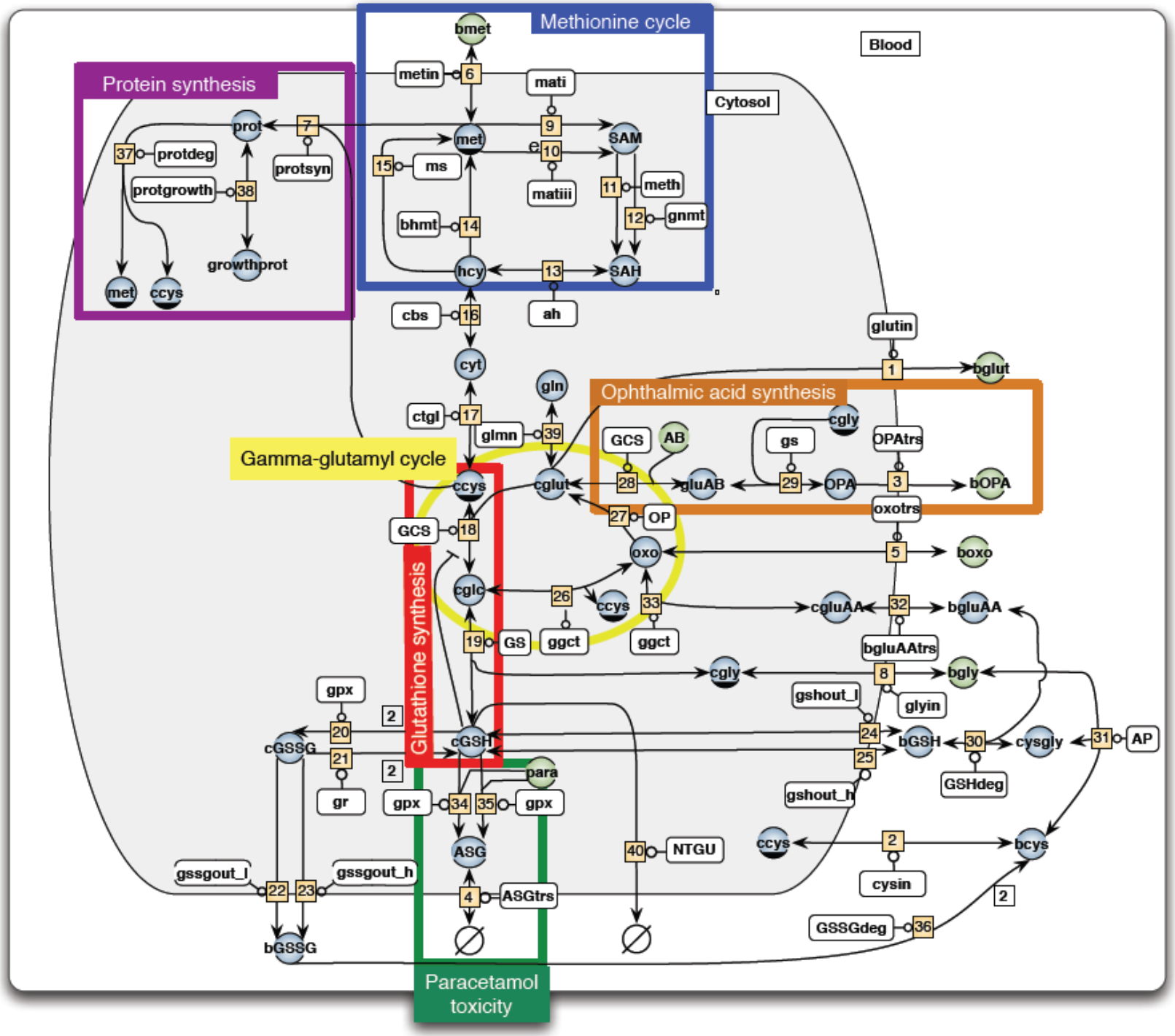


Opthalmic acid Biosynthesis



Why are these metabolites biomarkers?

- **Paracetamol is toxic via reactive metabolites formed by cytochrome P450 2E1**
- **The reactive quinoneimine metabolite is detoxified by conjugation with glutathione**
- **Depletion of glutathione is followed by damage to cellular macromolecules and cell death**
- **5-oxoproline (pyroglutamate) and opthalmic acid are involved in the biosynthetic pathways that relate to glutathione**
- **Why both metabolites? What are these biomarkers telling us? which is one is best? Or do you need both?**
- **Can we model it using systems biology?**


Methionine cycle
Protein synthesis
Blood
Cytosol
Ophthalmic acid synthesis
Gamma-glutamyl cycle
Glutathione synthesis
Paracetamol toxicity
bmet
metin
6
mati
prot
7
met
9
SAM
37
protdeg
protsyn
15
ms
10
matiii
protgrowth
38
11
meth
bhmt
14
12
gnmt
growthprot
hcy
13
SAH
met
ccys
cbs
16
ah
glutin
cyt
1
bglut
gln
ctgl
17
glmn
39
cgly
GCS
AB
gs
OPAtrs
ccys
cglut
28
gluAB
29
OPA
3
bOPA
27
OP
oxotrs
GCS
18
oxo
5
boxo
cglc
26
ccys
33
cgluAA
32
bgluAA
19
GS
ggct
ggct
bgluAAtrs
cgly
8
bgly
gpx
gshout_l
2
glyin
20
24
cGSSG
cGSH
bGSH
30
cysgly
31
AP
21
25
2
para
GSHdeg
gshout_h
gr
gpx
34
35
gpx
ASG
40
NTGU
ccys
2
bcys
gssgout_l
22
23
gssgout_h
4
ASGtrs
cysin
2
bGSSG
GSSGdeg
36

