Tools of "Omics"
Metabolomics

Dr. Rob J. Vreeken
Netherlands Metabolomics Centre
University of Leiden, the Netherlands

ESHRE conference on
System biology tools and preclinical models for translational research in endometriosis and adhesion formation
Sept 4&5, Liege, Belgium

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Outline

- **Metabolomics**
  - Why, what, how
- **Netherlands Metabolomics Centre**
  - Aim & objectives
- **Profiling of Lipids**
  - Application to study twins
- **Profiling of amines**
  - Application to CSF and Plasma
- **Identification of unknown metabolites**
  - MS trees for structure elucidation
- **Applications**
  - Biomarker discovery of disease models
  - Network analysis of drug intervention
- **Conclusions & Acknowledgements**

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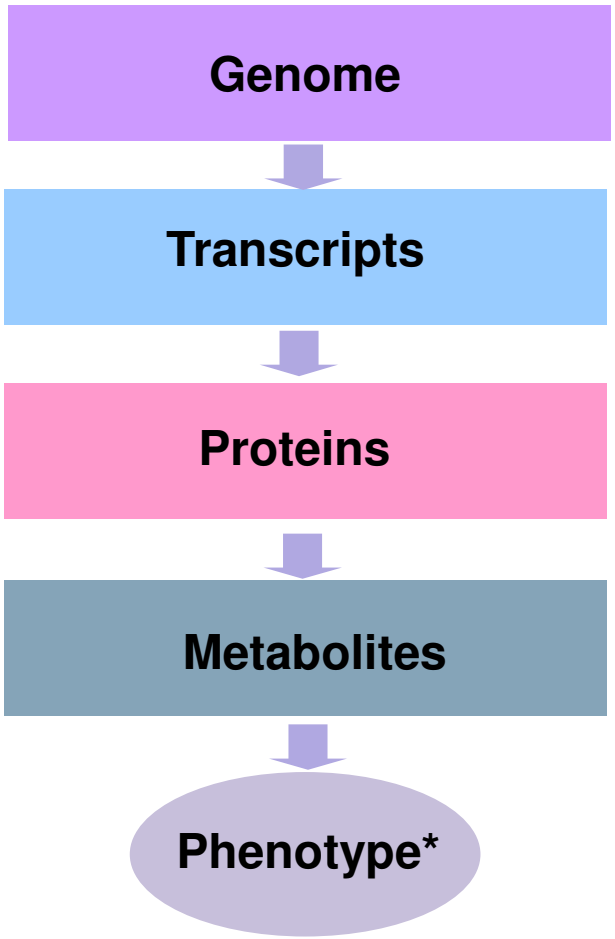
Why Metabolomics?



* the observable characteristics of an organism

- **Metabolome close to outward appearance (phenotype) in health & disease**
- **Metabolites essentially the same in humans & animals with often same biological functions**
- **In various chronic, multifactorial diseases metabolic disorders are involved (e.g. cardiovascular, diabetes, CNS diseases)**
- **Metabolite profiles of body fluids to discriminate disease & health**
- **Metabolomics essential part of Systems Biology**

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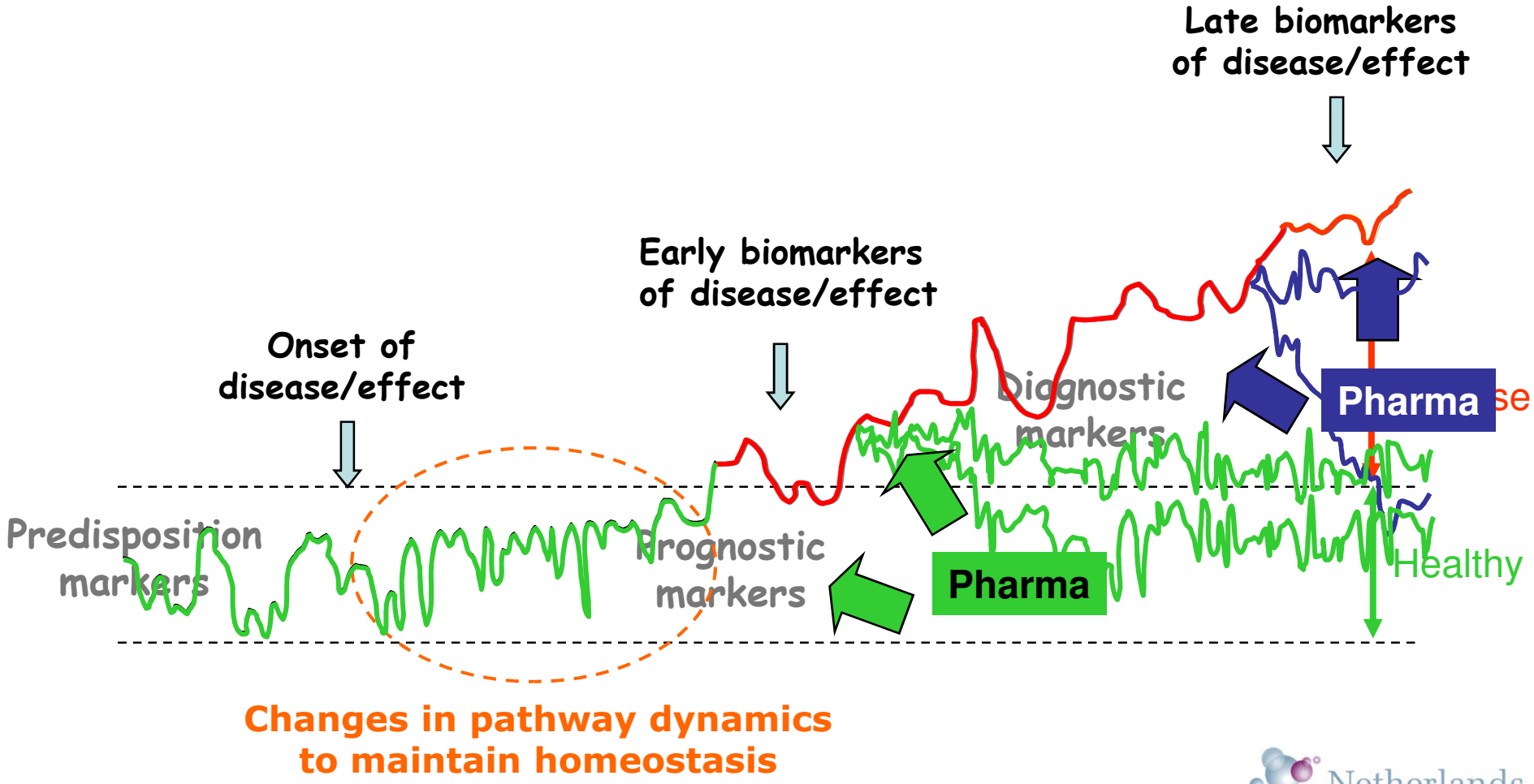
BIOMARKERS for health & disease
From normality, to dysfunction, to disease



