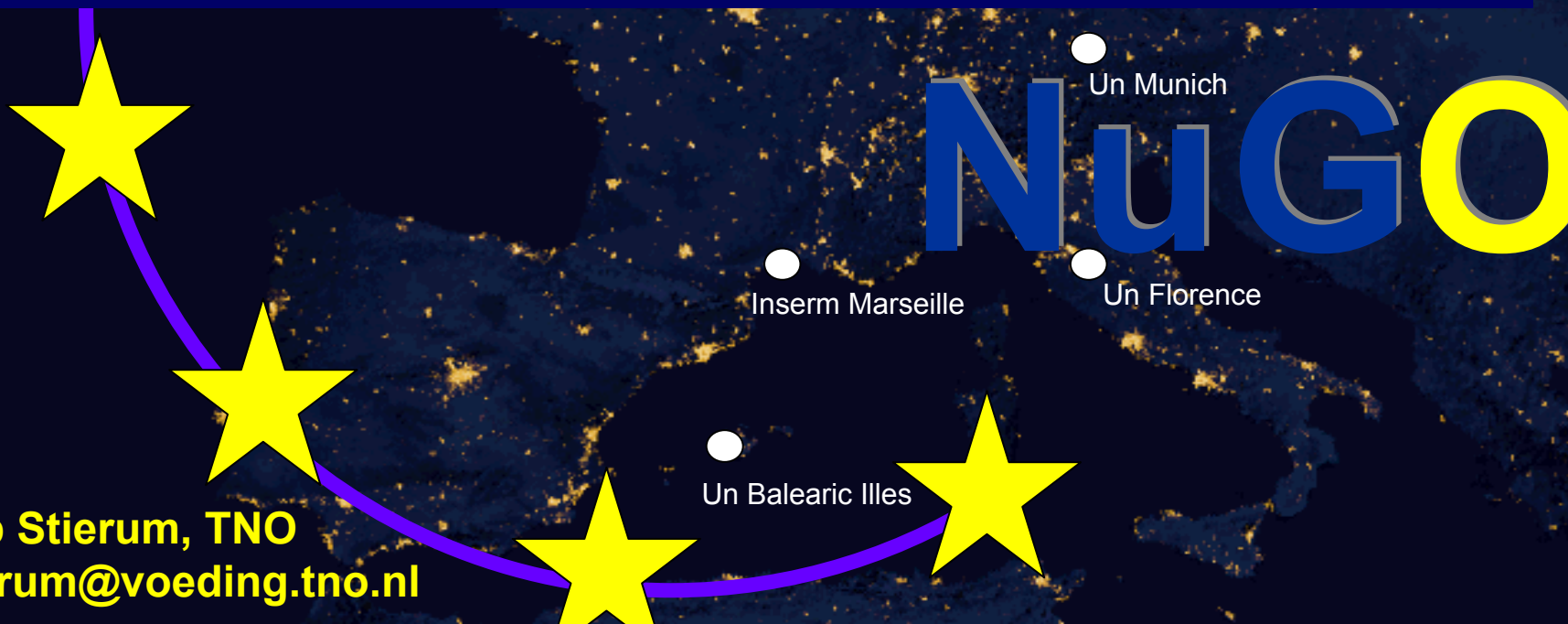


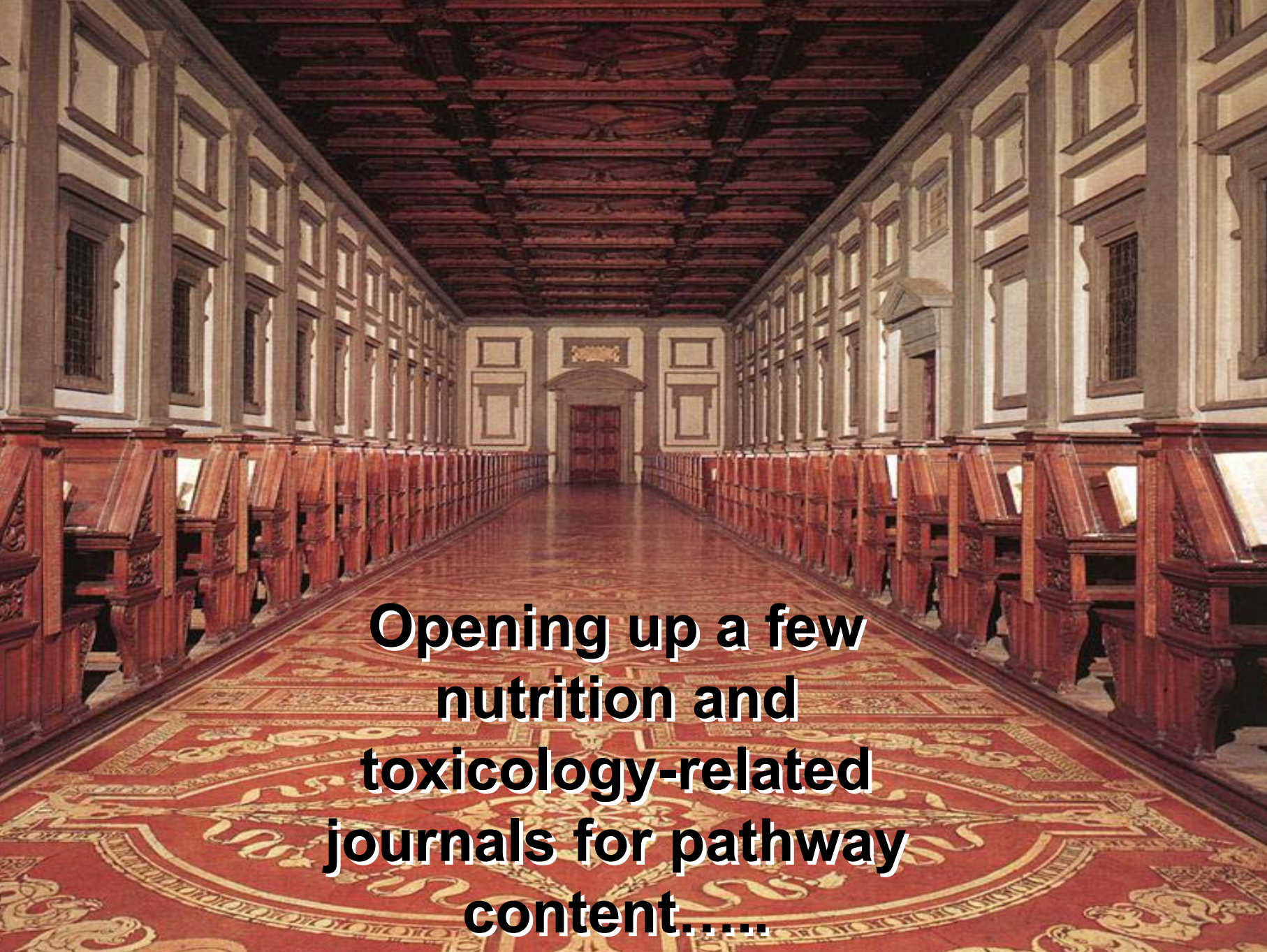


# Examples of pathway analysis in nutrigenomics and toxicogenomics studies



NuGO

Rob Stierum, TNO  
stierum@voeding.tno.nl

A photograph of a long, grand library hallway. The room features high ceilings with exposed wooden beams and intricate carvings. The walls are lined with tall, dark wood bookshelves that reach up to the ceiling. The floor is covered in a large, ornate red carpet with a complex gold and yellow geometric and floral pattern. At the far end of the hallway, there is a small, arched doorway. The lighting is warm and even, highlighting the rich wood tones and the detailed carpet design.

**Opening up a few  
nutrition and  
toxicology-related  
journals for pathway  
content.....**

## Alcoholic fermentation without yeast cells

1897 • *Eduard Buchner*

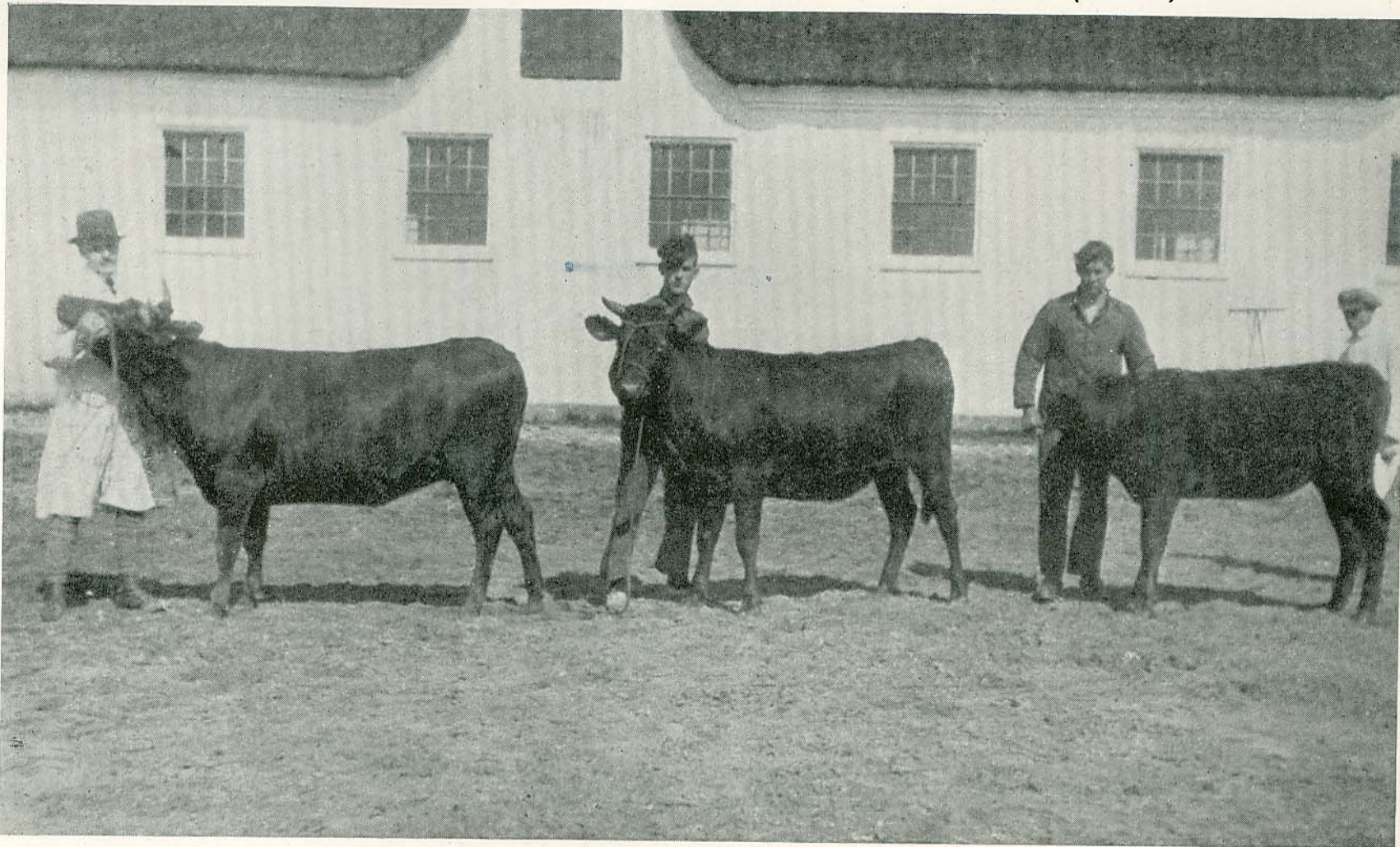
Buchner, Eduard. 1897. Alkoholische Gärung ohne Hefezellen. *Berichte der Deutschen Chemischen Gesellschaft*, Vol. 30, pages 117–124.

A SEPARATION OF THE FERMENTING power from living yeast cells has until now never been done. In the following a method is described which can be used to this end.

1000 g. of pure brewer's yeast cells, taken before the addition of potato starch, is mixed carefully with 1000 g. of quartz sand and 250 g. of Kieselguhr, and ground until the mass has

still... life seemed quite simple in the pre-genomics era...

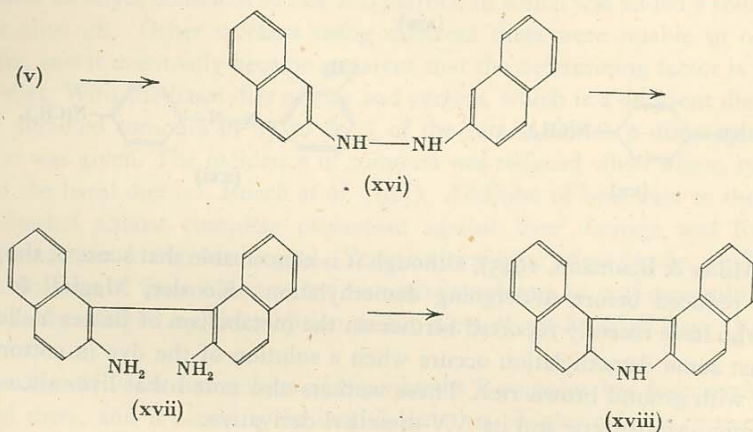
*British Journal of Nutrition*, (1947) 1, 245-253



Typical 15 months old heifers from groups on heavy (C), normal (A),

*British Journal of Nutrition, (1947) 1, 245-253**Mechanism of production of liver tumours by azo-compounds*

It has been generally assumed that the production of liver tumours by feeding or injecting azo-compounds is due to their transformation in the liver into other compounds which are the true carcinogenic agents. This assumption is largely based on the specific action which these azo-compounds have on the liver. It is true that the production of sarcomas at the site of injection of oily solutions of azo-dyes has been reported by several workers, but this cannot be dissociated from the influence of fatty solvents which are themselves known to produce similar effects. In the case of 2:2'-azonaphthalene, it was suggested (Cook *et al.* 1940) that the true carcinogen, formed in the liver, is 3:4:5:6-dibenzcarbazole, which might be formed in vivo by the following stages, which are readily brought about in the laboratory:



In support of this view it was shown that the postulated intermediate diamine (xvii) produced liver tumours of cholangiomatous type rapidly and in abundance when administered to mice. This di-amine is readily de-aminated to 3:4:5:6-dibenzcarbazole, which had previously been shown by Boyland & Brues (1937) to produce liver tumours in mice and also local tumours at the site of application. Further support was given to this view of the mechanism of carcinogenesis by 2:2'-azonaphthalene by some experi-

|    |      |      |        |
|----|------|------|--------|
| 平均 | 1.82 | 3.07 | 量は異なる。 |
|----|------|------|--------|