The model

- **Kinetic model - reversible Michaelis-Menten equations**

$$\frac{\mathrm{d}\left([\mathrm{cGSH}]\cdot V_{\mathrm{cell}}\right)}{\mathrm{d}t} = \frac{\mathrm{vGSf1}\cdot[\mathrm{cglc}]\cdot[\mathrm{cgly}]\cdot\left(1-\frac{[\mathrm{cGSH}]}{\mathrm{keqGS1}\cdot[\mathrm{cglc}]\cdot[\mathrm{cgly}]}\right)}{\mathrm{kGScglc}\cdot\mathrm{kGScgly}\cdot\left(1+\frac{[\mathrm{cgly}]}{\mathrm{kGScgly}}+\frac{[\mathrm{gluAB}]}{\mathrm{kGSgluAB}}\right)\cdot\left(1+\frac{[\mathrm{cglc}]}{\mathrm{kGScglc}}+\frac{[\mathrm{cGSH}]}{\mathrm{kGScGSH}}+\frac{[\mathrm{OPA}]}{\mathrm{kGSOPA}}\right)}$$

$$-2\cdot\frac{\mathrm{H2O2}\cdot\mathrm{vmGPX}\cdot[\mathrm{cGSH}]^2}{(\mathrm{H2O2}+9\cdot\mathrm{kmGPXH2O2})\cdot(\mathrm{kmGPXgsh}+[\mathrm{cGSH}])^2}$$

$$+2\cdot\frac{\mathrm{cNADPH}\cdot\mathrm{vmgr}\cdot[\mathrm{cGSSG}]}{\mathrm{kmgrGSSG}\cdot\mathrm{kmgrNADPH}\cdot\left(1+\frac{\mathrm{cNADPH}}{\mathrm{kmgrNADPH}}+\frac{[\mathrm{cGSSG}]}{\mathrm{kmgrGSSG}}+\frac{\mathrm{cNADPH}\cdot[\mathrm{cGSSG}]}{\mathrm{kmgrGSSG}\cdot\mathrm{kmgrNADPH}}\right)}$$

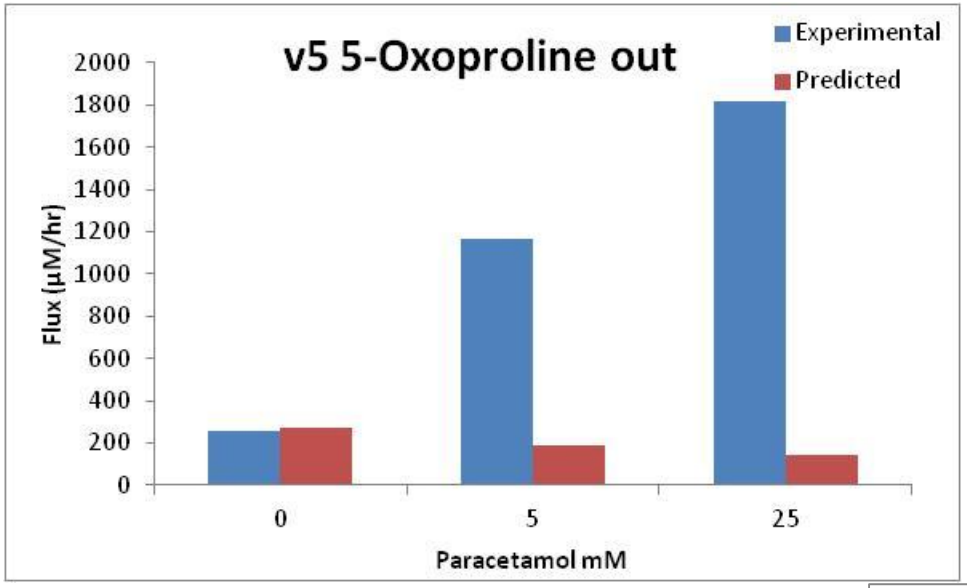
$$-\frac{\mathrm{vmgshoutl}\cdot[\mathrm{cGSH}]^3}{\mathrm{kmgshoutl}^3+[\mathrm{cGSH}]^3}-\frac{\mathrm{vmgshouth}\cdot[\mathrm{cGSH}]}{\mathrm{kmgshouth}+[\mathrm{cGSH}]}$$

