Metabolomics
Efficacy
Toxicity

Primary Molecules
Secondary Molecules

Adapted from D. Robertson, Pfizer Global Research and Development
Metabolomics

Comprehensive Definition:
The quantitative measurement of the time-related multiparametric metabolic response of living systems to pathophysiological exogenous or endogenous stimuli or genetic modification.

Operational Definition:
The systematic exploration of biofluid composition using NMR/pattern recognition technology in order to associate target organ toxicity with NMR spectral patterns and identify novel surrogate markers of toxicity.

Adapted from D. Robertson, Pfizer Global Research and Development
Metabolomics:
The study of the total metabolite pool (metabolome), metabolic regulation and fluxes in individual cells or cell types. Can be achieved through a wide spectrum of technologic methods including LC-MS, GC-MS, and nuclear magnetic resonance (NMR)

Metabonomics:
The study of the systemic biochemical profiles and regulation of function in whole organisms by analyzing a metabolite pool (metabolome) in biofluids and tissues. Usually implies that the study is done specifically through nuclear magnetic resonance profiling
Metabolome:
The quantitative complement of all the low molecular weight molecules present in cells in a particular physiological or developmental state

Biofluid:
A fluid sample obtained from a living system. The donor might typically be a human or an animal. Fluids can be excreted (such as urine, sweat), expressed or secreted (such as milk, bile), obtained by intervention (such as blood plasma, serum or cerebrospinal fluid), develop as a result of a pathological process (such as blister or cyst fluid), or be applied and collected (such as dialysis fluid)
Advantages of Metabolomics

- Identification of target organ, severity, onset, duration and reversal of the effects (time-course)
- Classify sample as “normal” vs. “abnormal”
- Determine mechanisms of action within the organ
- Potential for identifying novel biomarkers of toxic effect
- Non-invasive
- No a priori decisions about samples need be made
- No sample processing necessary other than cold collection
- Complete time course data can readily be obtained
- Minimization of compound requirements
- Relatively fast analysis (200-300 samples/day)
- Useful tool for modeling physiological variation and exposure conditions in animals and humans

Adapted from D. Robertson, Pfizer Global Research and Development
**NMR spectroscopy**

Spectroscopy deals with the interactions between electromagnetic radiation and matter. Spectroscopy is used to derive the properties of matter at the molecular level. Nuclear magnetic resonance (NMR) exploits the magnetic properties of atomic nuclei.

The method functions as follows: A substance is placed in a magnetic field. Some atomic nuclei (e.g., protons, nuclei of hydrogen atoms) then behave like microscopic compass needles, called nuclear spins. Each nuclear spin orientation corresponds to a different energy level. The spins may jump between the levels when the sample is exposed to radio waves whose frequency exactly matches the energy spacing. This is called resonance. One way of measuring the energy is to change the irradiation frequency. At resonance, the spins flip causing an electric signal. The strength of the signal is plotted as a function of frequency in a diagram, the NMR spectrum.

**In metabolomics**, it is the patterns that occur when many different biochemical entities are detected simultaneously in a mixture using $^1$H NMR that are interpreted.

From: www.nobel.se
NMR in Metabolomics:

Pro:
• Non-destructive
• Applicable to intact biomaterials
• More information rich in complex-mixture analyses
• No extraction/derivatization is necessary

Con:
• Less sensitive than MS

History:
• NMR has been used to study metabolites in biofluids for over a decade
• Metabolomics technology as it is known today (600 MHz \(^1\)H NMR) was pioneered by Jeremy Nicholson, Elaine Holmes and John Lindon of Imperial College in London
• Only recently have advances in flow-through NMR hardware and pattern recognition software made the possibility of “high”-throughput in vivo toxicity assessment a practical possibility
NMR Acquisition and Gilson 215 Control System

Varian Inova 600 Shielded magnet
120 ul flow probe

NMR flow probe

Refrigerated Metabolism Cage (0°C)