2) 含有する酸と還元糖量の比率は盛熟期にては、概ね 1:3、或は 1:4 なり。

3) A、果汁をそのまま放置する時は、破袋後6時間後迄、酸量は減少する。(但し  $t=10^{\circ}\text{C}$ )  
この減量現象はクロロホルムを加へても尙、続行するを以て、単なる細菌に依る醗酵作用ではなく、恐らく酵素の作用に依り枸橼酸が分解されるものと思れる。

$$\begin{array}{ccccc}
 \begin{array}{c} \text{CH}_2\text{COOH} \\ | \\ \text{C}(\text{OH})\text{COOH} \\ | \\ \text{CH}_2\text{COOH} \\ \text{Citronen Säure} \end{array} & \xrightarrow{-\text{CO}_2} & \begin{array}{c} \text{CH}_2\text{COOH} \\ | \\ \text{CHOH} \\ | \\ \text{CH}_2\text{COOH} \\ \beta\text{-Oxyglutal säure} \end{array} & \xrightarrow{-\text{H}_2\text{O}} & \begin{array}{c} \text{CH}_2\text{COOH} \\ | \\ \text{CO} \\ | \\ \text{CH}_2\text{COOH} \\ \text{Aceton dicarbon säure} \end{array}
 \end{array}$$

B、8時間以後、酸量は再び増加し、醋酸の存在を認める。クロロホルムの投入により、酸の増加を阻止し得るは、恐らく細菌の作用ならむと思れる。

$$\begin{array}{ccc}
 \begin{array}{c} \text{CH}_2\text{COOH} \\ | \\ \text{C}(\text{OH})\text{COOH} \\ | \\ \text{CH}_2\text{COOH} \\ \text{Citronen Säure} \end{array} & \longrightarrow & \begin{array}{c} \text{CH}_3\text{COOH} \\ \text{Essig Säure} \\ \text{COOH} \\ | \\ \text{CO} \\ | \\ \text{CH}_3 \\ | \\ \text{COOH} \\ \text{Oxalessig säure} \end{array}
 \end{array}$$

( 15 )

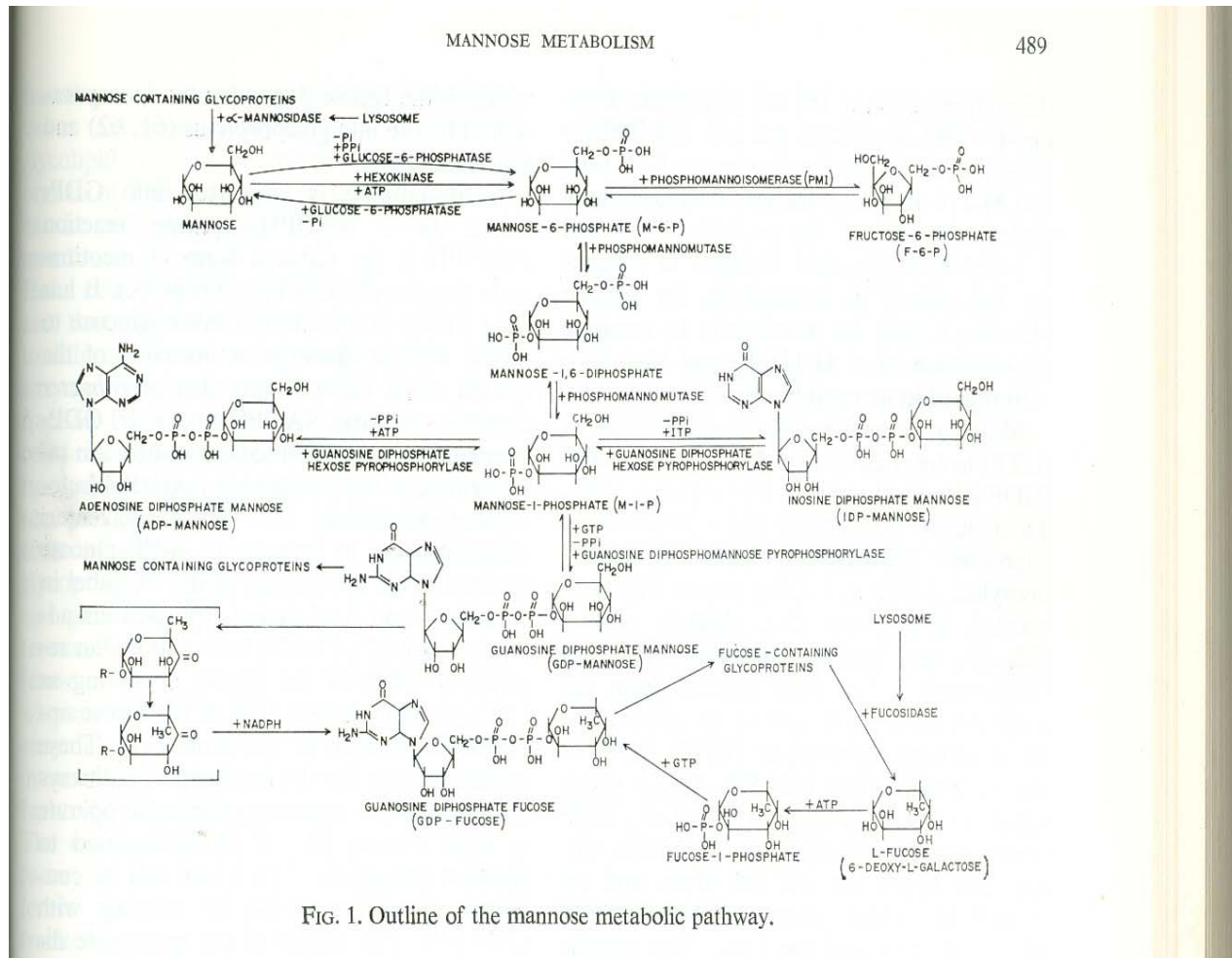
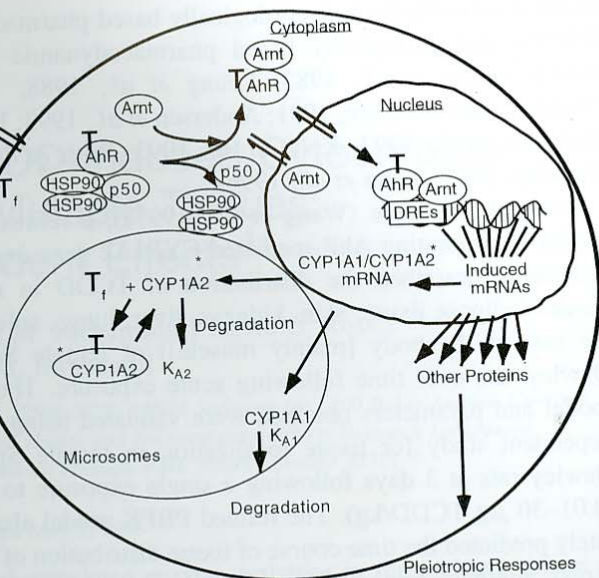
Mannose metabolism: *Am. J. Clin. Nut.* (1971), 24, 488-498

FIG. 1. Outline of the mannose metabolic pathway.



2 Liver only

1. Proposed AhR-mediated mechanism of the biologically based pharmacodynamic (BBPD) model for cytochrome P450 gene expression by TCDD. This proposed mechanism of TCDD-induced cytochrome P450 gene expression has been extensively reviewed (Safe, 1986; Whitlock, 1993; Birnbaum, 1994a; Hankinson, 1995). Ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-demethylase (MROD) activities are markers for CYP1A1 and CYP1A2 (Chaloupek *et al.*, 1995) gene expression,

## PHARMACODYNAMIC ANALYSIS OF CYTOCHROME P450

Assuming that the noise (both residual error and inter-individual variability) in the experimental data follows normal distribution, the lower and upper bound of the experimental results at each dose can be obtained, as shown below, when choosing a 95% confidence level:

$$\text{lower bound} = \text{mean value} - t_{0.025} * \text{SD}/(\text{number of animals})^{1/2}$$

$$\text{upper bound} = \text{mean value} + t_{0.025} * \text{SD}/(\text{number of animals})^{1/2},$$

where  $t_{0.025} = 3.2$  if  $n = 4$ ;  $t_{0.025} = 2.8$  if  $n = 5$ .

The judgment can also be made based on whether the hypothesis, the experimental data is close to model predictions at each dose, is acceptable or not. Based on the same assumption of normal distribution of experimental samples, the observed value of the test statistic is

$$\text{observed value of the test} = (\text{mean value-model pred.})/\text{SD}/(\text{no. of animals})^{1/2}.$$

The rejection region, with 5% level, is given by 3.2 for a group of 4 animals and 2.8 for a group of 5 animals. If the observed value  $< 3.2$  ( $n = 4$ ) or  $< 2.8$  ( $n = 5$ ), the hypothesis is acceptable.

### Mathematical Expressions of the Model

The mathematical expressions of the PBPK model were given previously (Wang *et al.*, 1997a). The mathematical expressions for CYP1A1 and CYP1A2 induction, as well as EROD and MROD activity, are given below.

**CYP1A1 and EROD induction.** The change of CYP1A1 with time in the liver was described by the model for a stimulation process proposed by Dayneka *et al.* (1993) and Andersen *et al.* (1993), as shown in Eq. 1.

=  $\text{EROD}_k/\text{Coe}_{A1}$ , where, subscripts. The induction of EROD activity is given by Eq. 4.

$$\frac{d\text{EROD}_k}{dt} = K_{\text{OERK}} \left( 1 + \ln_{A1k} \right)$$

where  $K_{\text{OERK}} = K_{\text{ER}} \text{EROD}_{\text{BSK}}$ ,  $K_{\text{ER}}$  is the first order degradation rate, and  $\ln_{A1k}$  is the induction factor. In addition, without TCDD,

therefore,  $K_{\text{OERK}} = K_{\text{ER}} \text{EROD}_{\text{BSK}}$ .

### Hepatic CYP1A2 and MROD

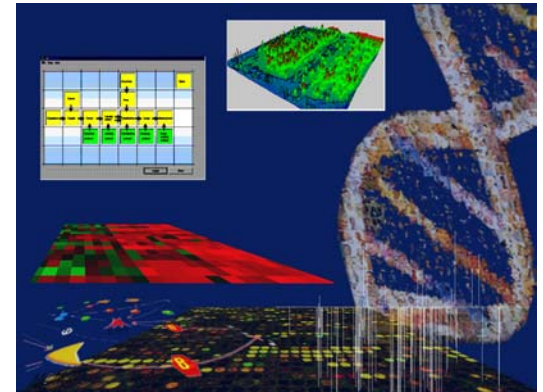
induction was presented previously (Wang *et al.*, 1997a).

$$\frac{dW_{L1}C_{A2t}}{dt} = K_{0A2} \left( 1 + \ln_{A2t} \right)$$

at  $t = 0$ ,  $C_{A2t} = C_{A2BS}$ .

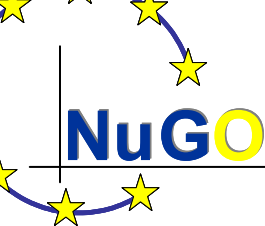
Since the concentration of CYP1A2 is low,  $dW_{L1}C_{A2t}/dt = 0$ . Therefore,  $K_{0A2}$  is the total and basal CYP1A2 concentration.  $K_{0A2}$  is the zero-order basal rate for CYP1A2 induction.  $\ln_{A2t}$  is the constant for CYP1A2 degradation.  $K_{0A2}$  is the CYP1A2 protein expression, and  $\ln_{A2t}$  is the induction factor, which equals  $\text{AhR} \cdot \text{TCDD}$ .

Establishing pathways in nutrition and toxicology from transcriptome, proteome and metabolome datasets



Several approaches:

- ‘manual’ : use your brain; current knowledge
- tools: pathway mapping and biological network tools
- statistical networks → biological inference



# Case I 'use your brain' + tool

---

Discovery of functional genomics-derived new biomarkers to assess the hepatotoxic effects at the mechanistic level of combined exposure to food additives.





**thiabendazole**

**Administration of additives through the diet for 28 days,  
male Sprague-Dawley rats (n=6)**

**control**

**thiabendazole (TB)  
(102-5077 ppm)**

**Clinical signs, clinical chemistry and liver histopathology**

**Total RNA isolation from liver**

**cDNA array**

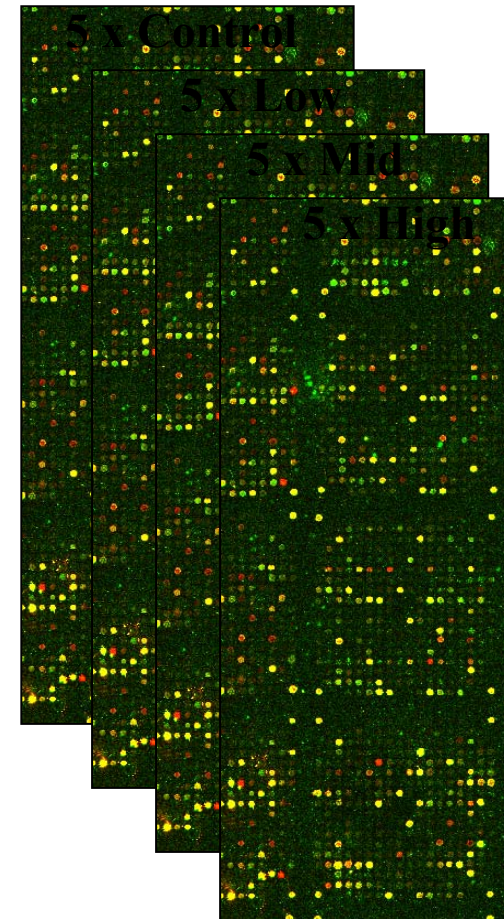
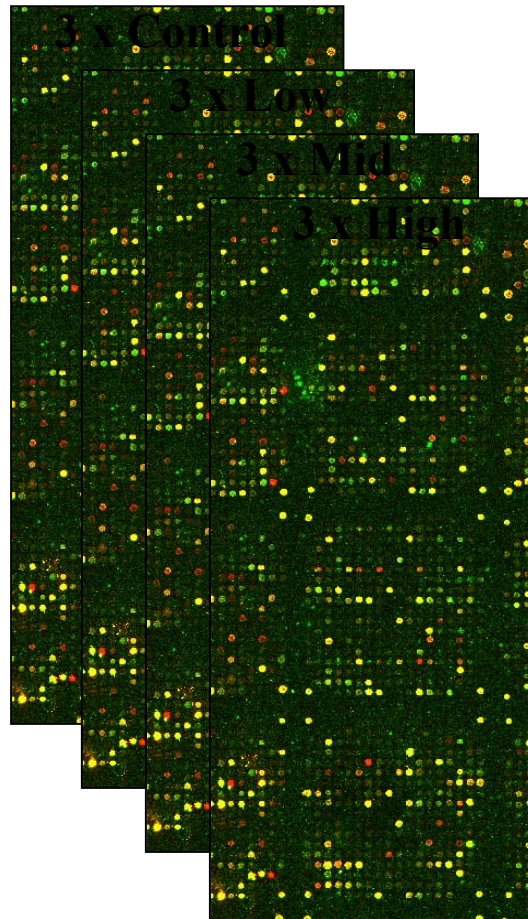
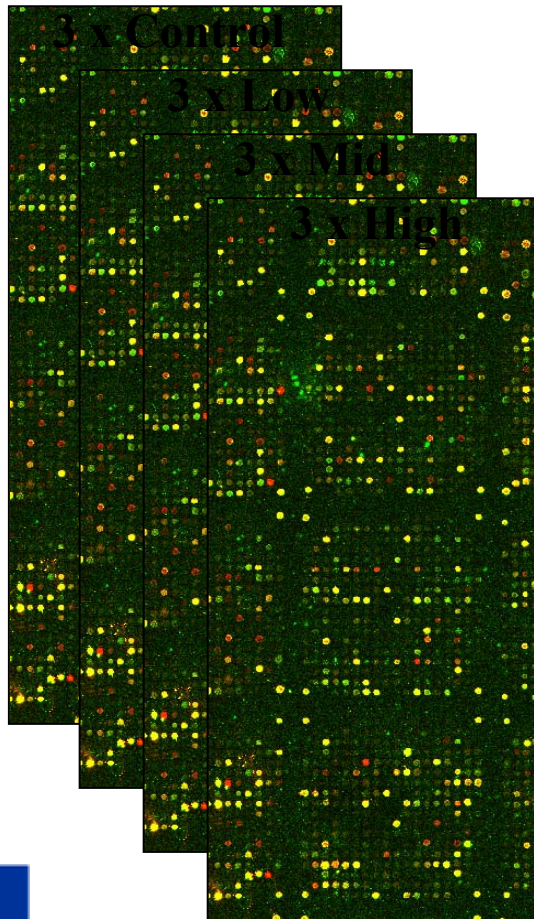
**Data analysis**