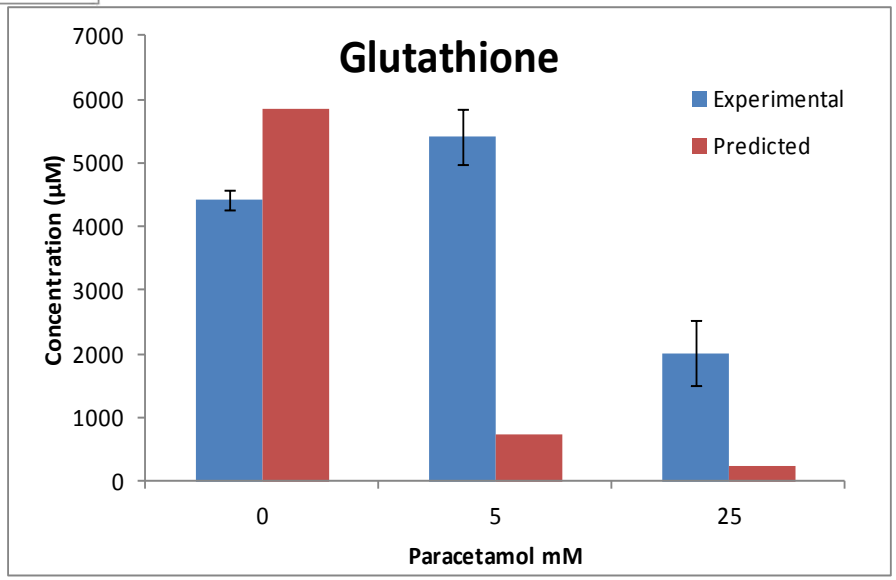
- **Used mostly literature values**
- **V_{max} and unknown parameters were fitted to experimental data**

Fitting to experimental data

- **Use an in vitro cell model (THLE-2E1cells)**
- **Incubate cells at three paracetamol concentrations: 0, 5mM and 25mM**
- **Measure (LC-MS) the concentration of metabolites in the glutathione pathway (intracellular and extracellular)**
  - **Measured 15 fluxes and concentrations using quantitative targeted metabolite determination by LC-MS**

Experiment vs Model

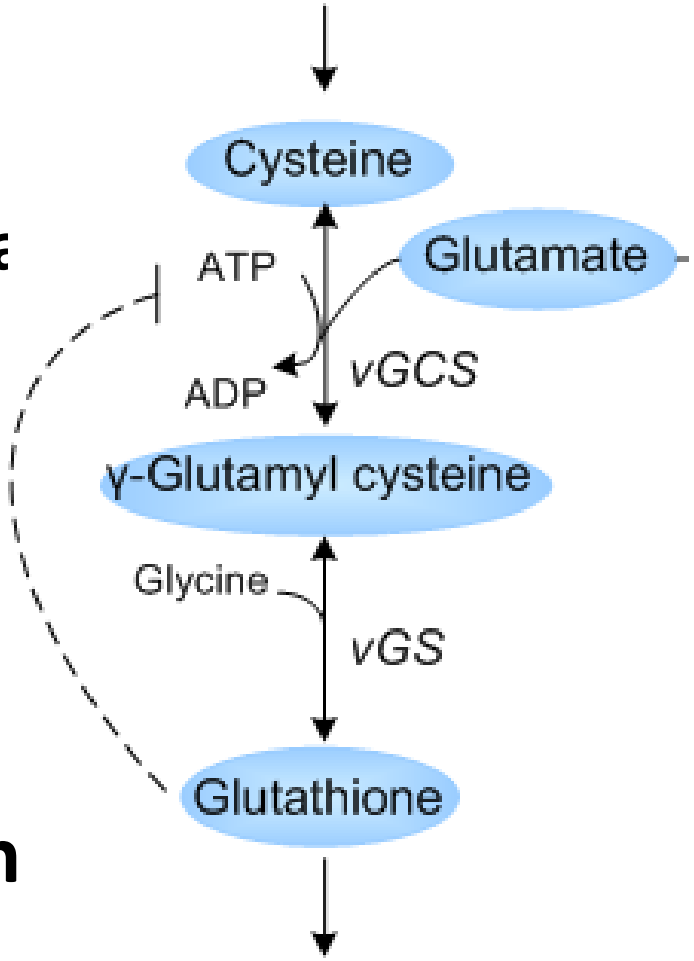




The model predicts the drug free conditions well, but not what occurs with increasing paracetamol exposure

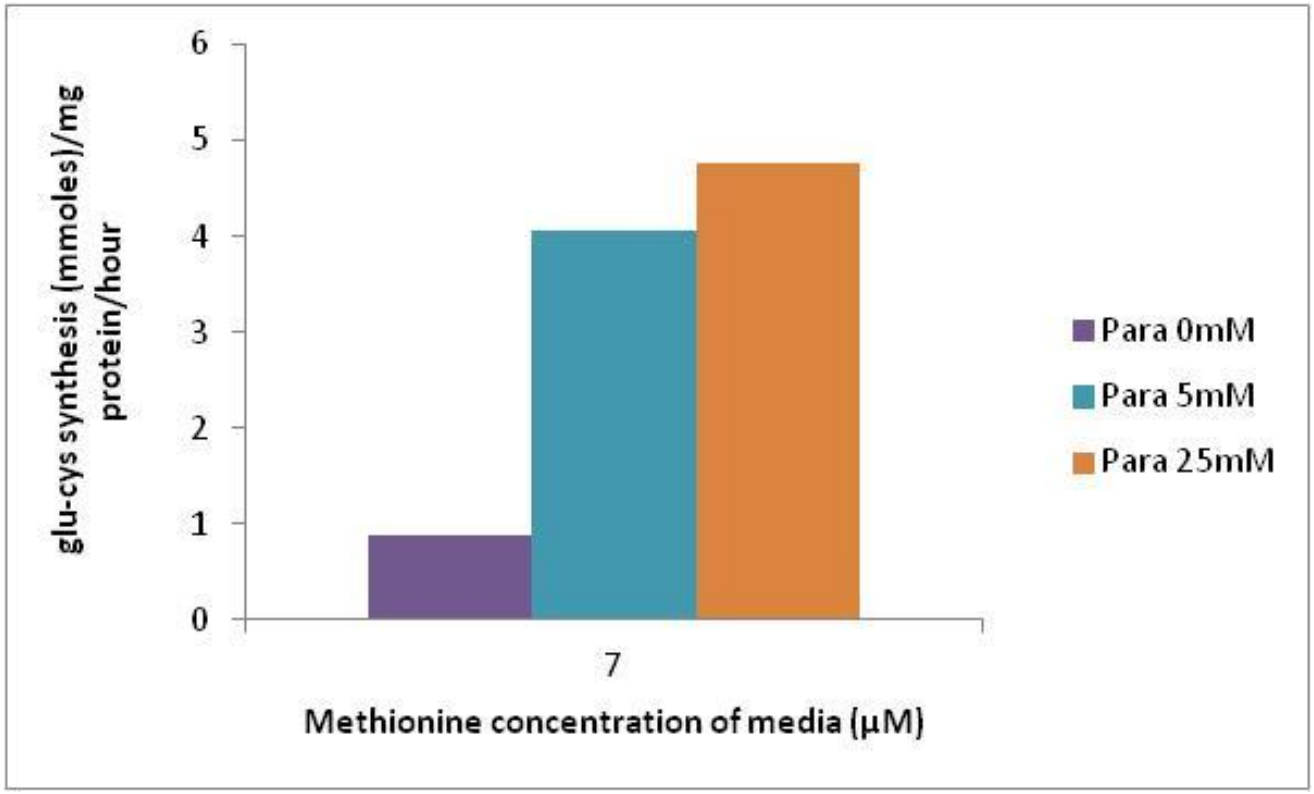
Model predicts control in glutamyl-cysteine synthetase (GCS)