+ NaN₃

Biomek® Robot

Deuterated Buffer ↔ TSP

Frozen Storage

Gilson 215 autosampler

Data Processing

Adapted from D. Robertson, Pfizer Global Research and Development
Normal Metabolic Profiles

Adapted from D. Robertson, Pfizer Global Research and Development
Functional NMR Spectrum of Rat Urine

“Biomarker Windows”

<table>
<thead>
<tr>
<th>Chemical Agent</th>
<th>Target Organ/Toxicity Type</th>
<th>Associated Biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>Heart and kidney (glomerulus)</td>
<td>↑ creatine, taurine, ↓ citrate, ↓ ΔαKG</td>
</tr>
<tr>
<td>Allyl alcohol</td>
<td>Liver (periportal)</td>
<td>↑ creatine, lactate, phenylacetylglucine, N-methyl nicotinamide, taurine, ↓ citrate, ΔαKG, ↑ phenylacetylglycine, DMG</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>Phospholipidosis (lung)</td>
<td>↓ succinate, TMAO</td>
</tr>
<tr>
<td>α-Naphthylisothiocyanate (ANIT)</td>
<td>Liver (cholestasis)</td>
<td>↑ acetate, bile acids, glucose</td>
</tr>
<tr>
<td>2-Bromoethanamine</td>
<td>Kidney (papilla) and mitochondrial dysfunction</td>
<td>↓ creatine, glucose, ↑ lactate, ↓ citrate, ΔαKG, succinate</td>
</tr>
<tr>
<td>Butylated hydroxytoluene</td>
<td>Liver</td>
<td>↓ glucose, taurine, ↑ creatine, ↓ citrate</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>Testicular</td>
<td>↓ creatine</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>Liver</td>
<td>↓ taurine, creatine, ↓ citrate, ΔαKG, succinate</td>
</tr>
<tr>
<td>2-Chloroethanamine</td>
<td>Kidney (papilla) and mitochondrial dysfunction</td>
<td>↑ acetate, diamide, DMG, glutaric acid, N-acetylglycine</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>Phospholipidosis and liver necrosis</td>
<td>↓ succinate, TMAO</td>
</tr>
<tr>
<td>S-(1,2-dichlorovinyl)-L-cysteine DCVC Z4</td>
<td>Kidney (S2/3 proximal tubular)</td>
<td>↑ citrate, succinate</td>
</tr>
<tr>
<td>S-(1,2-dichlorovinyl)-L-homocysteine DCVVC</td>
<td>Kidney (S2/3 proximal tubular)</td>
<td>↑ acetate, amino acids, glucose, organic acids</td>
</tr>
<tr>
<td>Ethionine</td>
<td>Liver</td>
<td>↓ glucose, taurine, ΔαKG</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>Liver (hepatitis-like lesion)</td>
<td>↑ acetate, betaine, bile acids, creatine, organic acids, taurine, urocanic acid</td>
</tr>
<tr>
<td>Hexachlorobutadiene</td>
<td>Kidney (S3 proximal tubular)</td>
<td>↑ acetate, amino acids, glucose, organic acids</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>Liver (steatosis)</td>
<td>↑ 2-amino adipate, β-alanine, creatine, N-acetyl-citrulline</td>
</tr>
<tr>
<td>Lanthanum nitrate</td>
<td></td>
<td>↓ creatine, fumarate, hippurate, TMAO</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ acetoacetate, alanine, aromatic amino acids, DMA, ethanol, glucose,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hippurate, ΔαKG, lactate, succinate, taurine, TMAO</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ allantoin, citrate, creatinine, glucose, urea</td>
</tr>
</tbody>
</table>
Techniques and Procedures in Metabolomics

NMR Spectra → Primary Data Processing → Unsupervised mapping of data in 3D space → Supervised classification and calculation of confidence intervals.