**•Criteria for selecting genes:**

- maximum of one missing value per dose response curve,
- maximum CV of difference between the dye swap of 0.5 (dye swaps should behave in similar direction
- at least 1.5 fold difference between any of the doses/compound

**Confirmation of microarray data findings for selected genes at the biochemical level:**

- BHT and TB: RT PCR for CYP2B1 and CYP2B1/2; 7-ethoxyresorufin O-deethylase (CYP1A) and 7-pentoxyresorufin O-depentylase (CYP2B) activity.
- CC: palmitoyl-CoA oxidation as marker for peroxisome proliferation
- TB: cellular tumor antigen p53 ELISA
- All additives: western blotting for CYP1A2 and CYP2B1/2



‘Manual’ network creation, concordance with a bioinformatics approach performed two years after

Network building around p53 protein, using Metacore<sup>TM</sup> software from GeneGO, comparison with manually created network from array results from thiabendazole experiment

## Systems Biology for Drug Discovery



**GeneGo, Inc**

**[www.genego.com](http://www.genego.com)**



### LEGEND



- Around p53 protein
- Making us of biological dB
- Filtered to reduce complexity:
  - for ‘rat ortholog’
  - for ‘transcriptional regulation’
  - for ‘liver’



edges while dragging

## Root Objects

## Objects

☒ Highlight on rolling over☒ Show effects

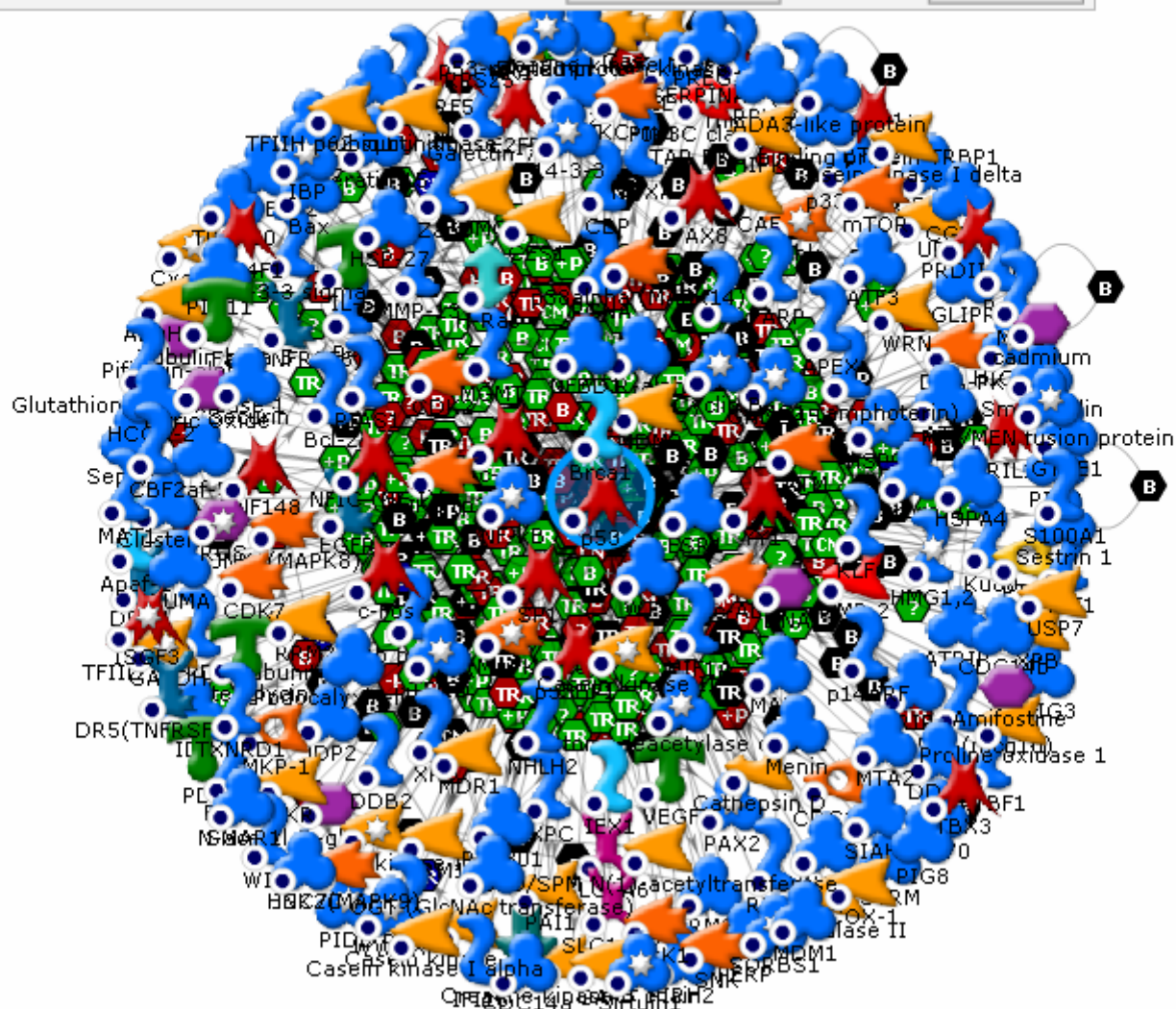
☐ Lock non-traced

☐ Drag neighbours☒ Show expression

Trace selected

☒ Show hints

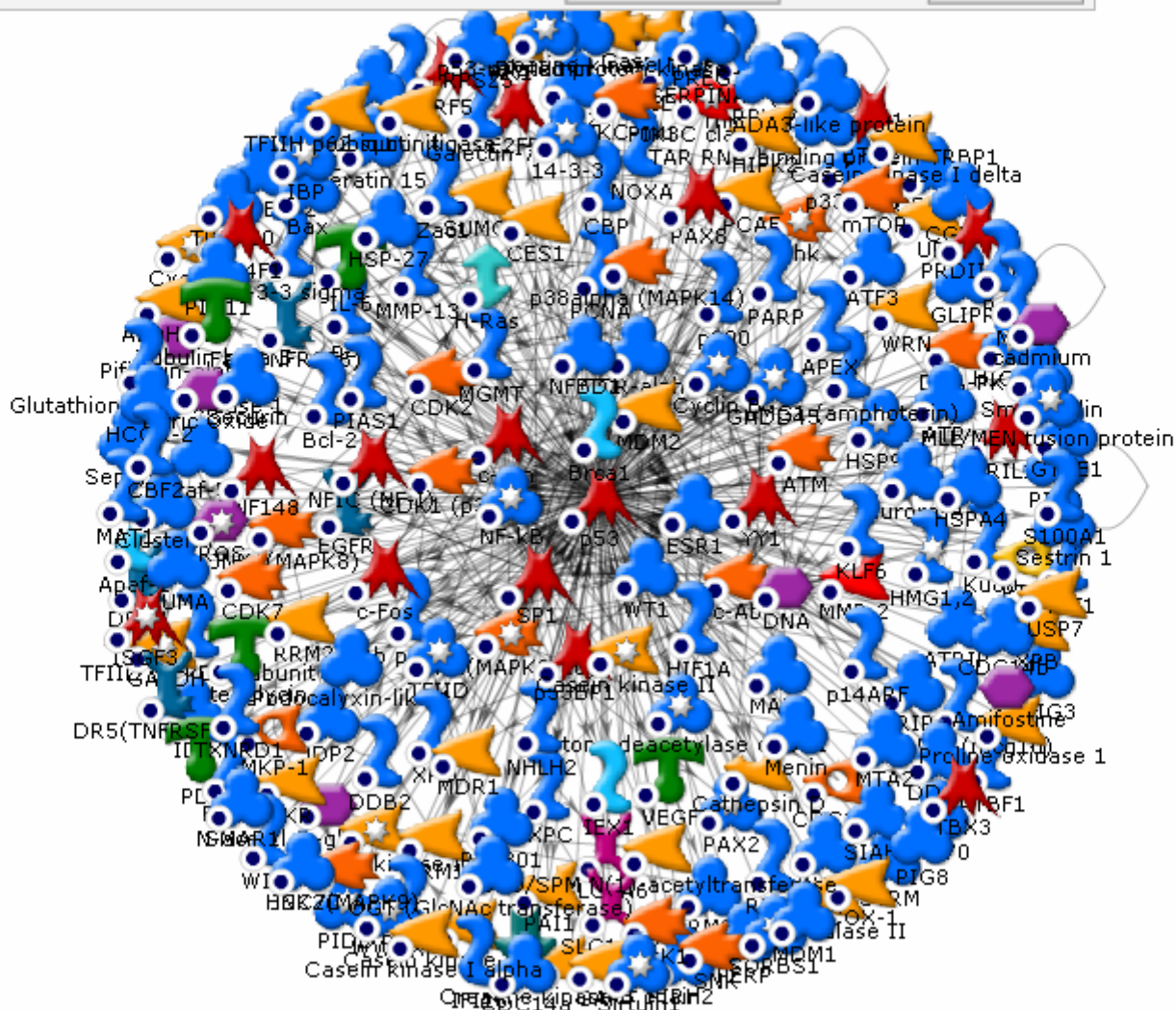
Reset





Edges while dragging  
Root Objects  
e Objects

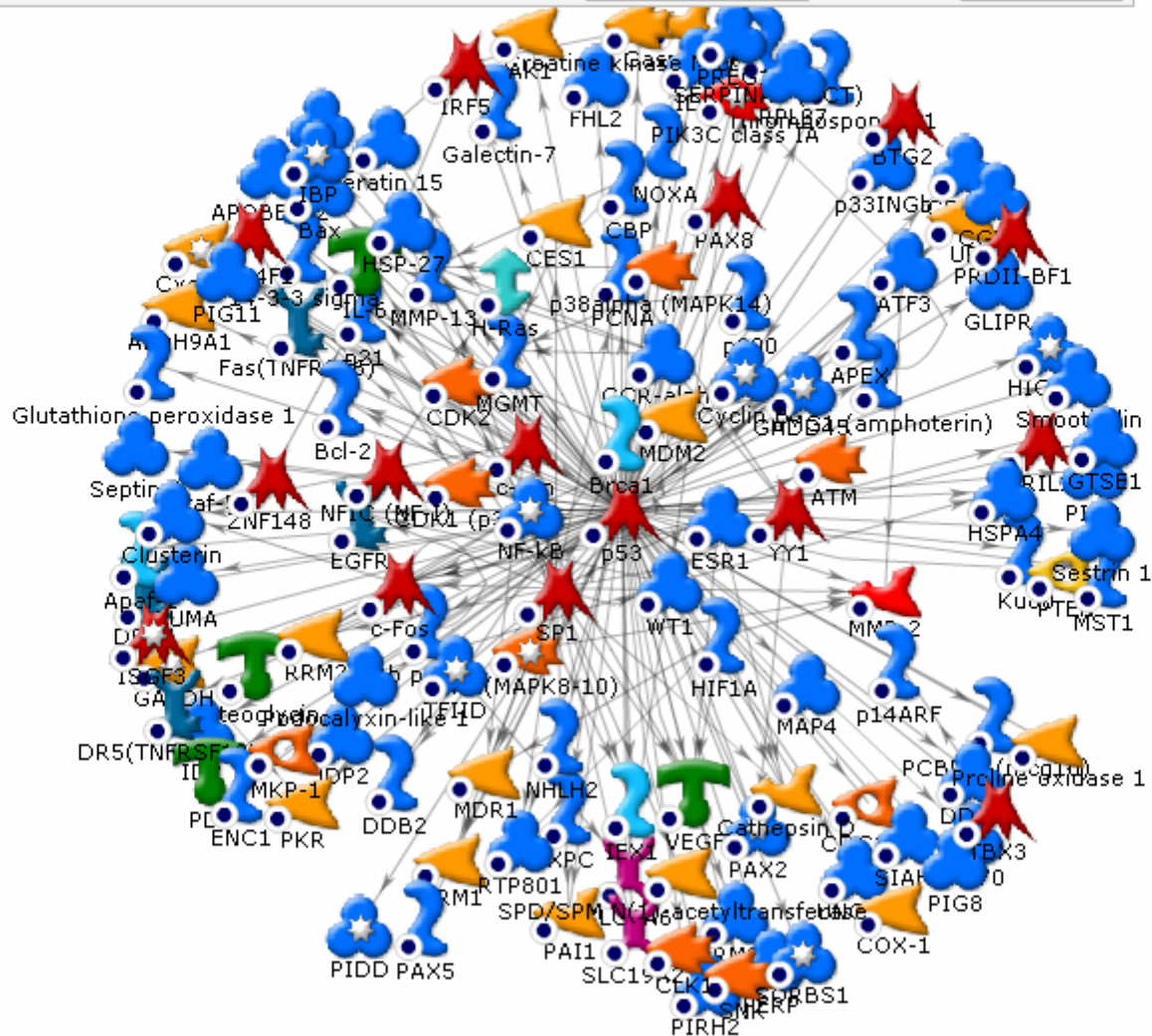
- ☐ Highlight on rolling over  
☐ Show effects  
☐ Lock non-traced
- ☐ Drag neighbours  
☒ Show expression
- ☐ Show hints