- **Metabolic control analysis**
- **At 25mM paracetamol V_{GCS} had a control of:**
  - **1 on 5-oxoproline flux**
  - **0.9 on GSSG synthesis**
  - **2 on GSH export**
- **Increases in GCS reported in the literature**



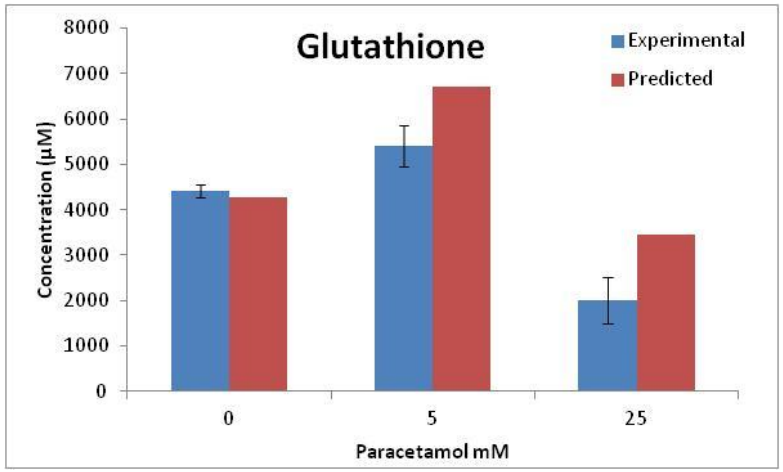
GCS activity vs paracetamol exposure

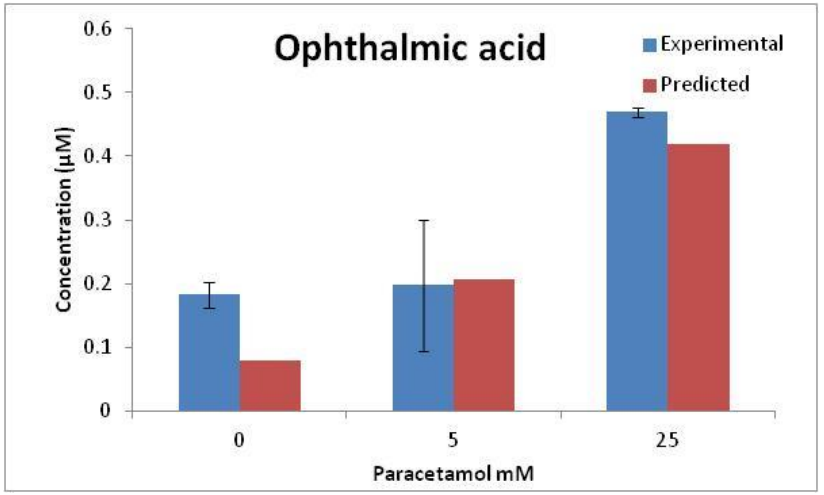
- **Enzyme activity assay**

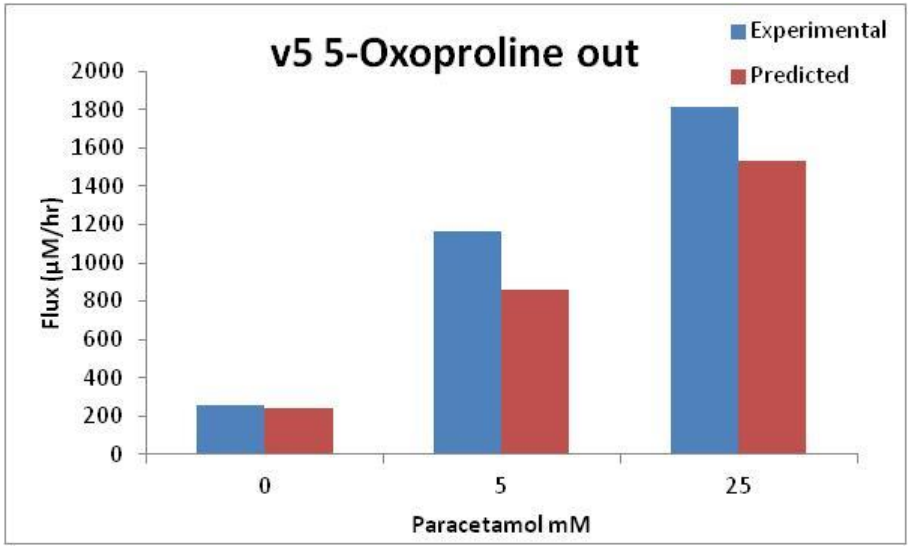


Add adaption of GCS to model

- **Good fits for GSH, OPA & 5-Oxo concentrations and fluxes**