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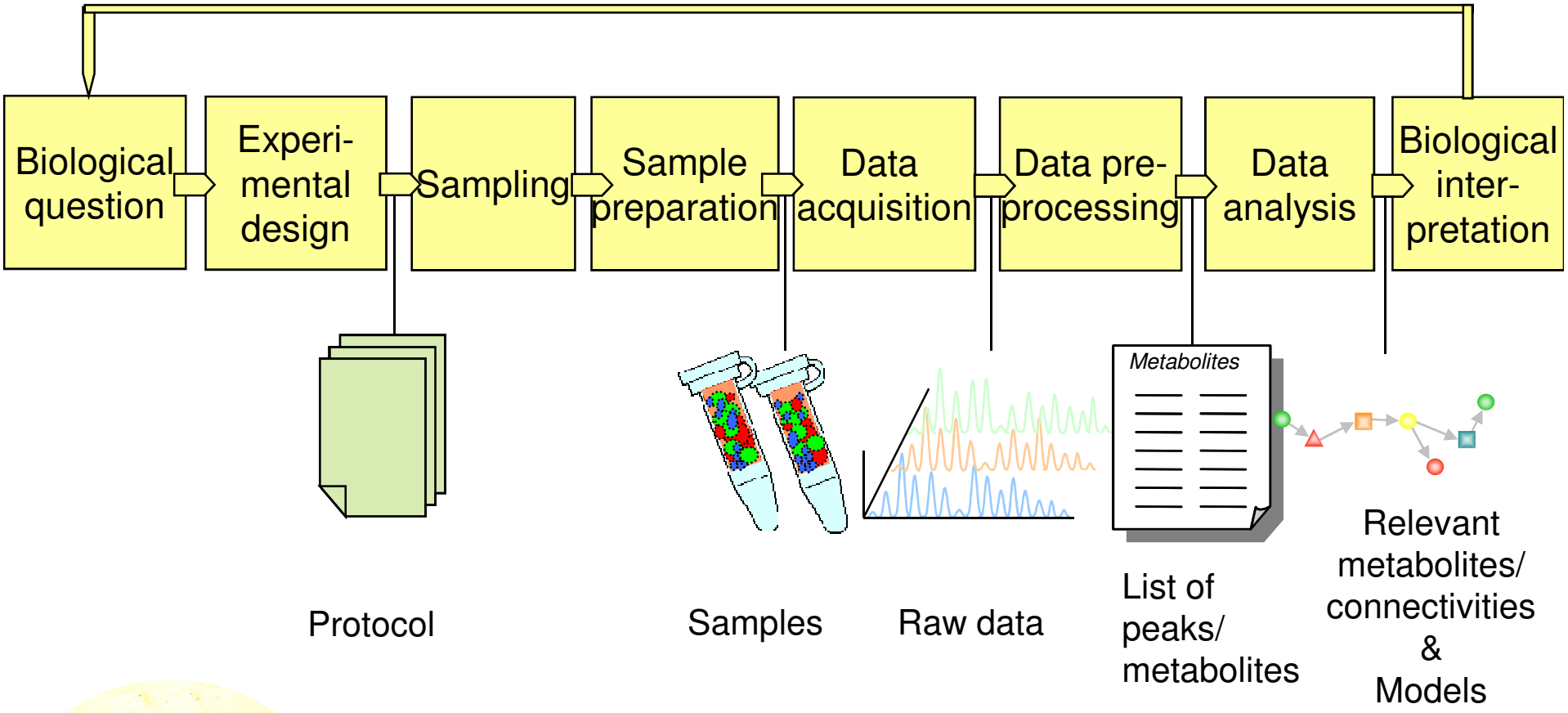
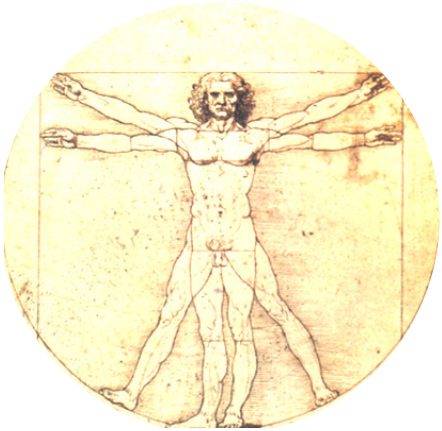
Metabolomics workflow





Requirement for clinical studies

- **Relevant metabolites (coverage)**
- **Quantitative data**
- **Identification of metabolites**

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Analytical Techniques used in metabolomics

GC-MS

- Highly reproducible retention times & High separation efficiency
- Electron Impact (EI) mass spectra

  Sensitivity, fingerprint & large databases available for identification

- Derivatization to include polar analytes & non-volatiles

  Robust procedure necessary ⇨ quality standards to control sample preparation and system performance as well as correct for variation of detector response

- Chemical Ionisation (CI) for increased coverage

  Spectra less informative (MS/MS; no databases)