☐ Highlight on rolling over
 ☐ Drag neighbours
 ☐ Show hints

☐ Show effects
 ☒ Show expression

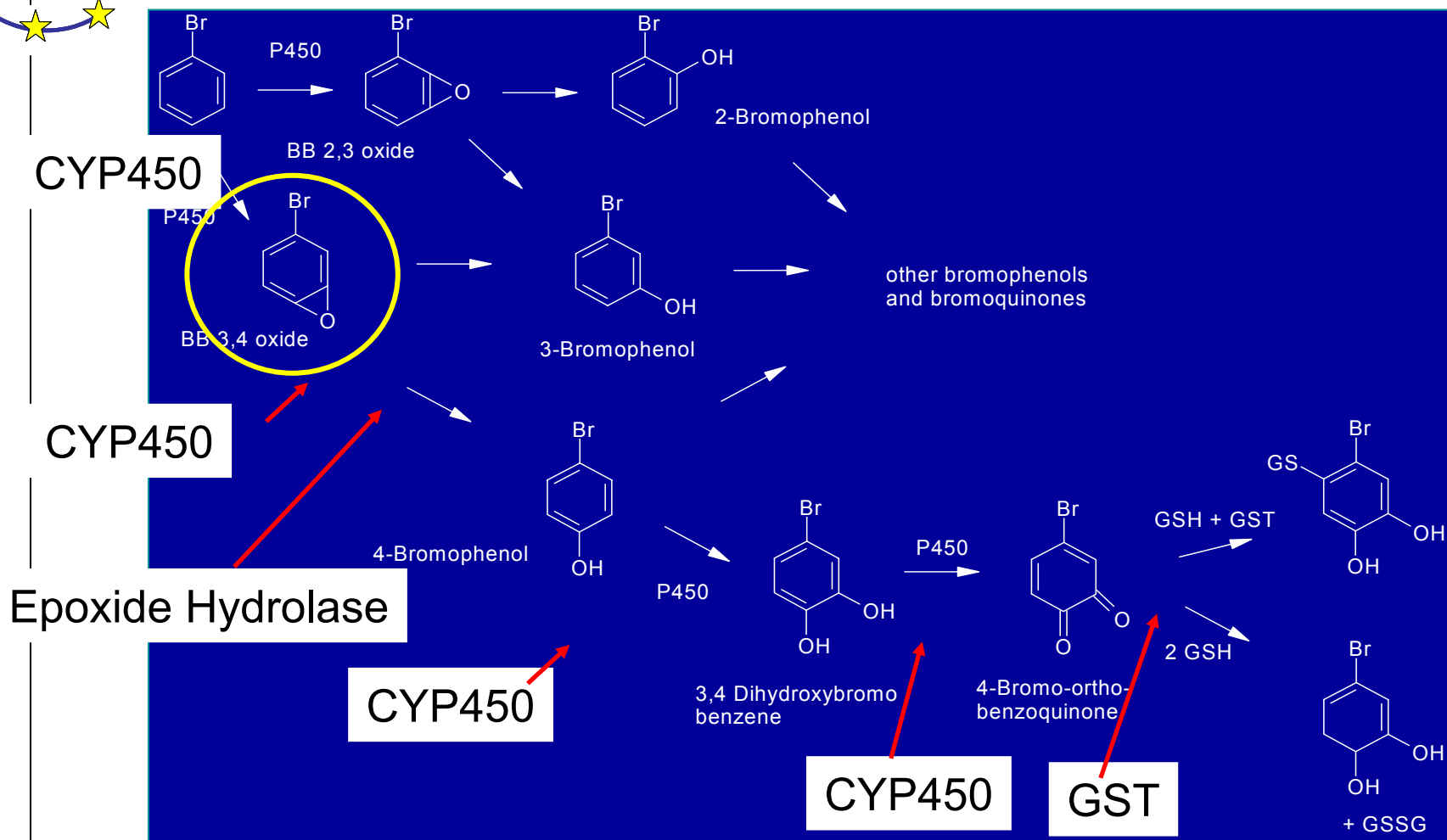
☐ Lock non-traced



- Transcriptome, proteome and metabolome changes in rats exposed to bromobenzene

- Based on work by Wilbert Heijne:
  - Wilbert H.M. Heijne, Rob H. Stierum, Monique Slijper, Peter J. van Bladeren and Ben van Ommen (2003) Toxicogenomics of bromobenzene hepatotoxicity: a combined transcriptomics and proteomics approach. *Biochemical Pharmacology*, 2003 Mar 1;65(5):857-75.
  - Heijne WH, Slitt AL, Van Bladeren PJ, Groten JP, Klaassen CD, Stierum RH, Van Ommen B. (2004) Bromobenzene-induced hepatotoxicity at the transcriptome level. *Toxicol Sci*. 2004 Jun;79(2):411-422.
  - Wilbert H.M. Heijne et al. Metabolome changes in bromobenzene treated rat liver, accepted

# Biotransformation of bromobenzene in NuGO rat liver



## I. Intraperitoneal dosing of rats

- **High (5 mmol/kg), untreated and solvent controls**
- **Liver isolation at 24 hrs.**
- **Transcriptomics and proteomics**

## II. Oral dosing of rats n=3

- **Low (0.5 mmol/kg)**
- **Mid (2 mmol/kg)**
- **High (5 mmol/kg)**
- **Untreated and solvent controls**
- **Liver isolation at 6, 24 and 48 hrs.**
- **Transcriptomics and metabolomics**

# BB upregulated (oral)

NuGO

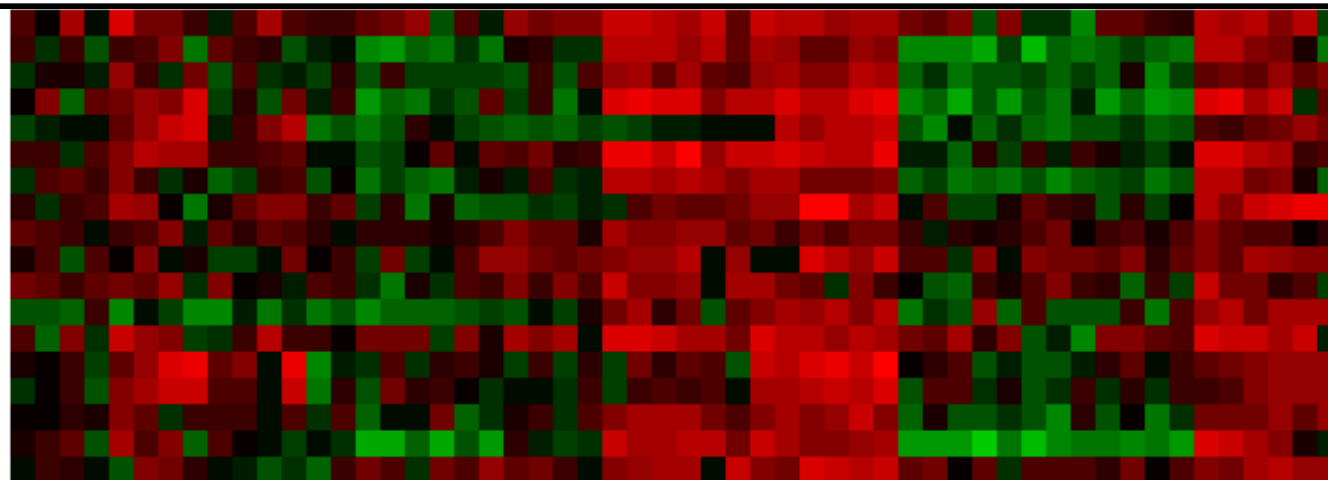
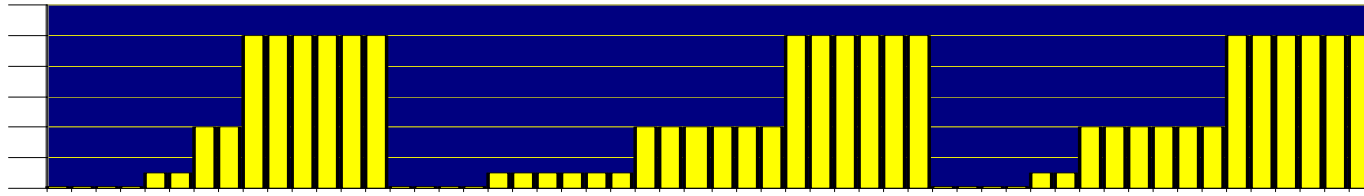
6 hours

24 hours

48 hours

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100

Dose (mmol/kg BW)



AA819472  
AA818339  
AA875245  
AA900551  
AA874884  
AA923966  
AA998734  
AI029162  
AI058620  
AI145442  
AI059976  
AA817701  
AA817693  
AA874884  
AA957593  
AA875245  
AA818339  
AI072634

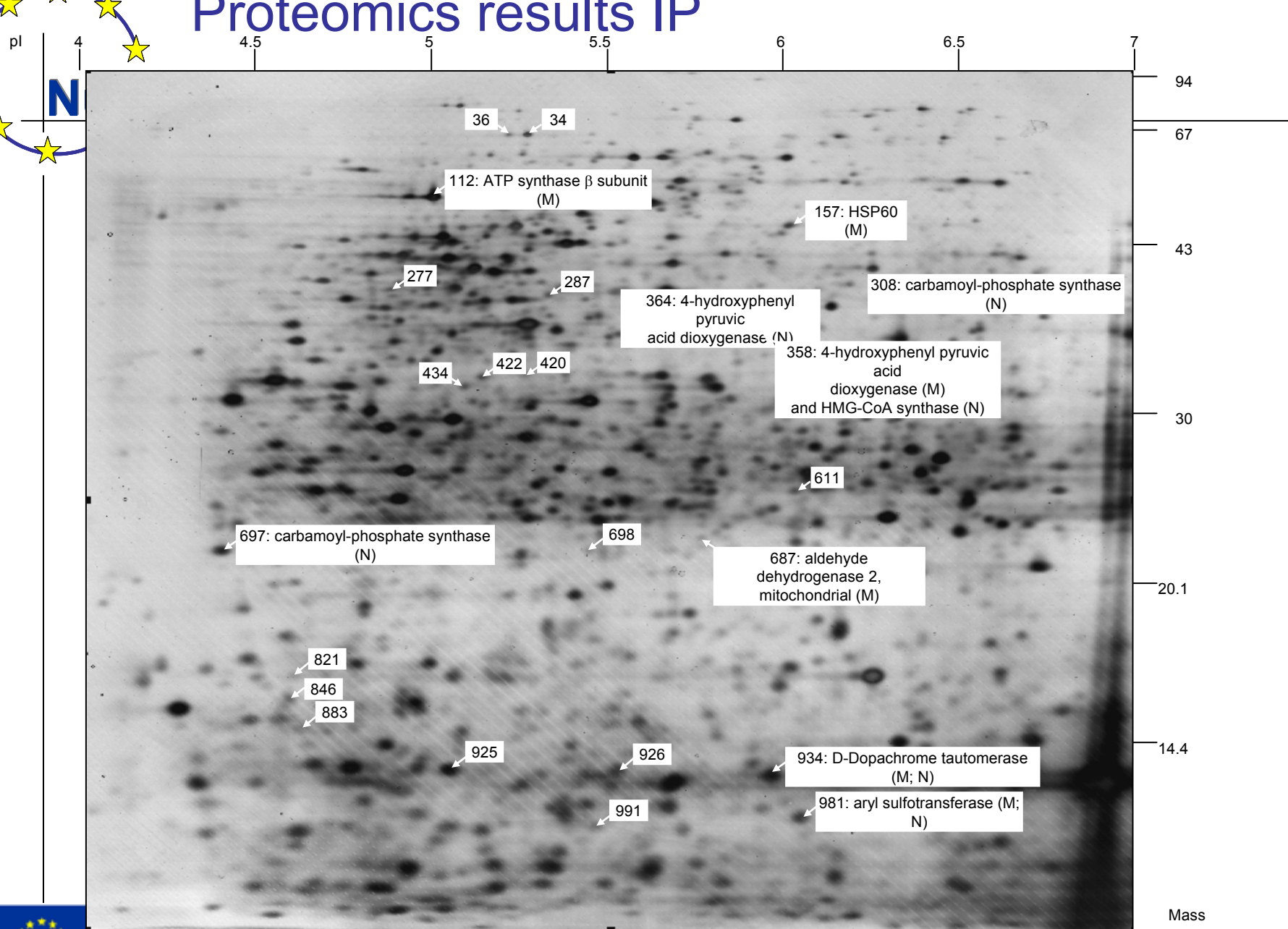
GST A  
Gamma-GCS  
Ferritin  
Heme oxygenase  
Peroxiredoxin1