Biomarking two stages of toxicology

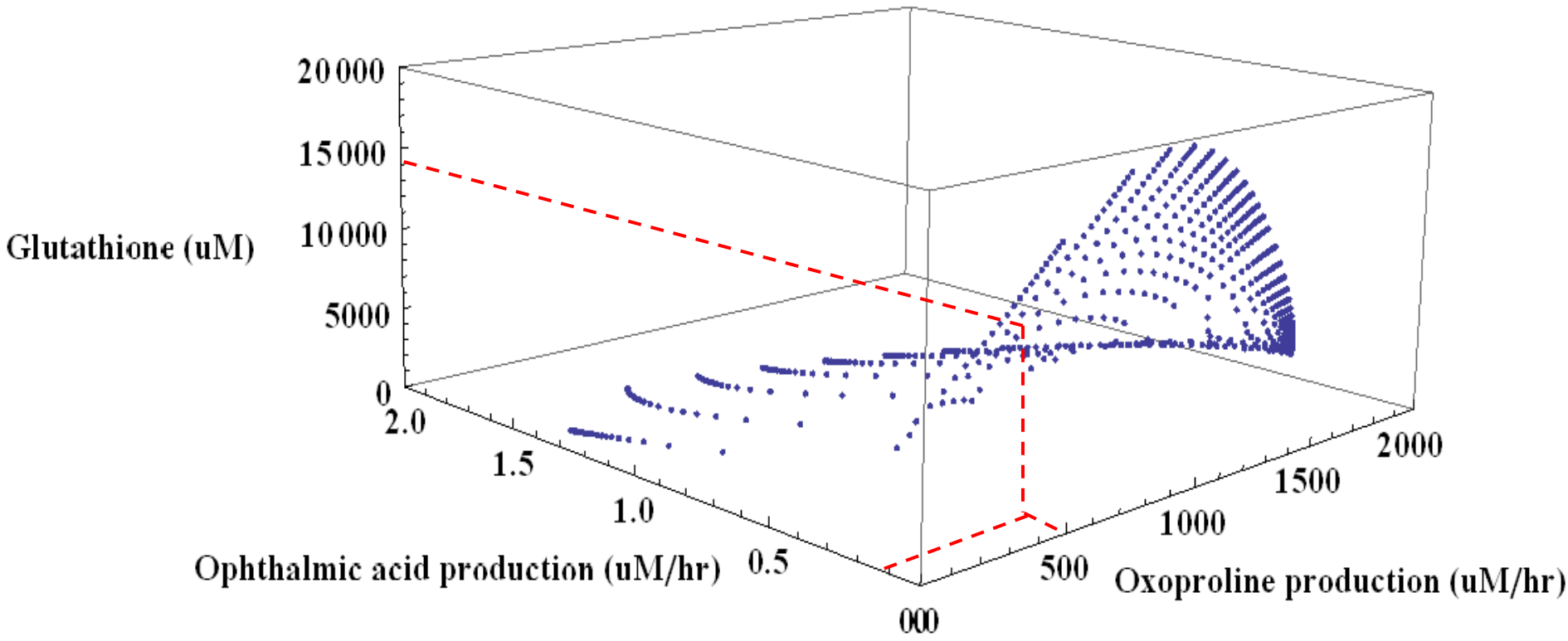
- Stage 1:
  - Paracetamol-conjugate formation
  - **Glutathione synthesis increases**
  - No depletion in cellular glutathione
- Stage 2:
  - Paracetamol-conjugate formation
  - Methionine and cysteine depleted
  - Glutathione synthesis decreases
  - **Depletion of cellular glutathione**

5-oxoproline

Ophthalmic acid

Biomarkers complement each other

- *In vitro* measuring biomarkers simultaneously will give unique glutathione concentration