LC-MS

- Wide variety of techniques covering a large range of polarities

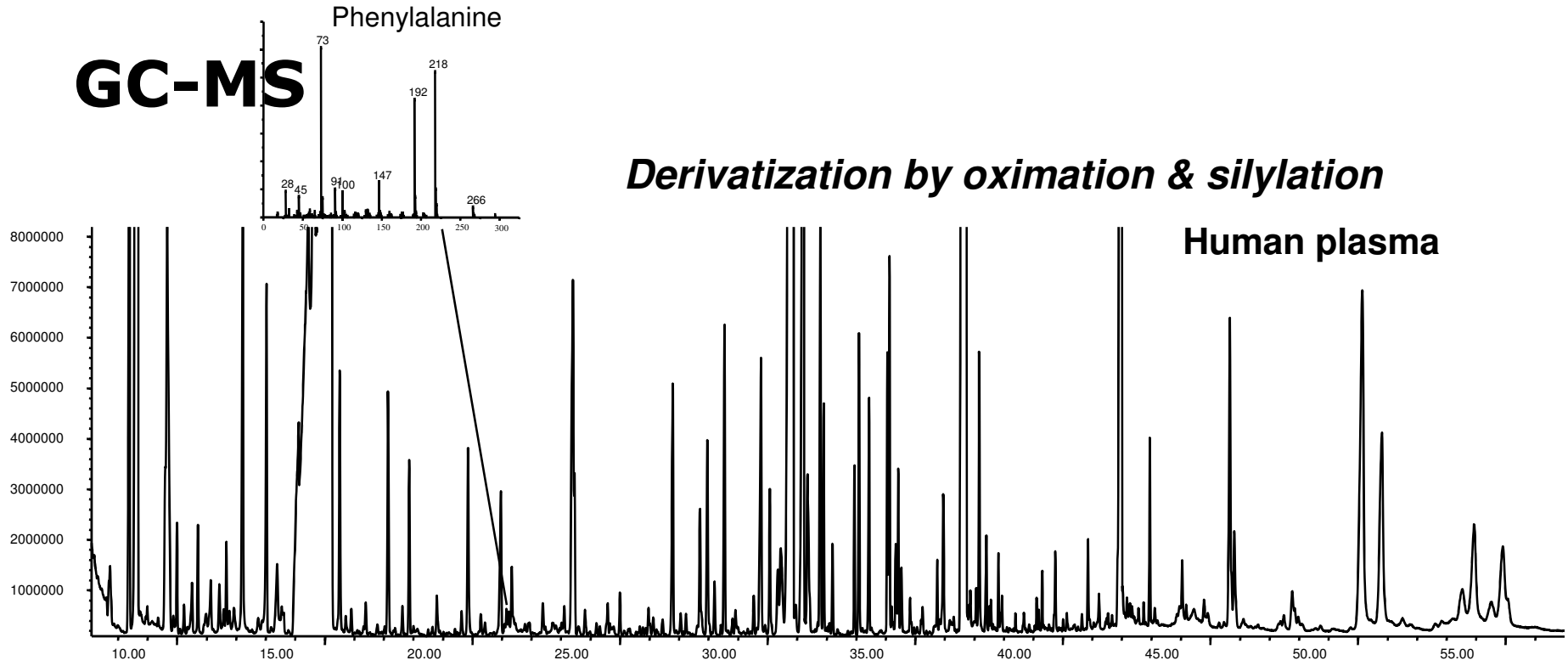
  Untargetted/screening/or targetted

- Easy of use and easy sample clean-up
- Requires MS^n for identification
- FIA-MS
- ESI, APCI, APPI, Maldi, DESI ?
- Q,QqQ, IT, FT, ToF, QToF ?

NMR

- High concentration metabolites
- Screening
- Semi-quantitative

GC-MS



- alcohols, aldehydes, cyclohexanols
- amino acids/small peptides
- amines
- aromatic compounds
- organic acids/fatty acids
- phospho-organic acids
- sugars, sugar acids, sugaramine
- sugar phosphates
- steroids
- up to about 40 C-atoms

Robust method is key!

Koek et al, Anal. Chem., (2006) 78:1272

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Derivatization efficiency (standards)

Compound	RSD (%)	Efficiency (%)	Compound	RSD (%)	Efficiency (%)
Alanine	5	110	Fructose	2	95
Asparagine	7	30	Glucose	4	85
Aspartic acid	10	70	Ribose	3	95
Glutamic acid	9	50	Xylitol	5	115
Glutamine	11	40	Fructose-6-phosphate	6	45
Glycine	3	100	Glucose-6-phosphate	5-10	50-65
Leucine	2	85	Fumaric acid	2	60
Lysine	7	55	Lactic acid	1	90
Methionine	11	65	Malic acid	3	60
Phenylalanine	5	80	Oxaloacetaat	2	80
Serine	7	80	Pyruvic acid	2	70
Threonine	3	70	5-Fluorocytosin	6	25
Valine	4	105	Glycerol-3-phosphate	4	30

- **Hydroxyl and carboxyl functional groups: performance very satisfactory**
- **Amine and phosphoric functional groups: performance satisfactory**
- **Amide, thiol or sulfonic functional groups: critical compounds, performance satisfactory when analytical system in good shape**

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Metabolite profiling with LC-MS: choice of detection mode

LC

CE

direct infustion

ESI
electrospary ionization

APCI
atmospheric pressure chemical ionization

APPI
atmospheric pressure photo ionization

Quadrupole

Ion-trap

Time-of-flight

Quadrupole

Ion-trap

Time-of-flight

ICR-FTMS

Orbitrap

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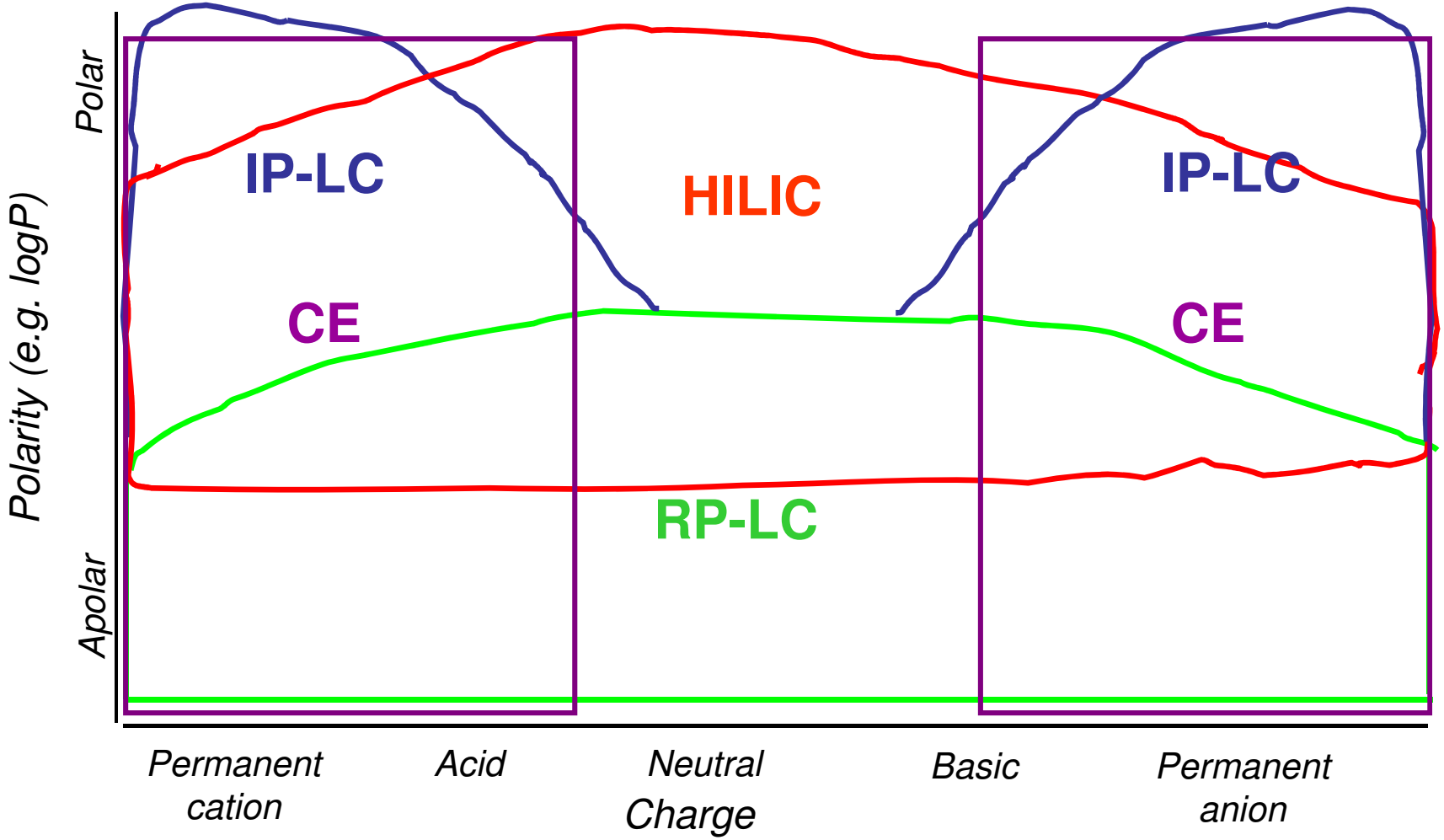
The coverage challenge: liquid-phase separation