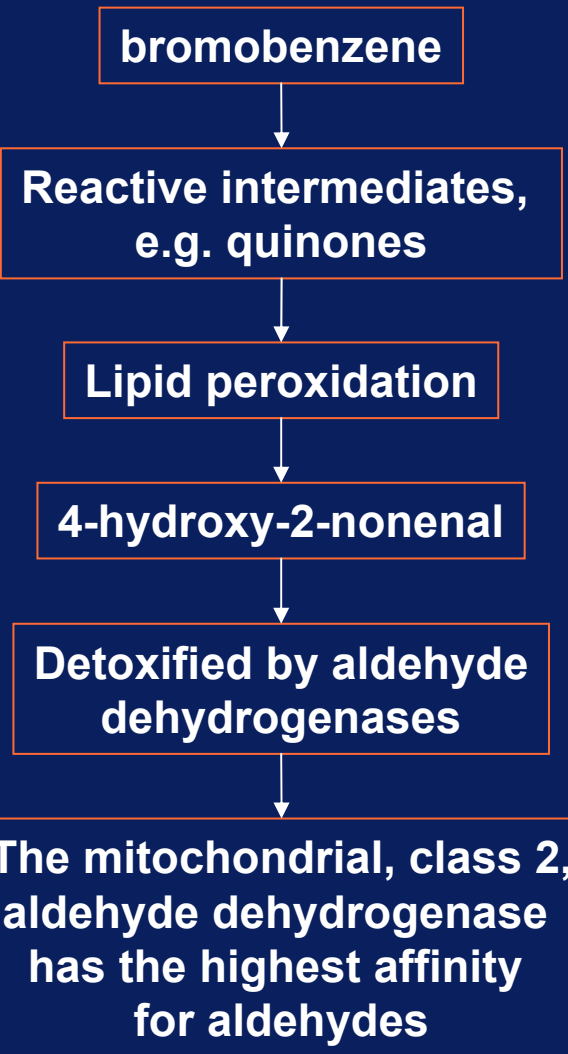
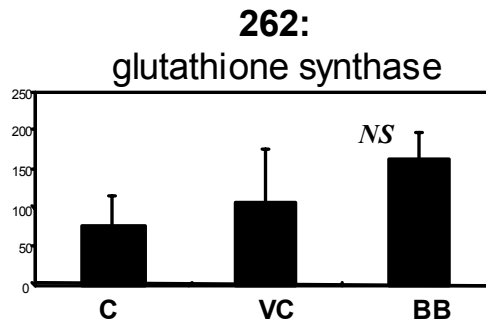
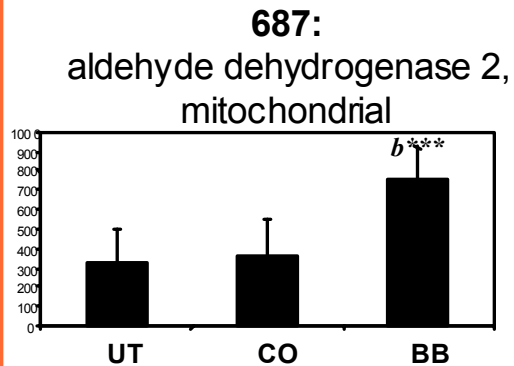
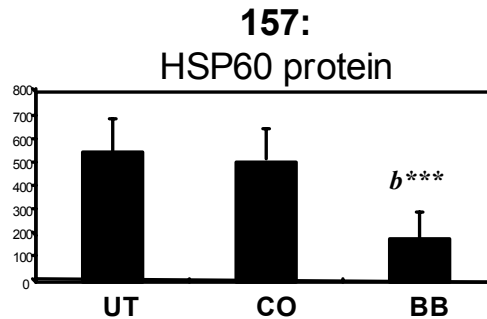
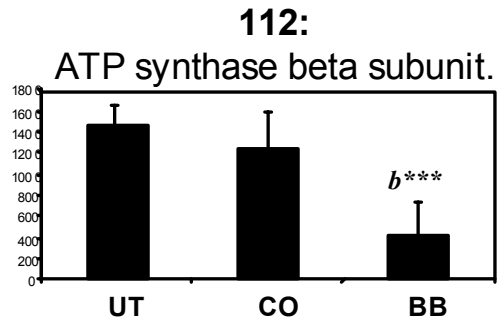
Electrophile response element (EpRe; ARE)

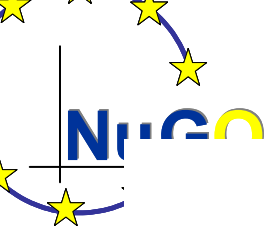


# Proteomics results IP



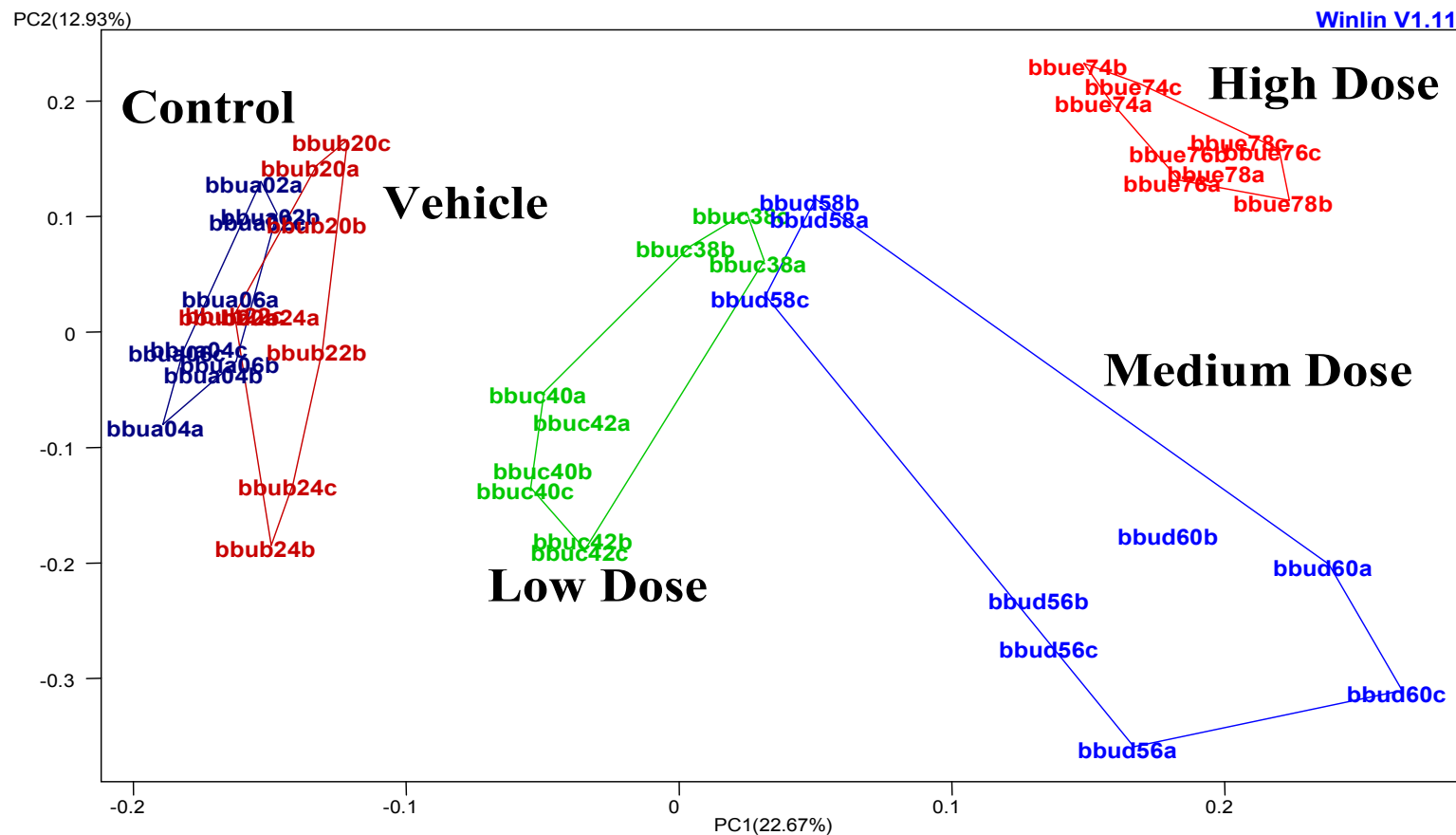
# Change in the expression of individual rat liver proteins upon exposure to bromobenzene--> additional explanation of bromobenzene-mediated hepatotoxicity

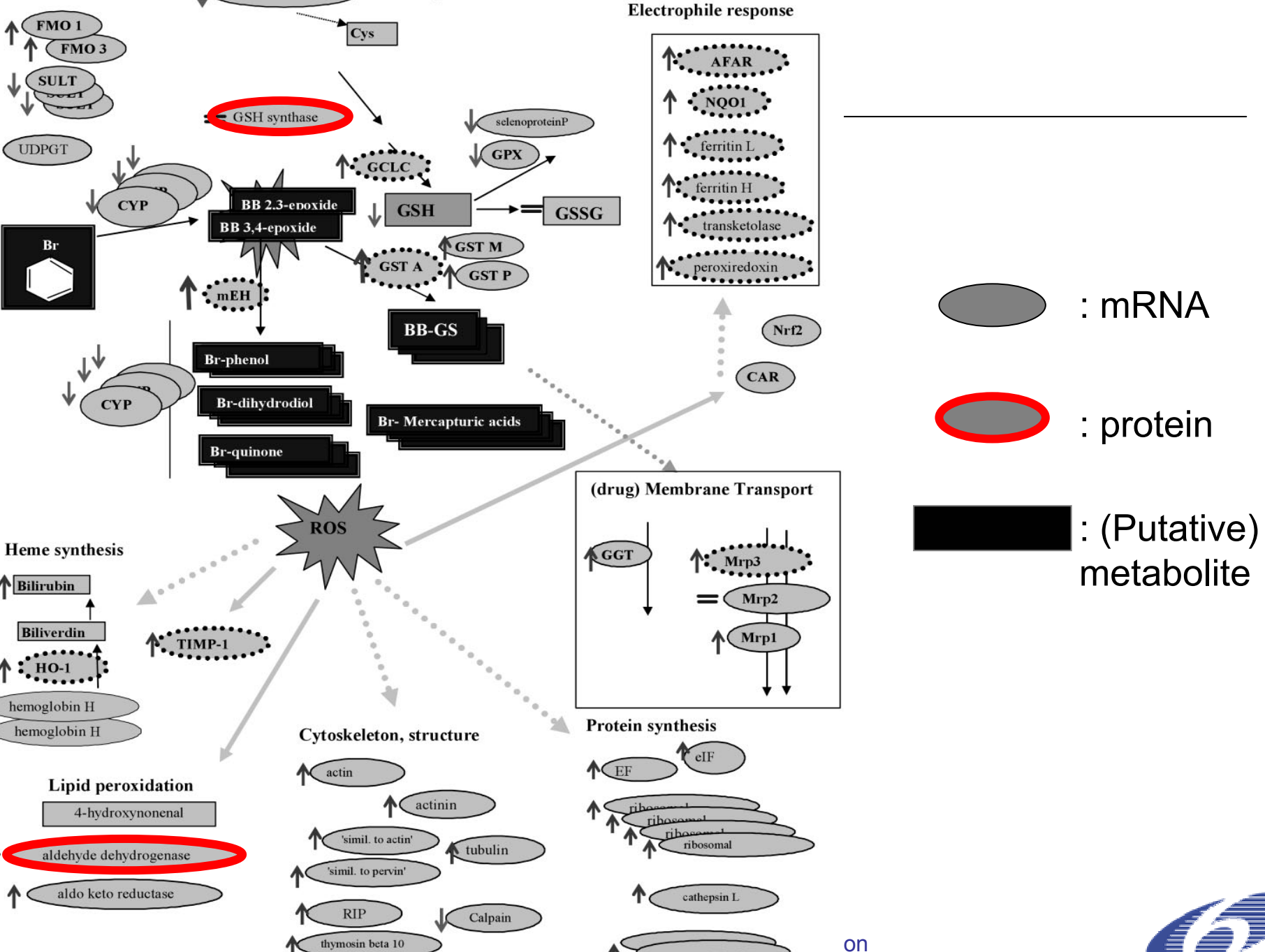




# Metabolomics (oral)

## PCA Score plot PC1 vs PC2 urine 6 hours





## *Time- and dose-dependent effects of curcumin on gene expression in human colon cancer cells.*

*Van Erk MJ, Teuling E, Staal YC, Huybers S, Van Bladeren PJ, Aarts JM, Van Ommen . Time- and dose-dependent effects of curcumin on gene expression in human colon cancer cells. J Carcinog. 2004 May 12;3(1):8.*

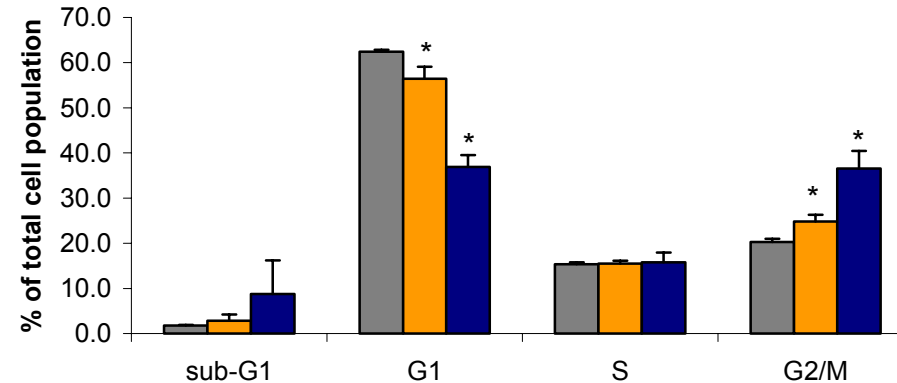
- Curcumin incubation of colorectal cancer cell line HT29
- Time course experiment: 3h, 6h, 12h, 24h, 48h
- Two datasets: low dose and high dose
- Select genes from expression data set present in at least 3 or more time points
- Use GenBank accession numbers to find up-to-date Unigene Clusters
- Final dataset: ~2600 genes for both low-dose and high-dose dataset



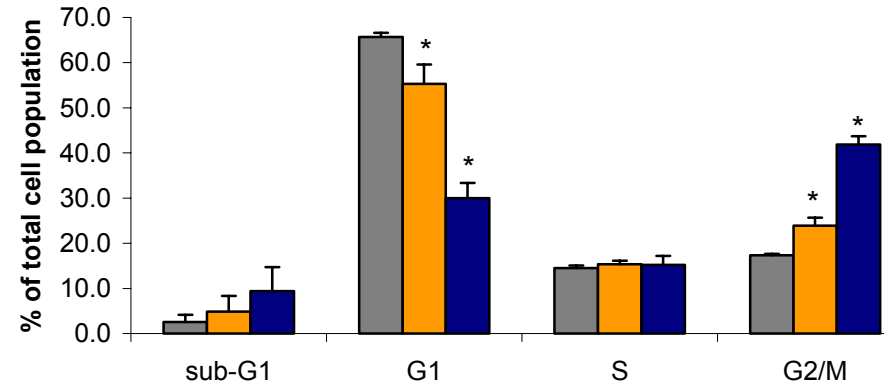
# Effect of curcumin in colon cancer cells: effect on cell cycle parameters

■ control  
■ lower concentration  
■ higher concentration

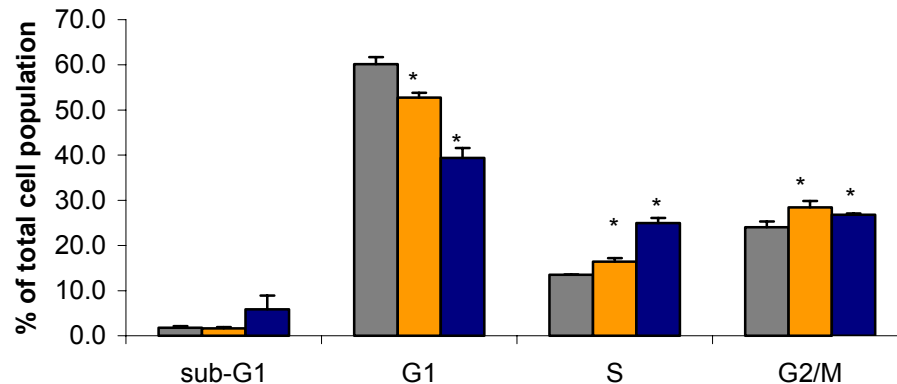
3h



6h



24h



Changes in cell cycle distribution in  
response to curcumin:

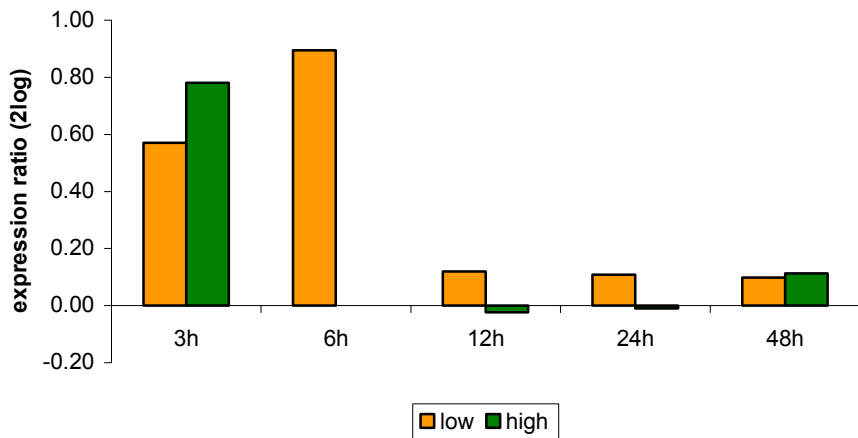
G2/M phase arrest

- decrease in % of cells in G1 phase,
- increase in % of cells in G2/M phase

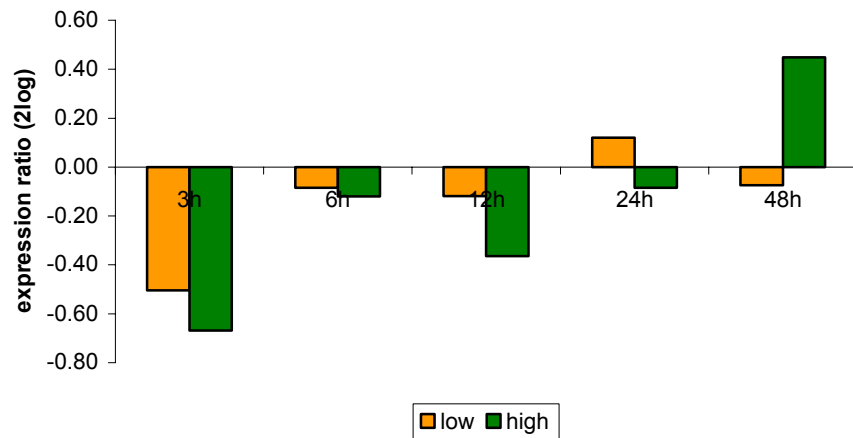
Van Erk et al., 2004

# Microarray data: Effect on cell cycle genes

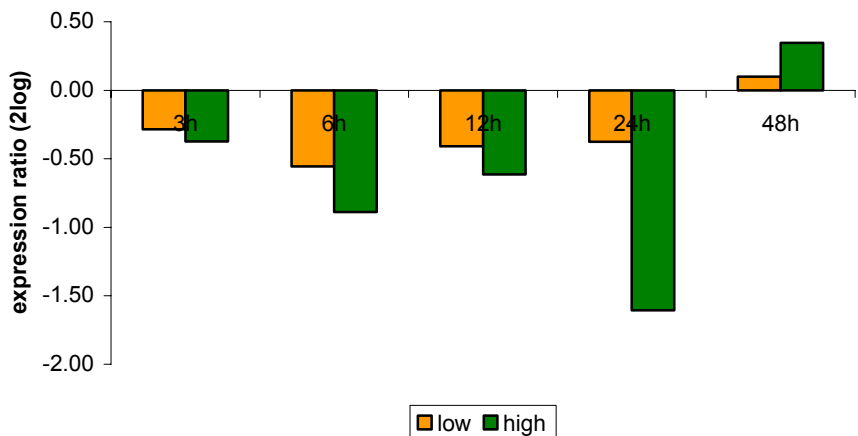
p16-INK4 (CDKN2A)



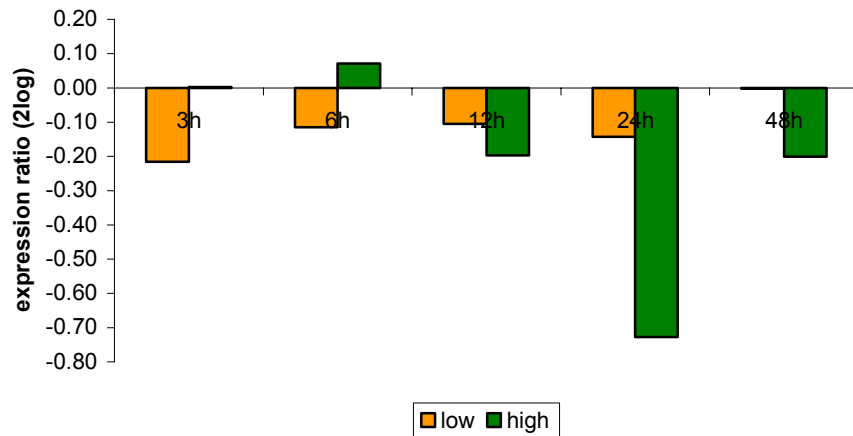
PCNA

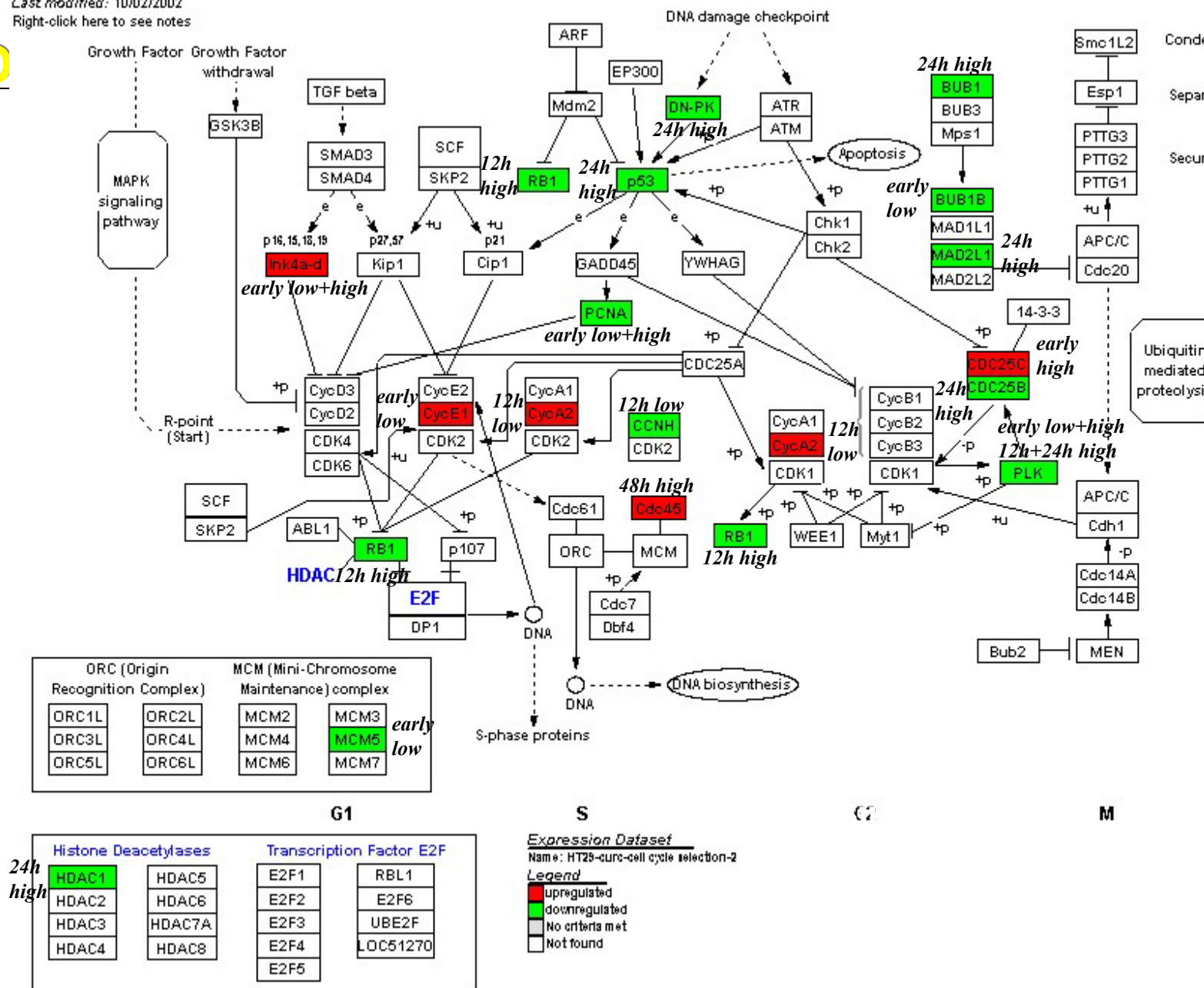


polo-like kinase

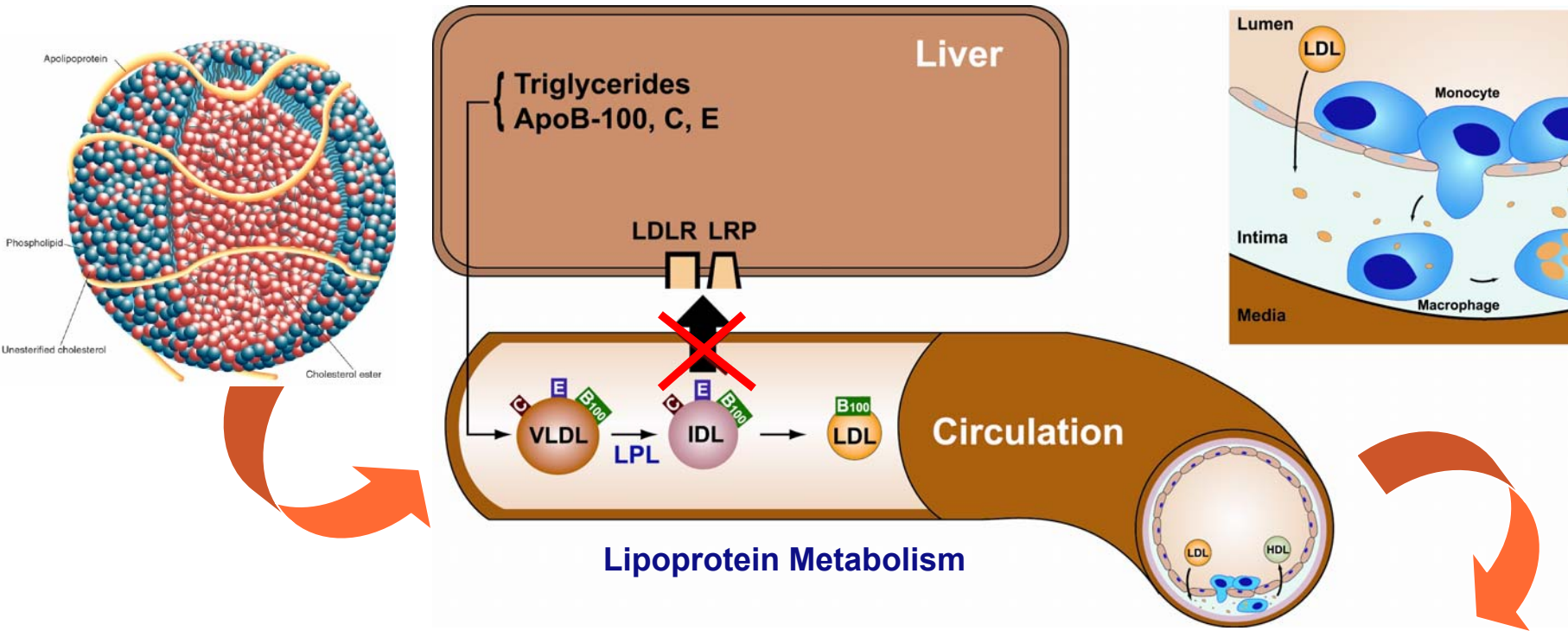


histone deacetylase 1





# Case 4: statistical networks → biological inference: Nutritional systems biology on ApoE3 Leiden Model



## ApoE3 Transgenic Mouse Model

Over-expressed human mutant apolipoprotein gene

Apolipoprotein changes prevent binding to liver receptors

Clearance of plasma lipoproteins inhibited

Mice are pre-atherosclerotic at 9 weeks...