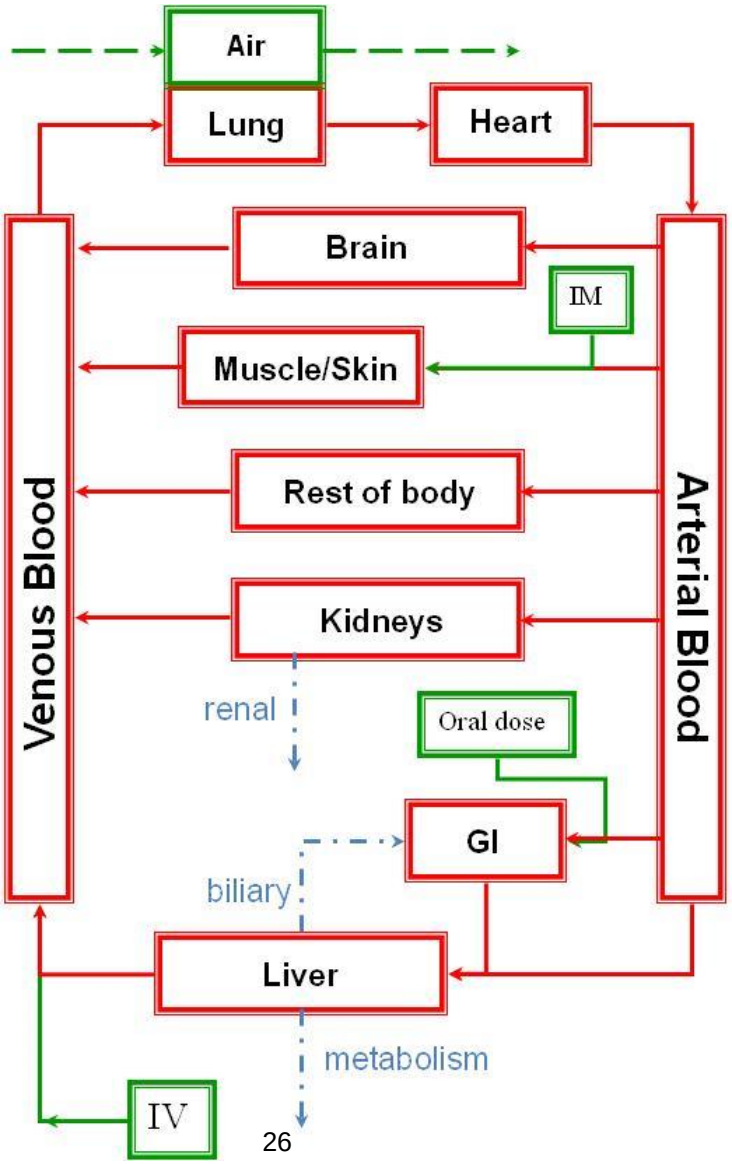


In vitro to *in vivo* –

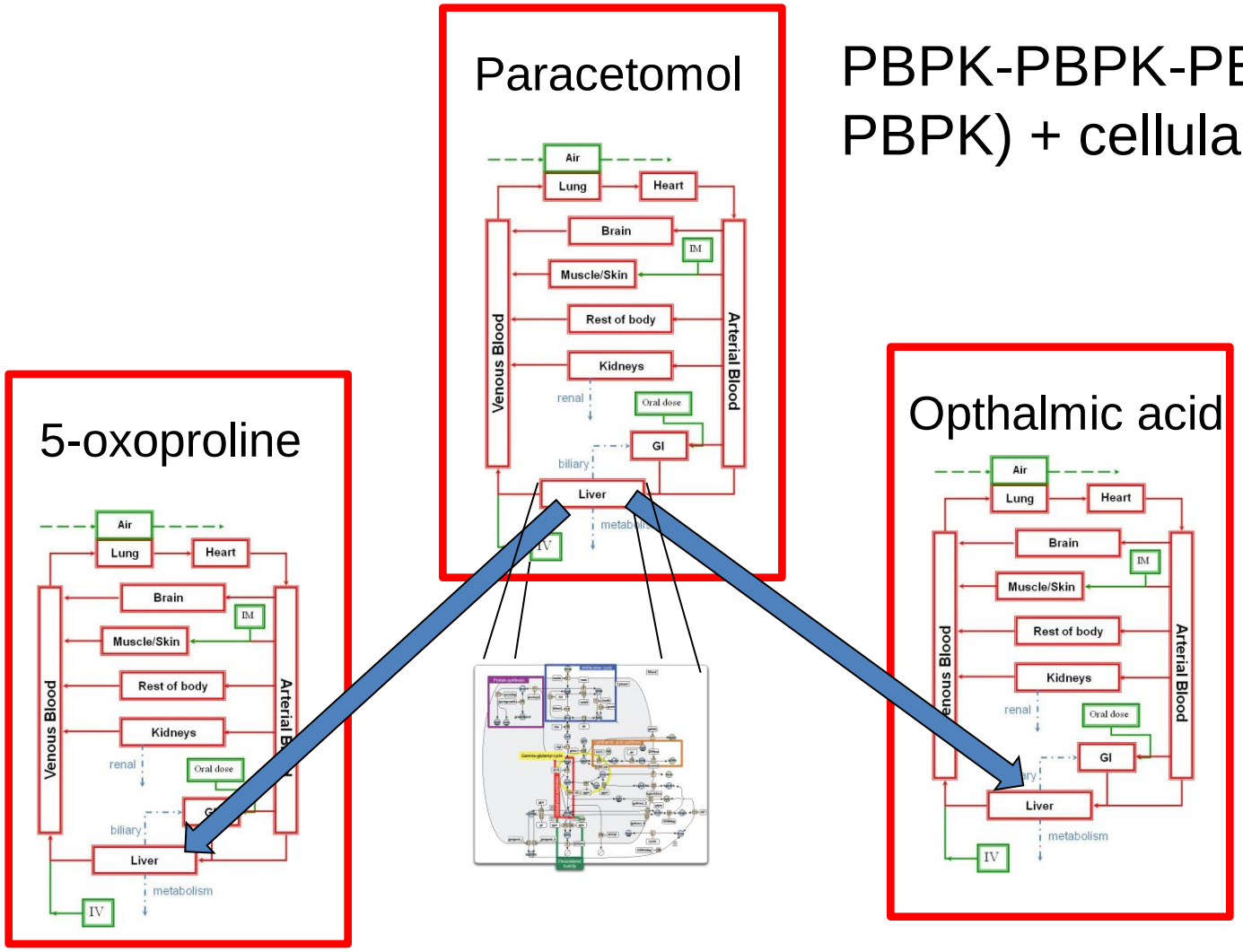
Physiologically Based PK Modelling (PBPK)