Note: derivatization can be used to change polarity, charge and detectability

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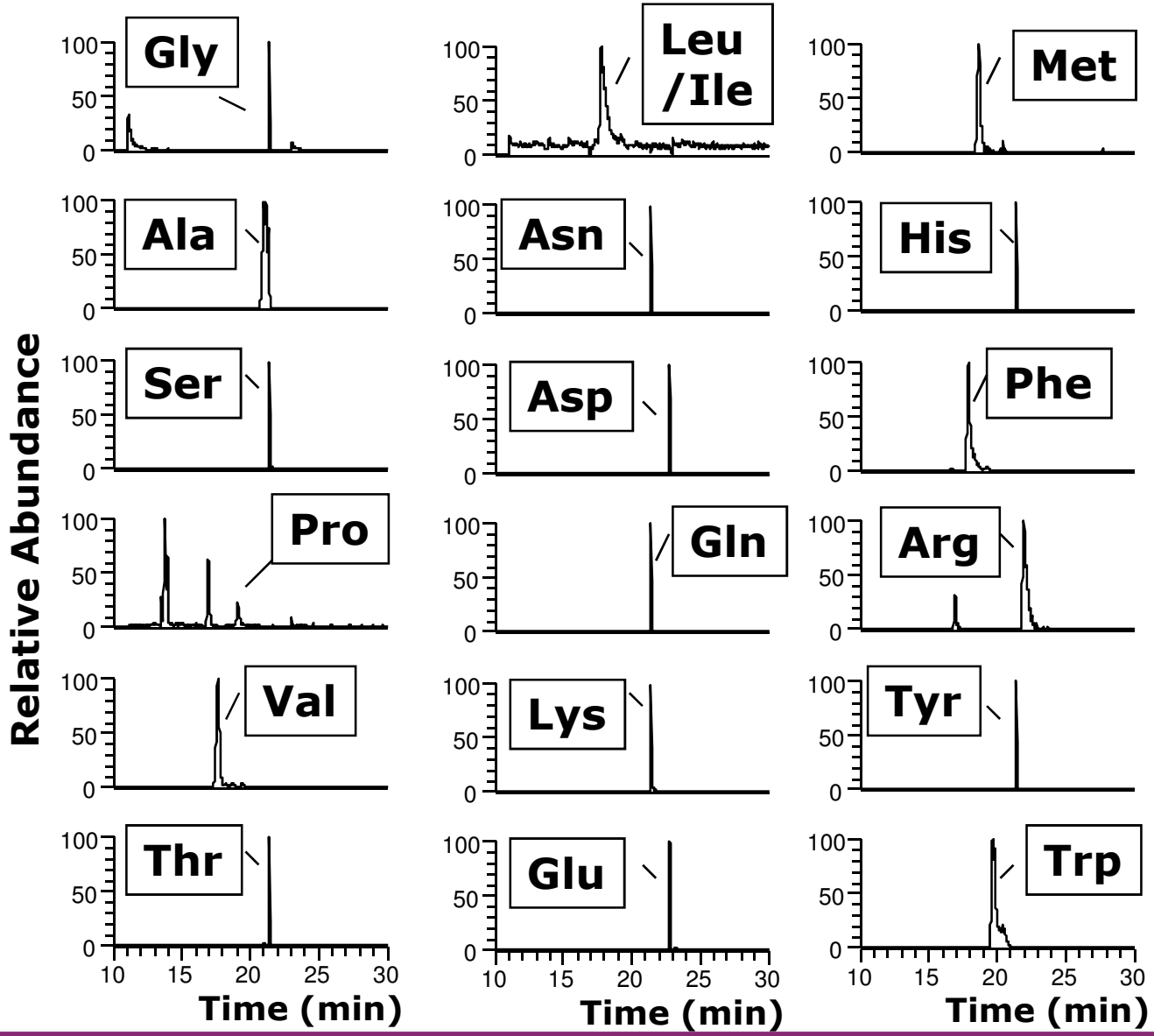
Endogenous amino acids in 0.5 μL aliquot of human urine