Atherosclerotic Change

# Methodology

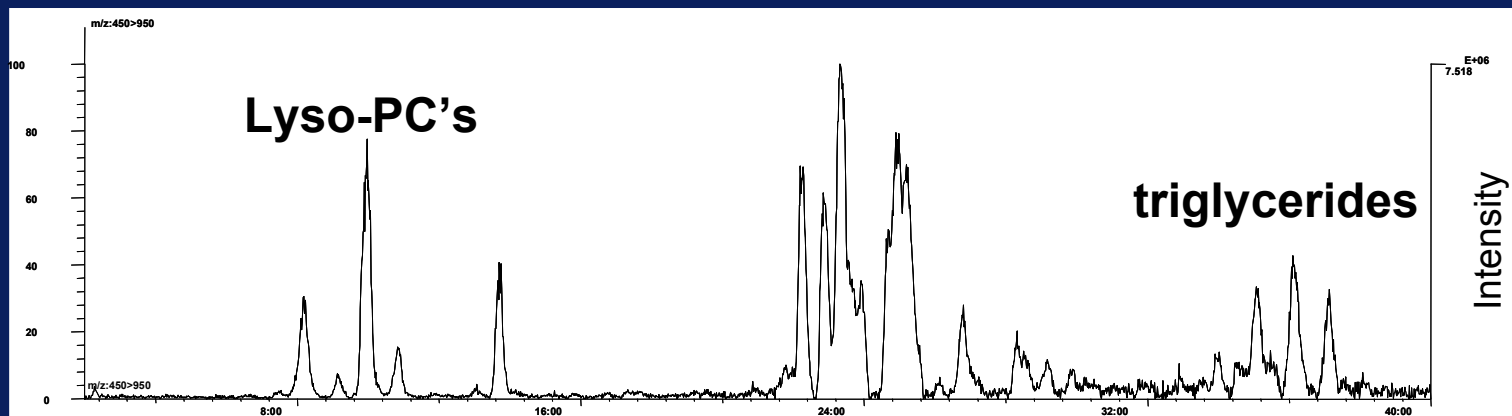
- **Specimens: 10 male ApoE3 Leiden /10 male wild type controls (WT)**
- **Normal chow diets/environmentally similar**
- **Sacrificed at 9 weeks**

|               | <b>Genomic Analysis</b> | <b>Proteomic Analysis</b> | <b>Metabolomic Analysis</b> |
|---------------|-------------------------|---------------------------|-----------------------------|
| <b>Liver</b>  | <b>Complete</b>         | <b>Complete</b>           | <b>Complete</b>             |
| <b>Plasma</b> | <b>NA</b>               | <b>Complete</b>           | <b>Complete</b>             |
| <b>Urine</b>  | <b>NA</b>               | <b>NA</b>                 | <b>Complete</b>             |

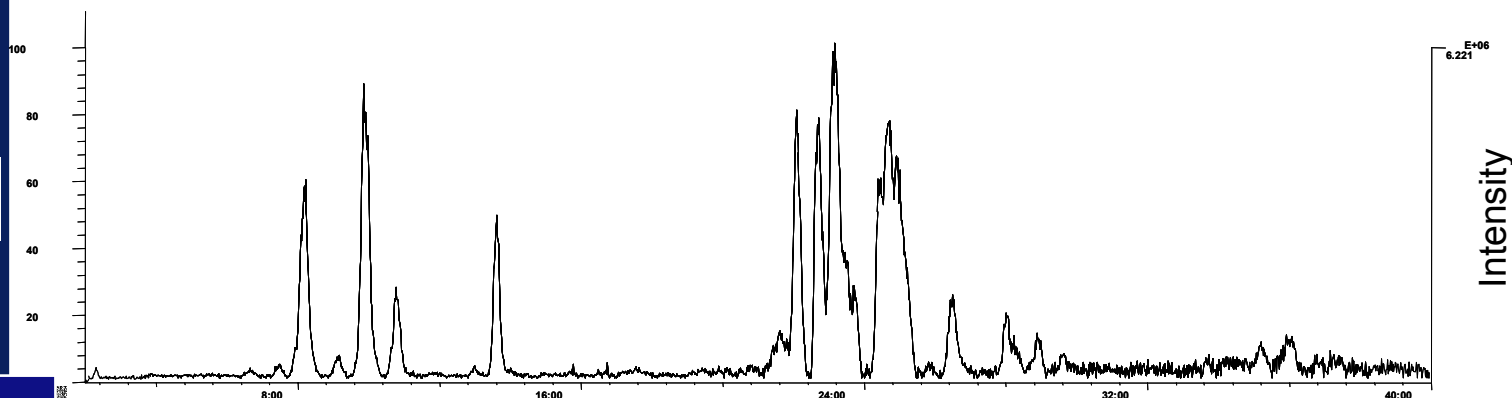
# Metabolite Analysis

## - ApoE3 vs. WT: LC-MS Plasma Lipid Profiles

**ApoE3**



**Wildtype**

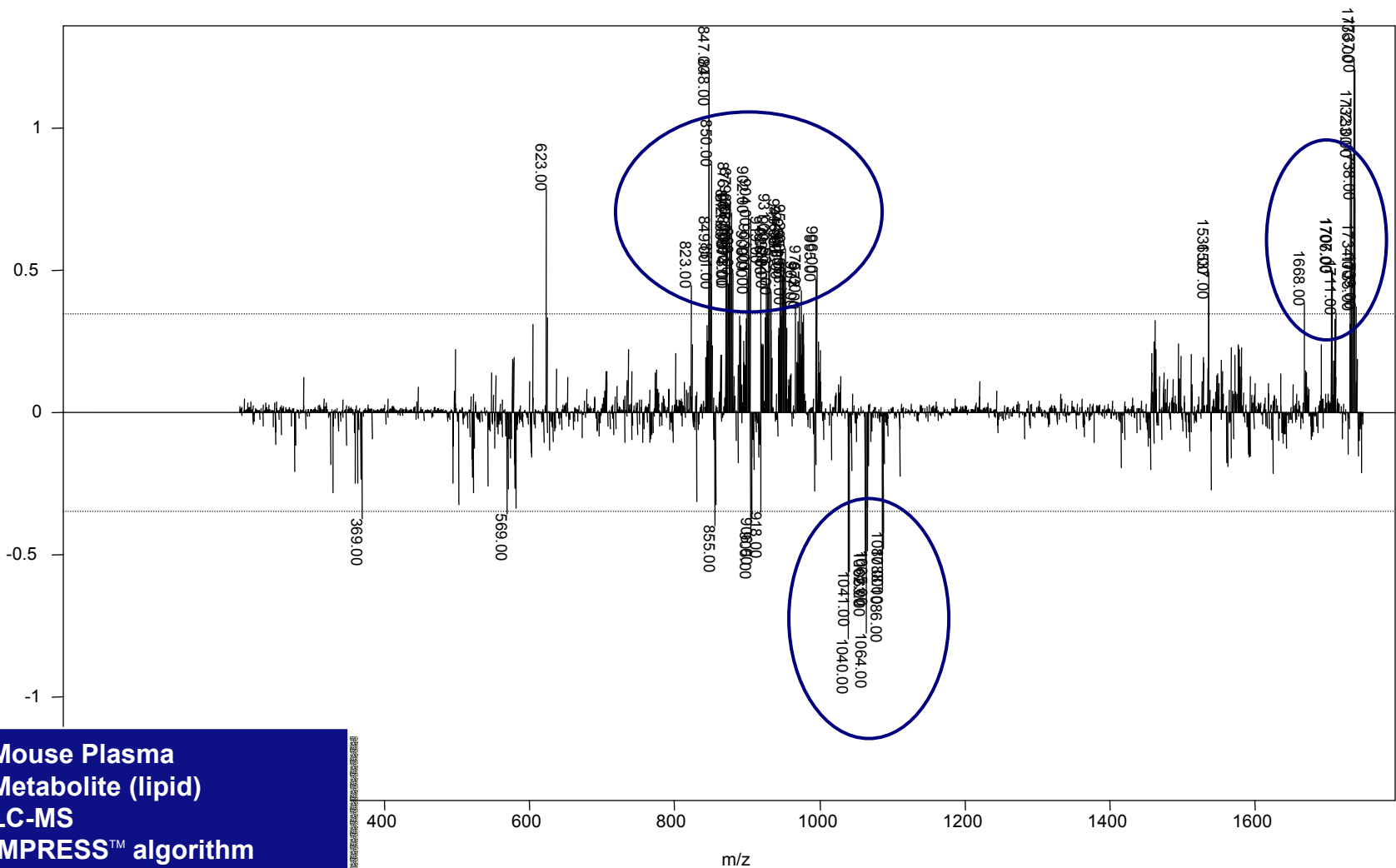


Mouse Plasma  
Metabolite (lipid)  
LC-MS  
IMPRESS™ algorithm

• Mass Chromatograms of Plasma Lipids

# Metabolite Analysis

## - ApoE3 vs. WT: Plasma Lipid Difference Factor Spectrum



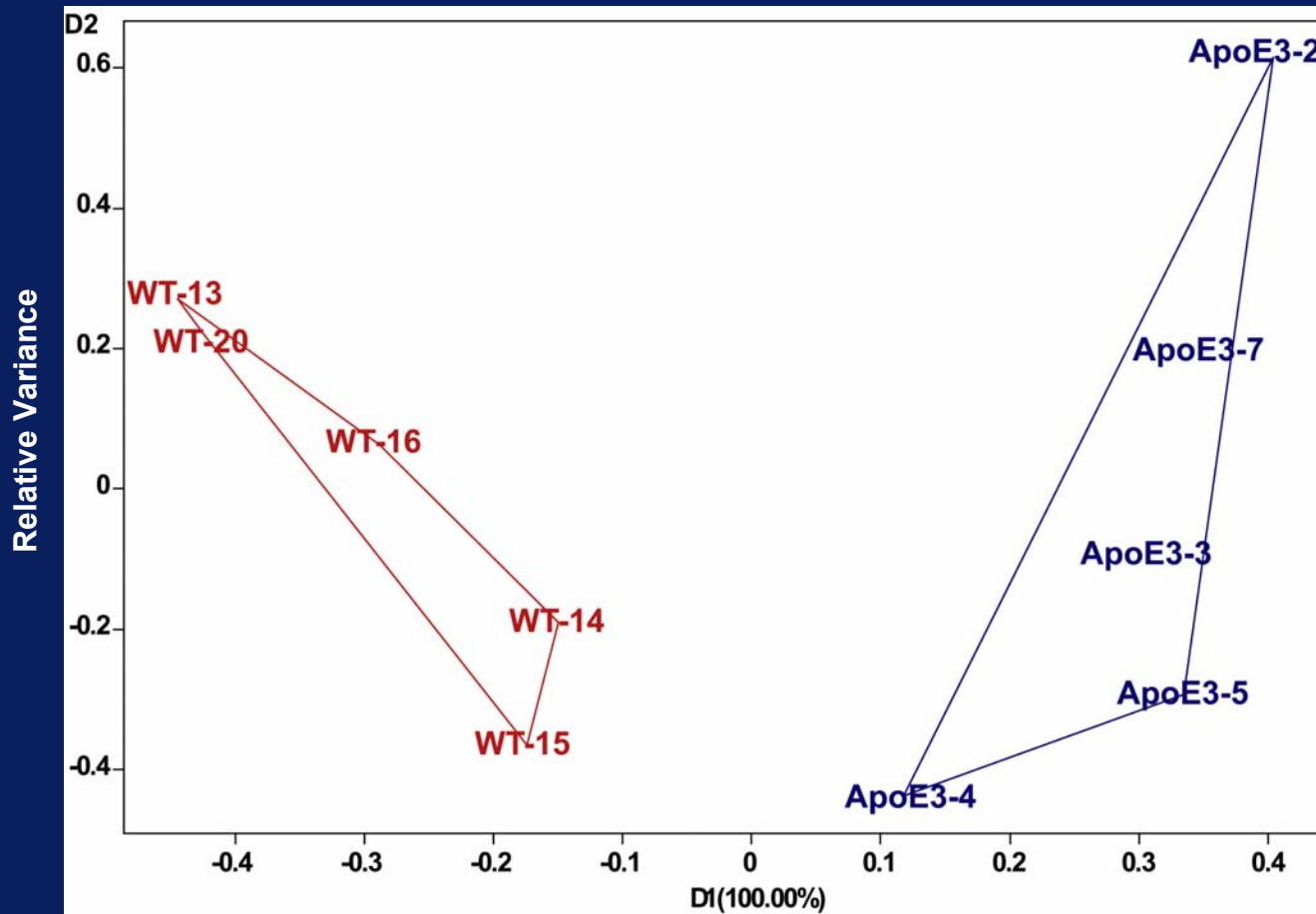
More abundant

Less abundant

Mouse Plasma  
Metabolite (lipid)  
LC-MS  
IMPRESS™ algorithm  
PARC™ pattern recognition

# Proteomic Analysis

## - ApoE3 vs. WT: Principal Component and Discriminant Analysis

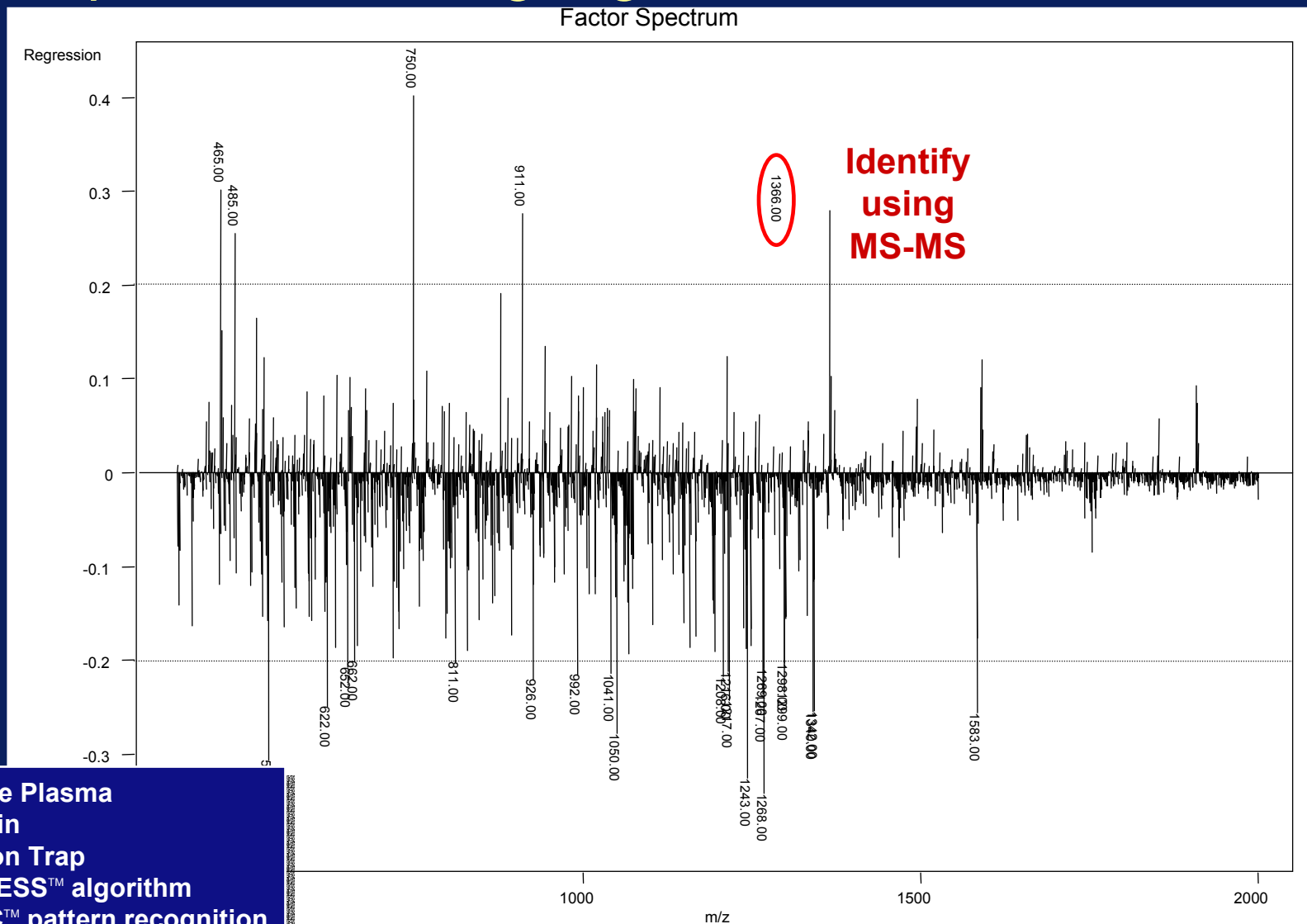


Mouse Plasma  
Protein  
ESI-Ion Trap  
IMPRESS™ algorithm  
PARC™ pattern recognition