**Pattern Recognition (PR) Methods:**

PR and related multivariate statistical approaches can be used to discern significant patterns in complex data sets and are particularly appropriate in situations where there are more variables than samples in the data set. The general aim of PR is to classify objects (in this case $^1$H NMR spectra) or to predict the origin of objects based on identification of inherent patterns in a set of indirect measurements. PR methods can reduce the dimensionality of complex data sets via 2 or 3D mapping procedures, thereby facilitating the visualization of inherent patterns in the data.

**Principal Components Analysis (PCA):**

This is a data dimension reduction method that involves a mathematical procedure that transforms a number of (possibly) correlated variables into a (smaller) number of uncorrelated variables called *principal components*. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. Use of PCA enables the "best" representation, in terms of biochemical variation in the data set to be displayed in two or three dimensions.
Rat d

Hr Post Dose

ALT (IU/L)

0 100 200 300 400 500

Rat a
Rat b
Rat c
Rat d

Upper Limit of Normal

PC1

-50 -40 -30 -20 -10 0 10 20

PC2

-40 -30 -20 -10 0 10 20

67

138

298

43

117

55

267

4409

484

67

17

Control ALT = 33-94 (Normal Reference Range)

24 hr ALT = 1228 ± 1061

48 hr ALT = 994 ± 884

96 hr ALT = 161 ± 108

Adapted from D. Robertson, Pfizer Global Research and Development
Adapted from D. Robertson, Pfizer Global Research and Development
PCA analysis of vehicle effect on rat urine NMR spectra

Adapted from D. Robertson, Pfizer Global Research and Development
ANIT 100 mg/kg

day 4

day 3

day 2

day 1

creatinine

hippurate

TMAO

citrate

2-oxoglutarate

succinate

creatinine

predose

Adapted from D. Robertson, Pfizer Global Research and Development
Number in parentheses = mean serum total bilirubin (mg/dL)

Adapted from D. Robertson, Pfizer Global Research and Development
ANIT (50 mg/kg) in Mouse

Adapted from D. Robertson, Pfizer Global Research and Development
Allyl Alcohol (12 mg/kg)

Concurrent Controls

Note: Numbers next to symbols are individual ALT levels (IU/L)

Slightly elevated ALP (260-280 IU/L)

Adapted from D. Robertson, Pfizer Global Research and Development
Allyl Alcohol (120 mg/kg)

Concurrent Controls

Elevated 24, 48Hr ALP
359-732 IU/L

Elevated Bilirubin
(1.1 mg/dL)

Normal ALP
(< 206 IU/L)

Pretest

24 Hr

48 Hr

72 Hr

96 Hr

Necrosis Elevated ALT

Adapted from D. Robertson, Pfizer Global Research and Development
Allyl Alcohol
Combined Data, 12 (▲) & 120 (○) mg/kg

Concurrent Controls

Biliary Toxicity
Elevated ALP & Bilirubin

Necrosis
Elevated ALT

PC 1

PC 2
Metabolomic Detection of Liver Toxicity

Metabolomic Detection of Kidney Toxicity

Adapted from D. Robertson, Pfizer Global Research and Development
Limitations of Metabolomics

- Specialized equipment is required
- Extensive expertise is required
- Information is limited to time- and dose-points taken
- High risk of false positive data: a compound causes significant metabolism changes without associated toxicity
- Difficulty in separation of physiological (adaptive) and toxicological (adverse) effects
- Sensitivity of the assay
- Certain pathological states have negligible effects on biofluids: liver fibrosis may go undetected until damage is severe
- Availability of biofluids for certain organ toxicity: CNS vs. urine
- Distinguishing effects of multi-organ toxicants: biomarkers in different biofluids are different and in one biofluid are inter-mixed
Future Directions in Metabolomics

• Develop comprehensive metabonomic database
• Expand metabonomics applications to many species
• Evaluate cryoprobe technology for increased sensitivity or increased throughput
• Expand technology to novel targets:
  – Cardiac toxicity
  – Adrenal toxicity
• “Grand Unification” of Genomic/Proteomic and Metabonomic technologies