Sample:
1/40 diluted urine
injection: 20 μL

Validation

Repeatability (RSD): 1-9%

Linearity (R^2): 0.998-0.999

LOD (ng/ml): <1

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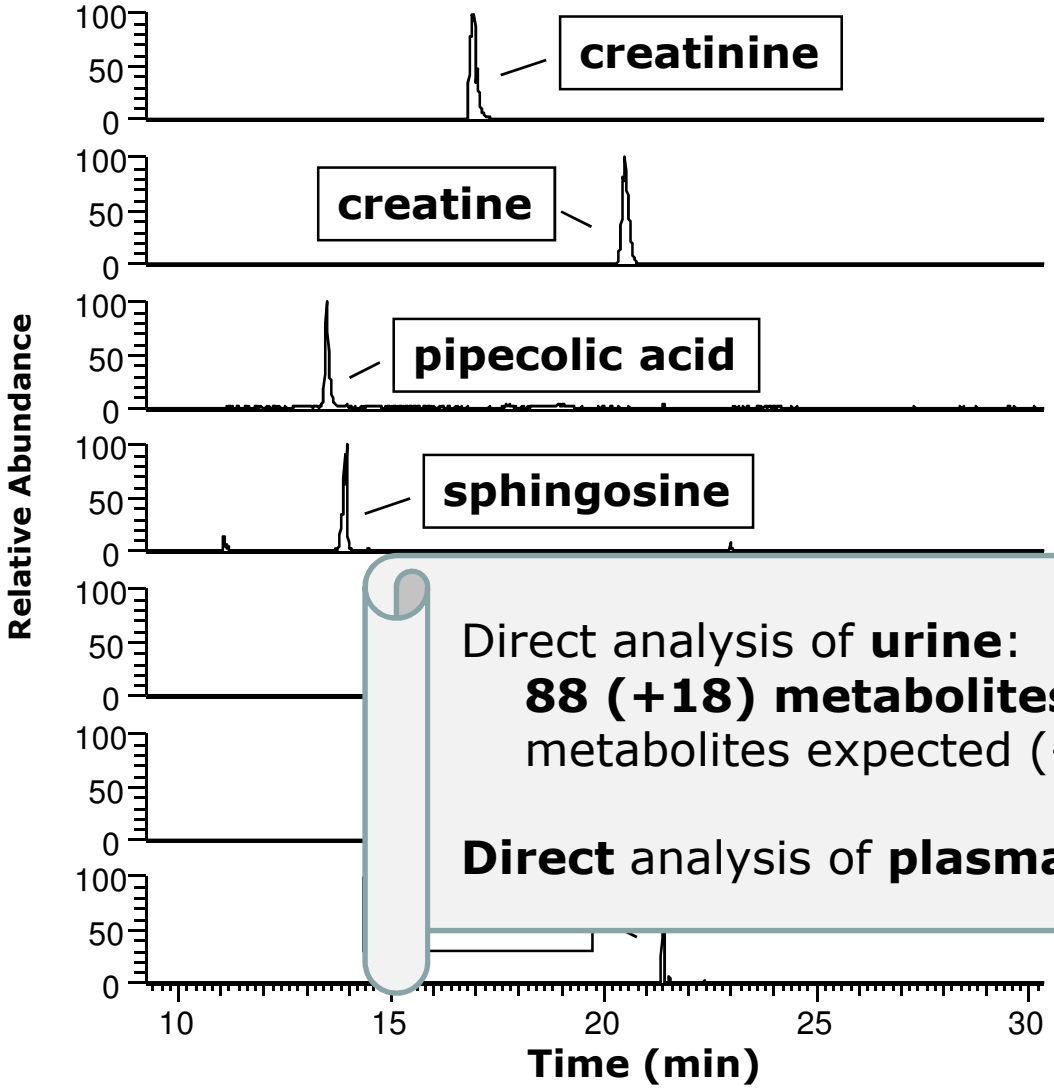
SCX-HILIC-MS: extracted ion chromatogram of possible metabolite candidates in urine



Possible metabolites were searched with the mass window of 1 millimass unit by using calculated mass.

Direct analysis of **urine**:

88 (+18) metabolites detected of **130 (+20)** cationic metabolites expected (+ isomers) according to HMDB

Direct analysis of **plasma** & **CSF**

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Ambition

Creation of a world-class metabolomics knowledge infrastructure to improve personal health and quality of life

Strategy

- Generic technology development in the core programme
- Technology translation together with associate partners

Funding

- Governmental grant (53 MEuro's) for 5 yrs.

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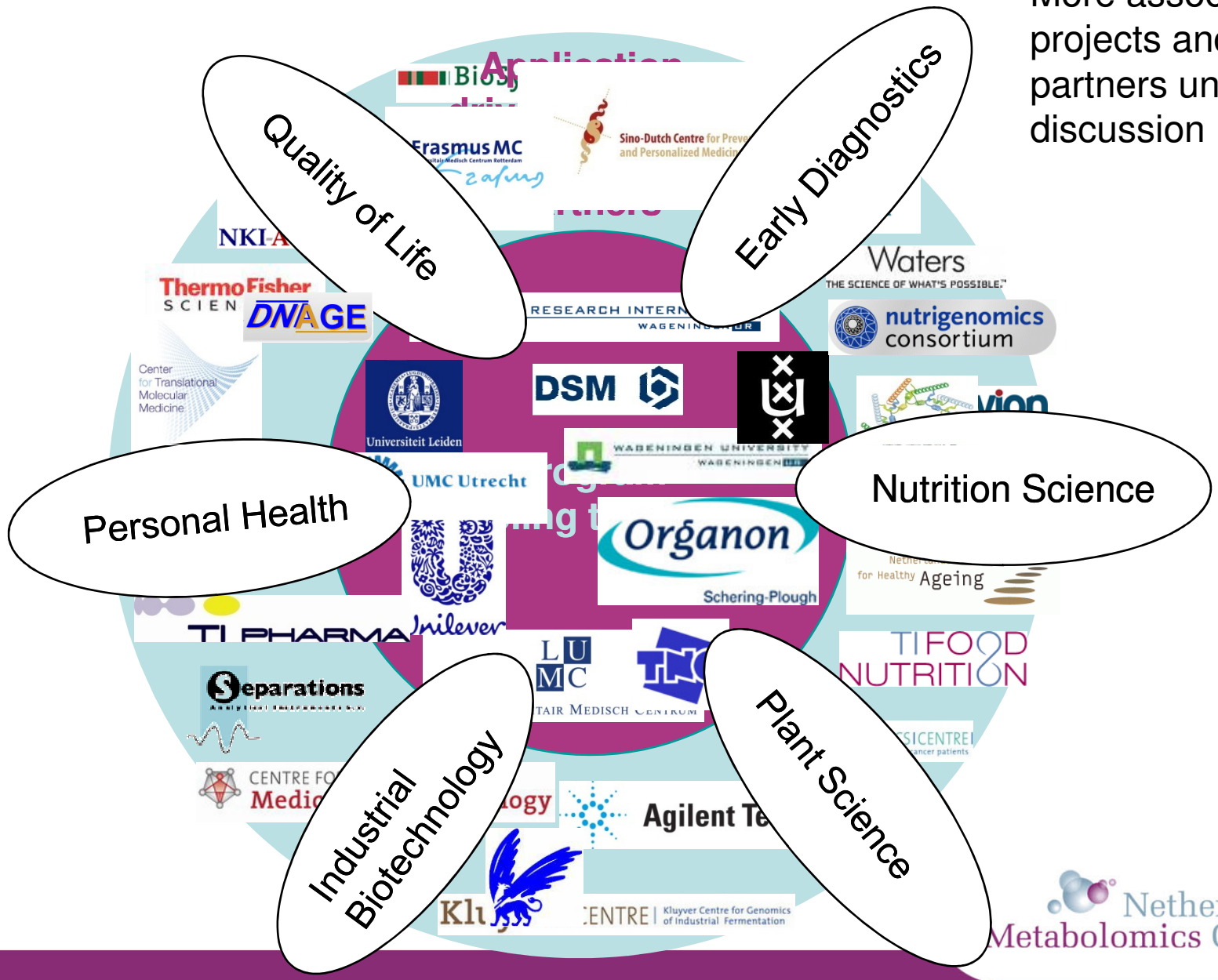
NMC networking partners