- *Unsupervised clustering reveals segregation*
- *Hundreds of components contribute to observed differences*  
→ *can be used as powerful, selective and specific biomarkers*

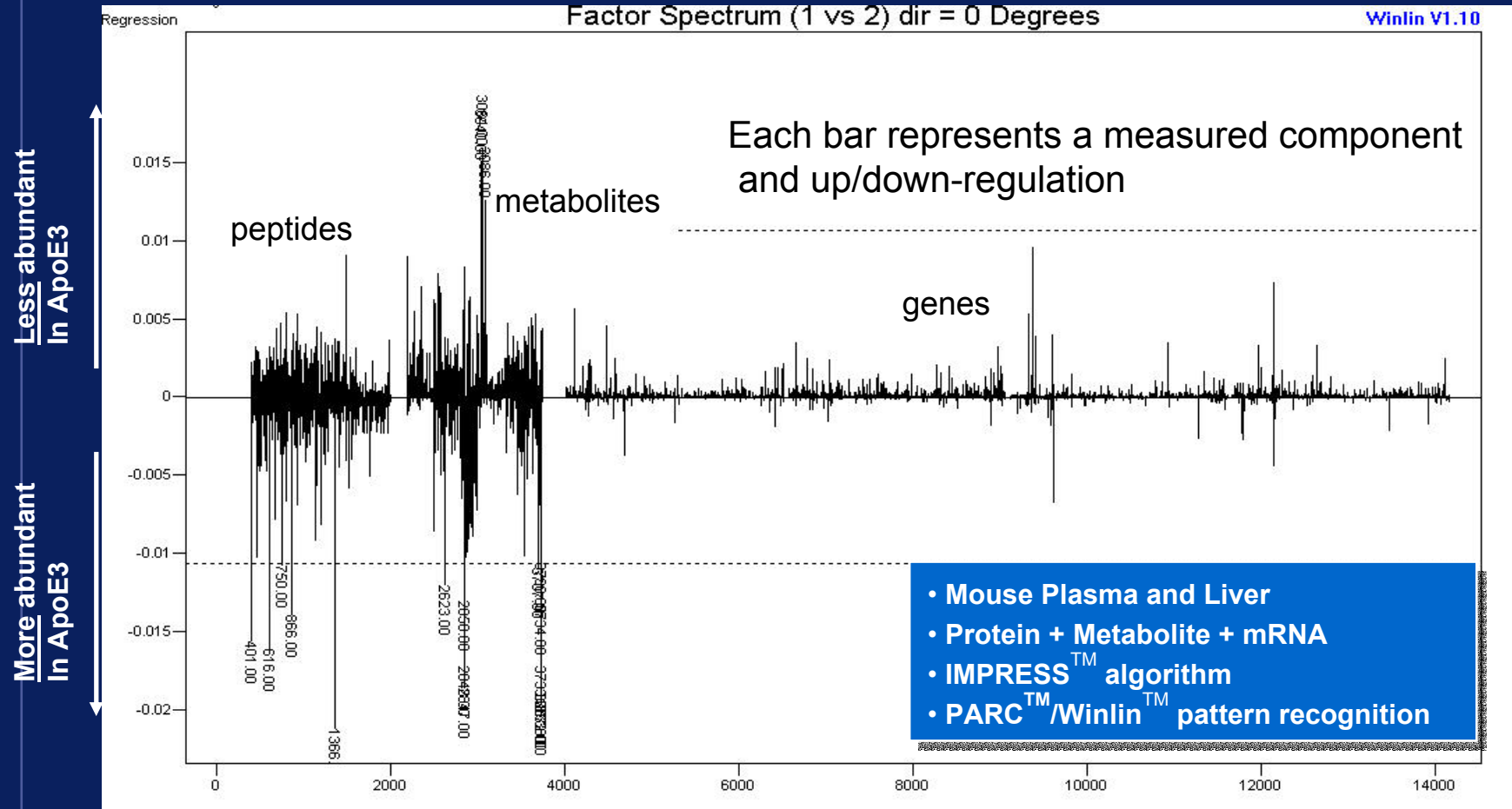
# Proteomic Analysis

## - ApoE3 vs. WT: Difference Factor Spectrum Peptides Exhibiting Significant Differences



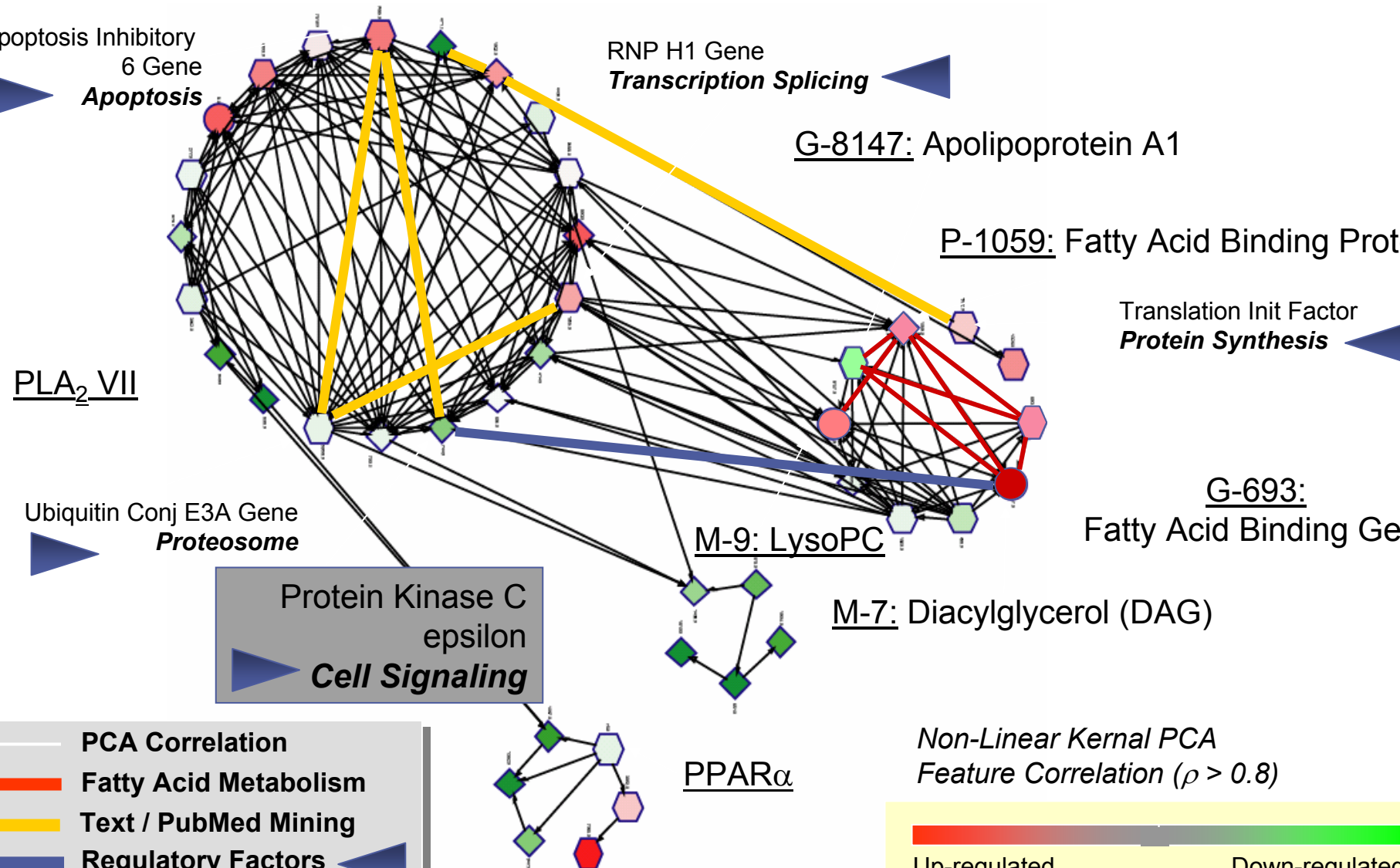
# Proteomic, Genomic, Metabolomic Data: Identifying / Mining Differences

- ApoE3 vs. WT: *Difference Spectrum*

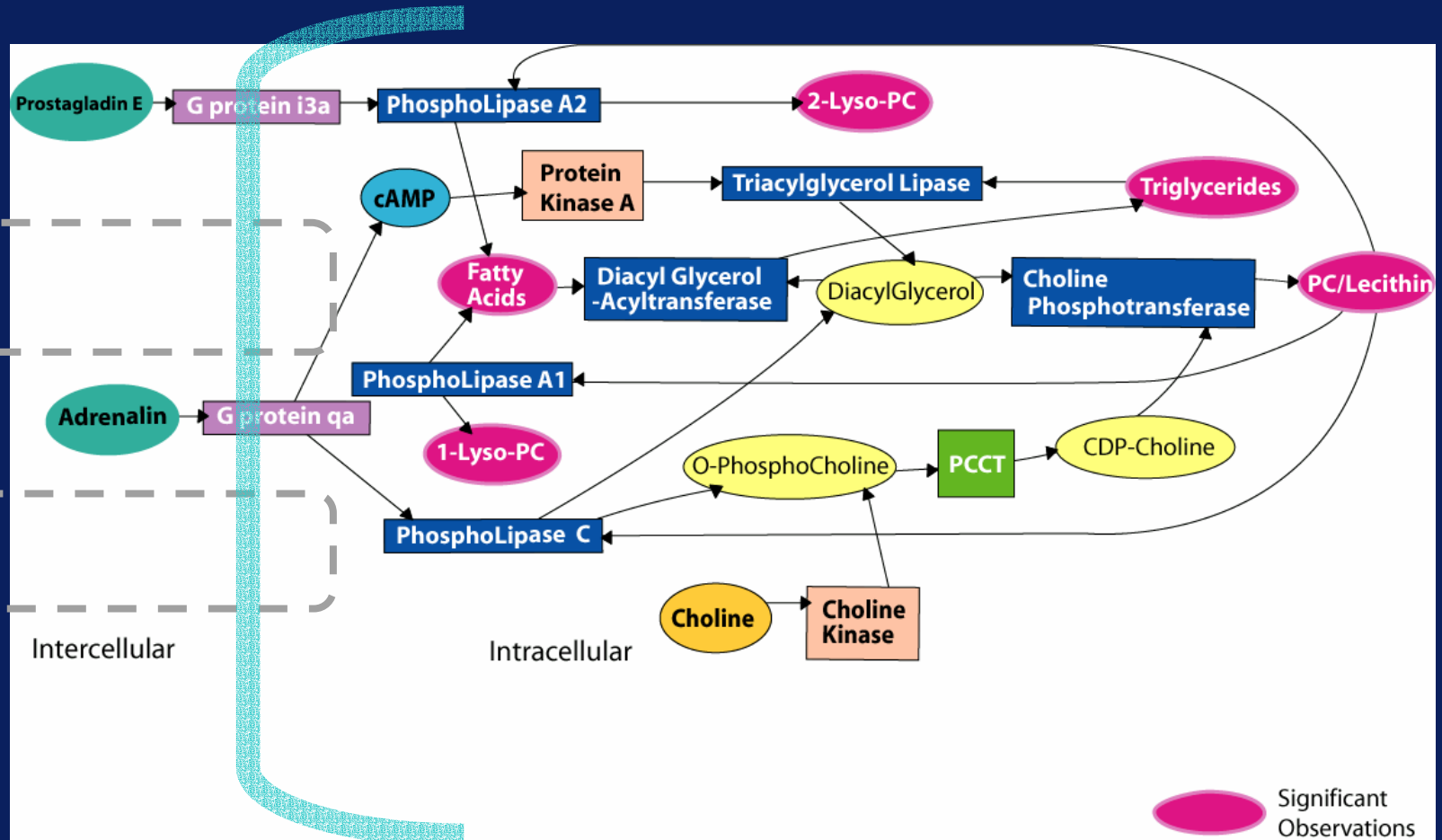


# BioSystematics™

## - Mapping Known Relations onto Non-Linear PCA Correlations



# Phosphatidylcholine Lipid Metabolism Pathway Model Suggested by Results



protein

transmembrane

small molecule

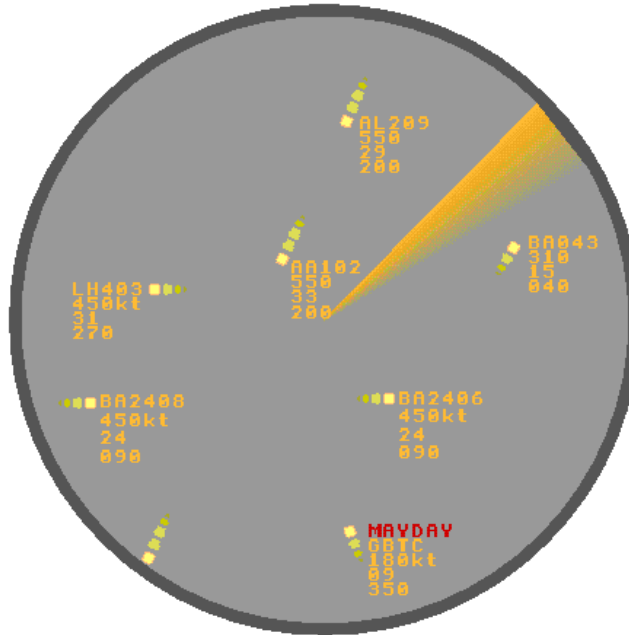
• New insights in targets/biomarkers in the ApoE3 system

- **TNO, NL:**
  - Dr. Marjan van Erk: curcumin in vitro studies
  - Ing. Marinus Dansen: array quality control
  - Prof. Dr. Louis Havekes and Prof. Dr. Jan van de Greef + colleagues from Beyond Genomics Medicine, Waltham, MA, USA: APOeE3 systems biology
  - Prof. Dr. John Groten: food additives project
  - Dr. Wilbert Heijne: bromobenzene studies
  - Dr. Robert Jan Lamers and Dr. Joop van Nesselrooij: metabolomics of bromobenzene
  - Dr. Ben van Ommen: coordinator of The European Nutrigenomics Organisation, group leader Genomics
- **BIBRA INTERNATIONAL LTD, UK**
  - Prof. Dr. Brian Lake, Dr. Clive Meredith + colleagues thiabendazole project
- **EUROPEAN BIOINFORMATICS INSTITUTE, HINXTON, UK**
  - Dr. Susanna Assunta Sansone and Dr. Phillipe Rocca-Serra: support with ArrayExpress
- **UNIVERSITY OF UTRECHT, NL**
  - Dr. Frank Holstege group: microarrays fabrication
  - Prof Dr. Albert Heck group: BB proteomics mass spectrometry





Nederlandse  
Spoorwegen  
(Static, delay!)



Meridiana Airlines  
(Dynamic)



Taxi in the  
ancient city  
of Florence  
(Conserved)