Organism mathematically described as a series of compartments that represent tissues and organs

- **Arranged to reflect anatomical layout**
- **Connected by the arterial and venous pathways**
- **Defined using literature tissue volumes and blood flow rates**
- **Progress of drug through the body can be followed by defining the discrete ADME properties**



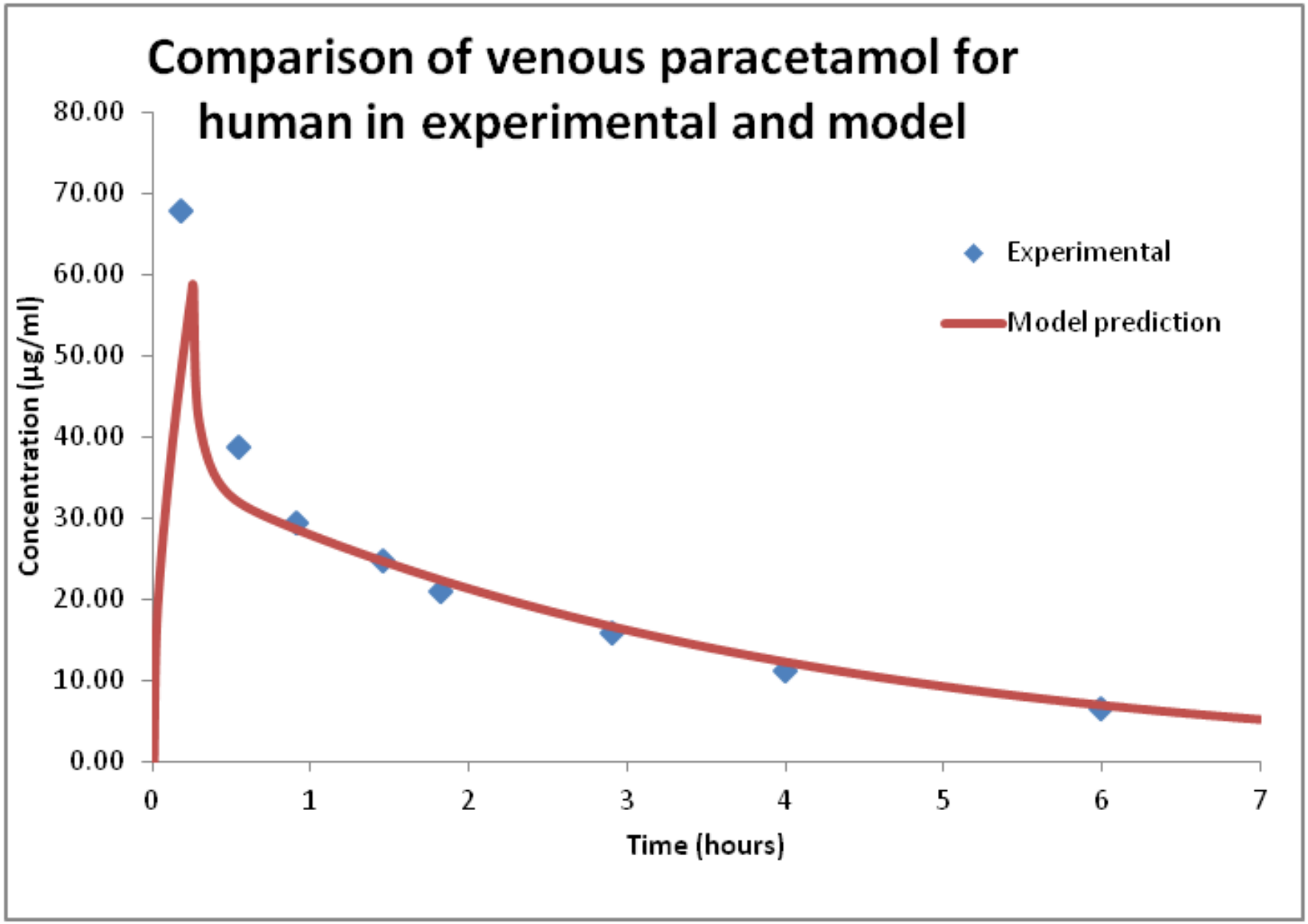
Model for Both Drug and Biomarkers



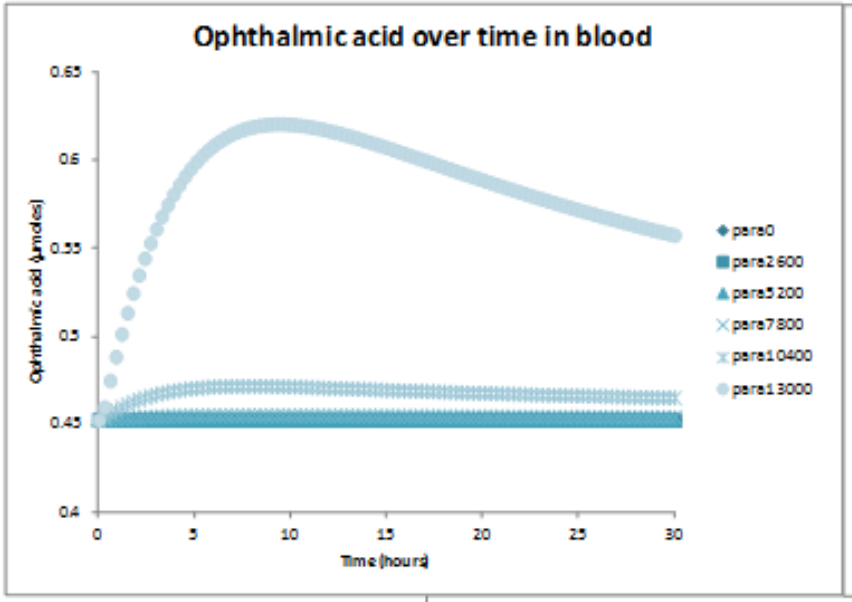
PBPK-PBPK-PBPK (Triple PBPK) + cellular model.

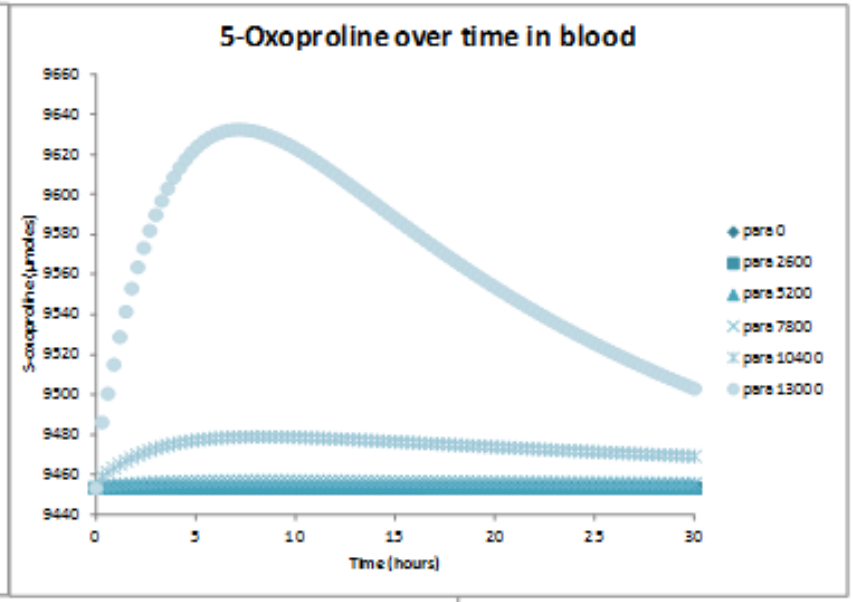
Before simulating the effects of paracetomol on the biomarkers we need to know that the PBPK models reproduces the literature results.

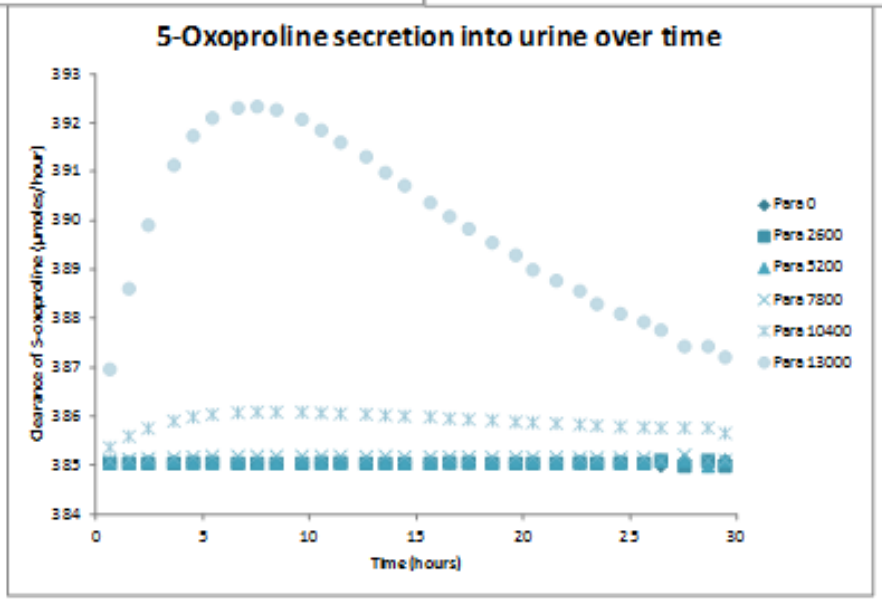
Paracetamol in Humans: Actual vs Predicted



Simulating the effects on 5-oxo and OPA after dosing paracetomol







Biomarkers raised in blood and urine

Conclusions

- **Biomarkers – discovering metabolites as *potential* biomarkers is easy**
- **But they only become real biomarkers when they have been validated**
- **And are only useful when you understand what they are telling you**
- **Biomarker validation requires targeted methods and bespoke studies**
- **Systems Biology should be a key to deeper understanding**

Acknowledgements

- Robert S. Plumb, Elaine Holmes, Jeremy K. Nicholson
- Eleni Gika, Filippos Michopoulos, Georgios Theodoridis
- Suzanne Geenen, Hans Westerhof
- Neil Loftus, Neil Kaplowitz, Cheng Ji