More associated projects and partners under discussion



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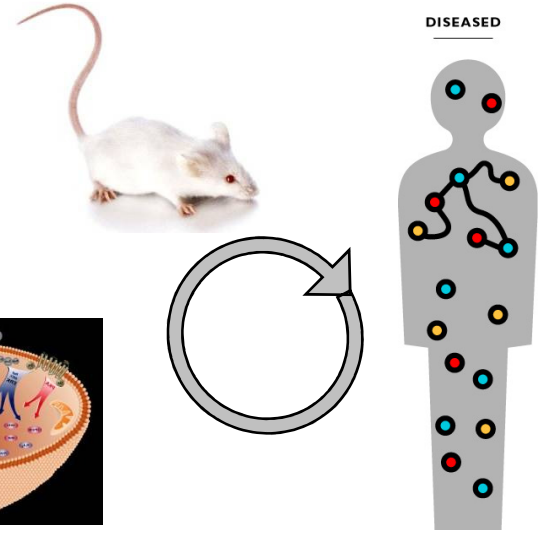
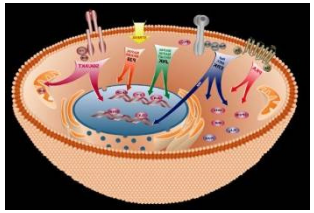
Examples of Core Tech Dev. projects

- **Qp projects**
  - *Biology driven improved coverage of the metabolome*



"From cell systems to animal models to humans, and vice versa"



  - A generic steroid platform (adipose tissue as a model system)
  - Generic peptide-platform (incl. PTM's)
  - Metabolic biomarkers for oxidative stress
  - Inflammation platform
  - Nano/micro sampling for local metabolomics
  - Comprehensive LC-MS metabolite profiling: quantitative and sensitive detection of small differences

Examples of Core Tech. Dev. projects

- **MI projects**
  - Identification strategies using LC-SPE-NMR-MS^n and HR-MS^n

    novel tools for data acquisition and spectral tree interpretation
  - Development of algorithms or rule-based tools for identification of human metabolites from HR MS^n data
  - NMR/MS based prediction
  - Top down identification of unknown metabolites

    structure generation and candidate rejection
  - De-novo identification of polar metabolites with GC-EI/CI-MS^n:

    from measurement to structure
- **BS projects**
  - Analyzing complex-structured metabolomics data

    Data fusion & Global dynamic modelling
  - Power analysis and experimental design

    Power analysis & Experimental designs for time-resolved metabolomics
  - Incorporating a priori information
  - From metabolomics data to networks and back

    Emerging time-resolved metabolomics data structures from in-silico networks

Core projects linked to associate projects via deliverables

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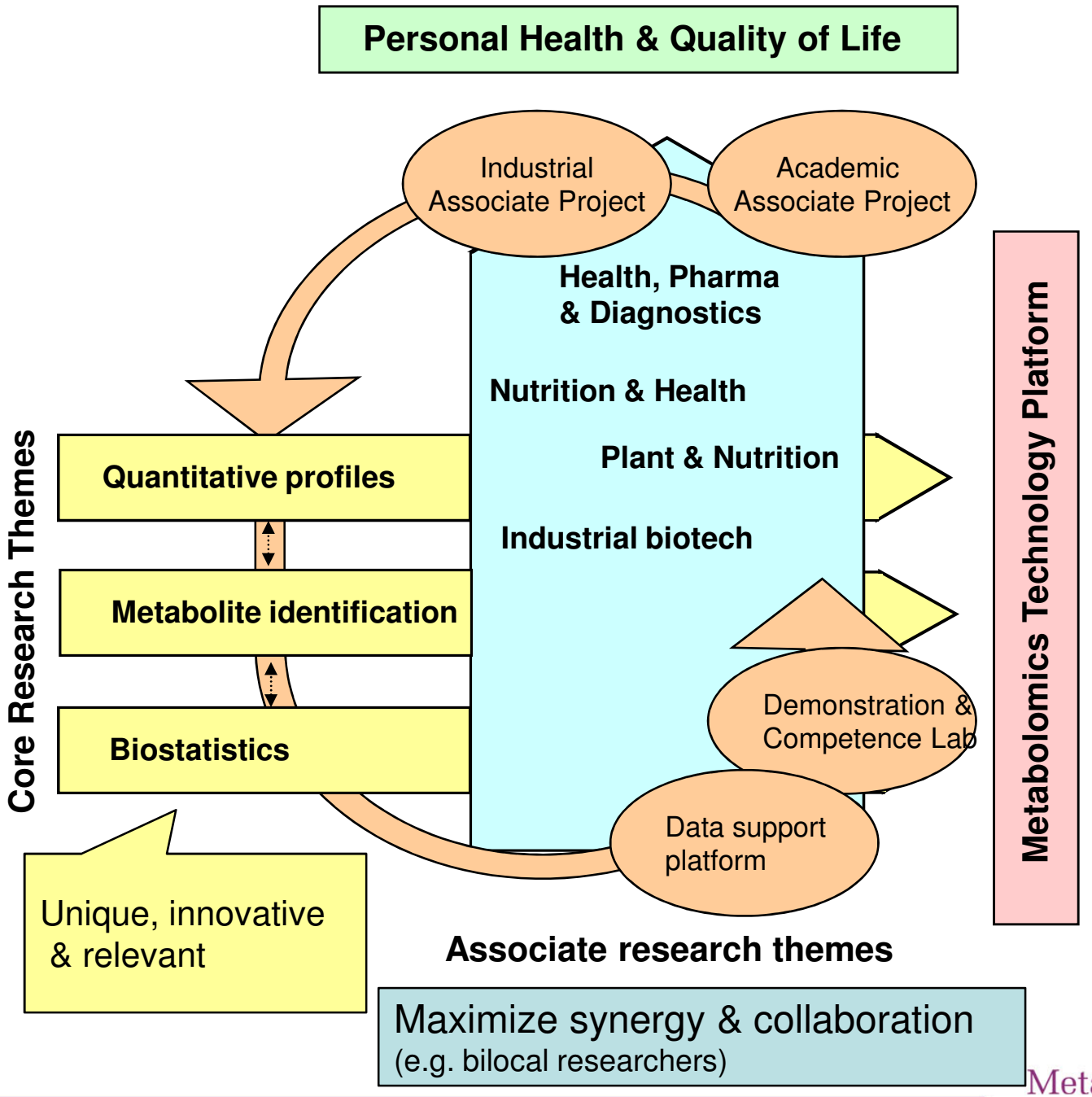

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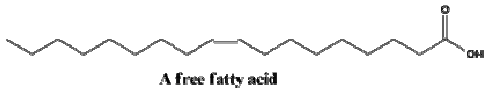
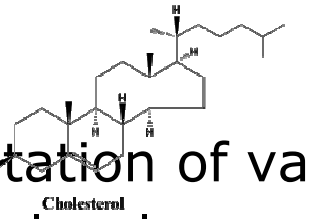
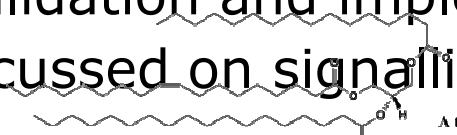
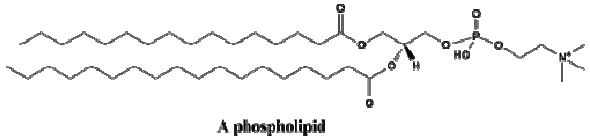
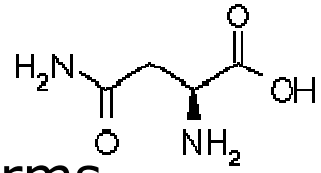
Demonstration & Competence Lab (1)

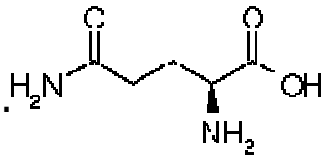






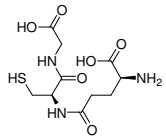


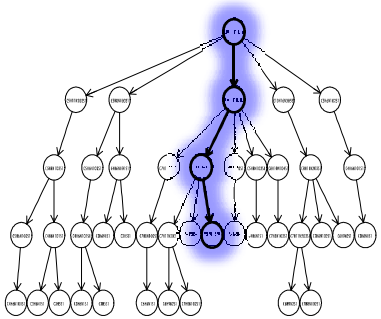


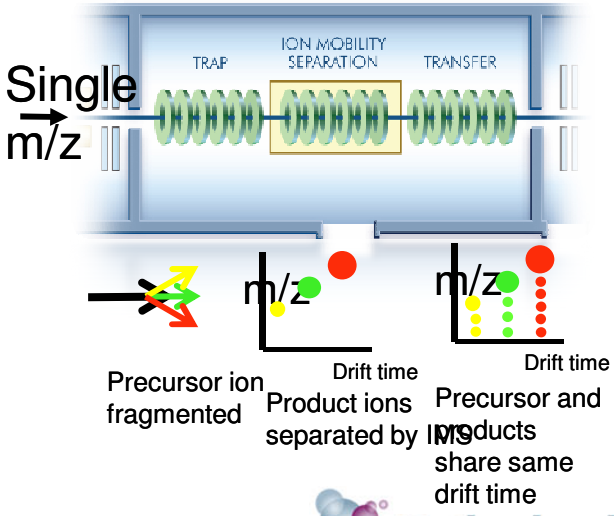


- Validation and implementation of various platforms focussed on signalling molecules
  - E.g. lipids, amino acids, catecholamines, fatty acids, eicosanoids, etc.

- Identification -pipeline & -service
  - Ion mobility, MS^n and MS Trees







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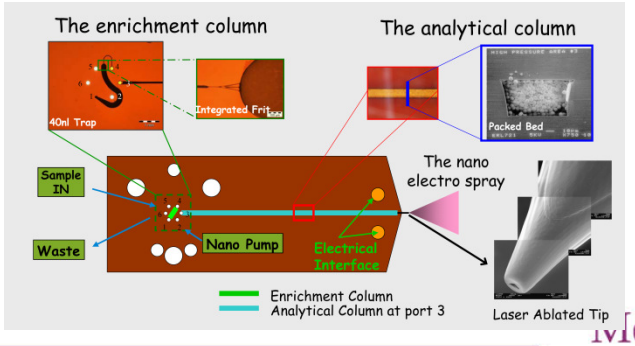
Demonstration & Competence Lab (2)

- **Platforms based on High throughput & High resolution analysis**
  - "UPLC" methods
  - Q-ToF and high resolution trap instruments

- **Methods based on Miniaturized systems using micro/nano-fluidics for small sample volumes**
  - µ-LC and Nano-LC, chip LC



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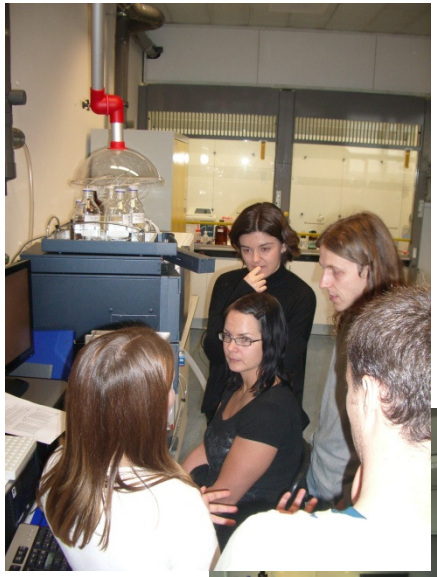
Instrumentation & facilities



- Demonstration & feasibility studies for members/collaborators (NGI)

- Trainings/workshops
  - Hands-on Metabolomics workshop Nov 24-27, 2008
  - 2010 planning 2 workshops

- Meeting place for (inter)national scientists having acces to “State of the Art” instrumentation

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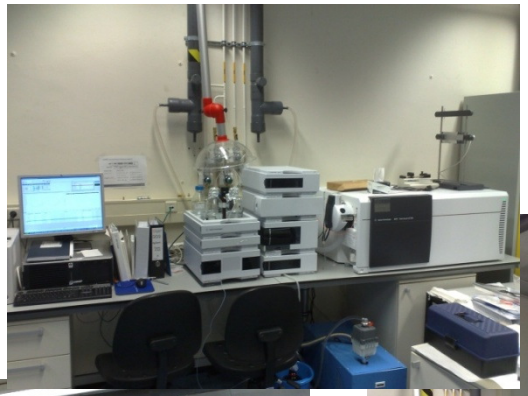
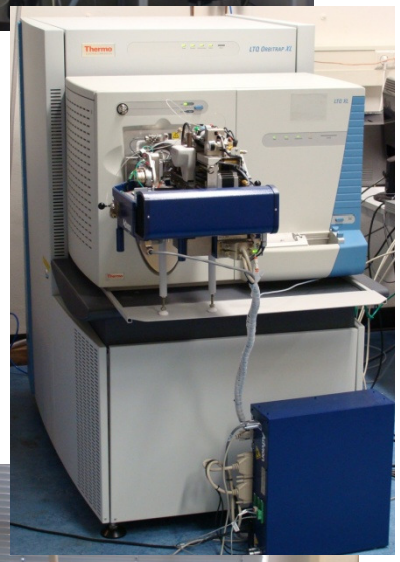
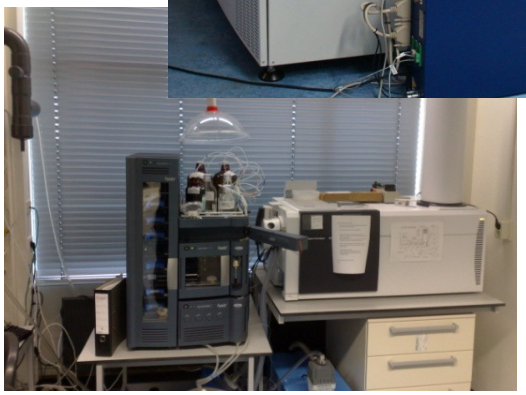
Instrumentation & Facilities







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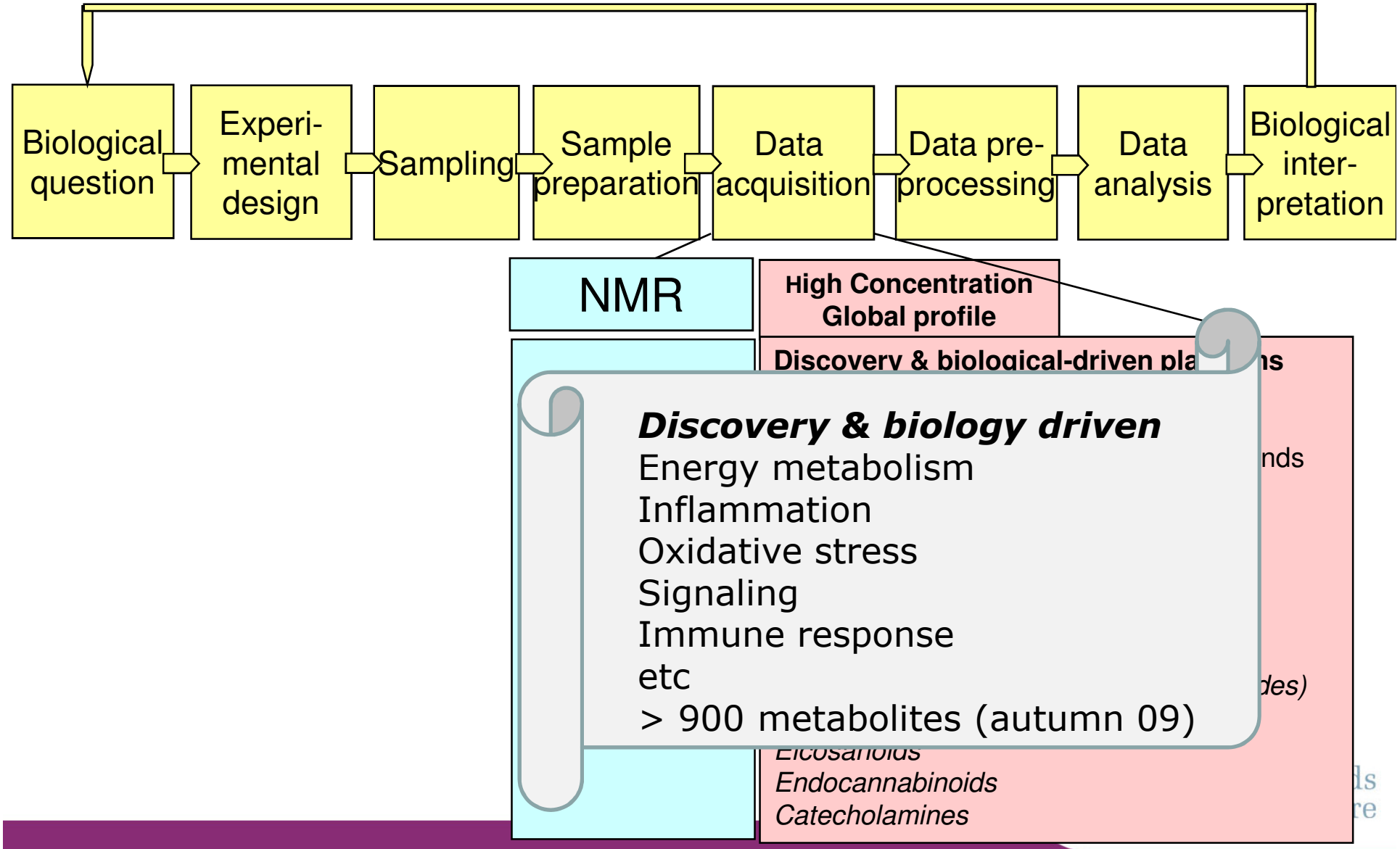
Metabolomics workflow



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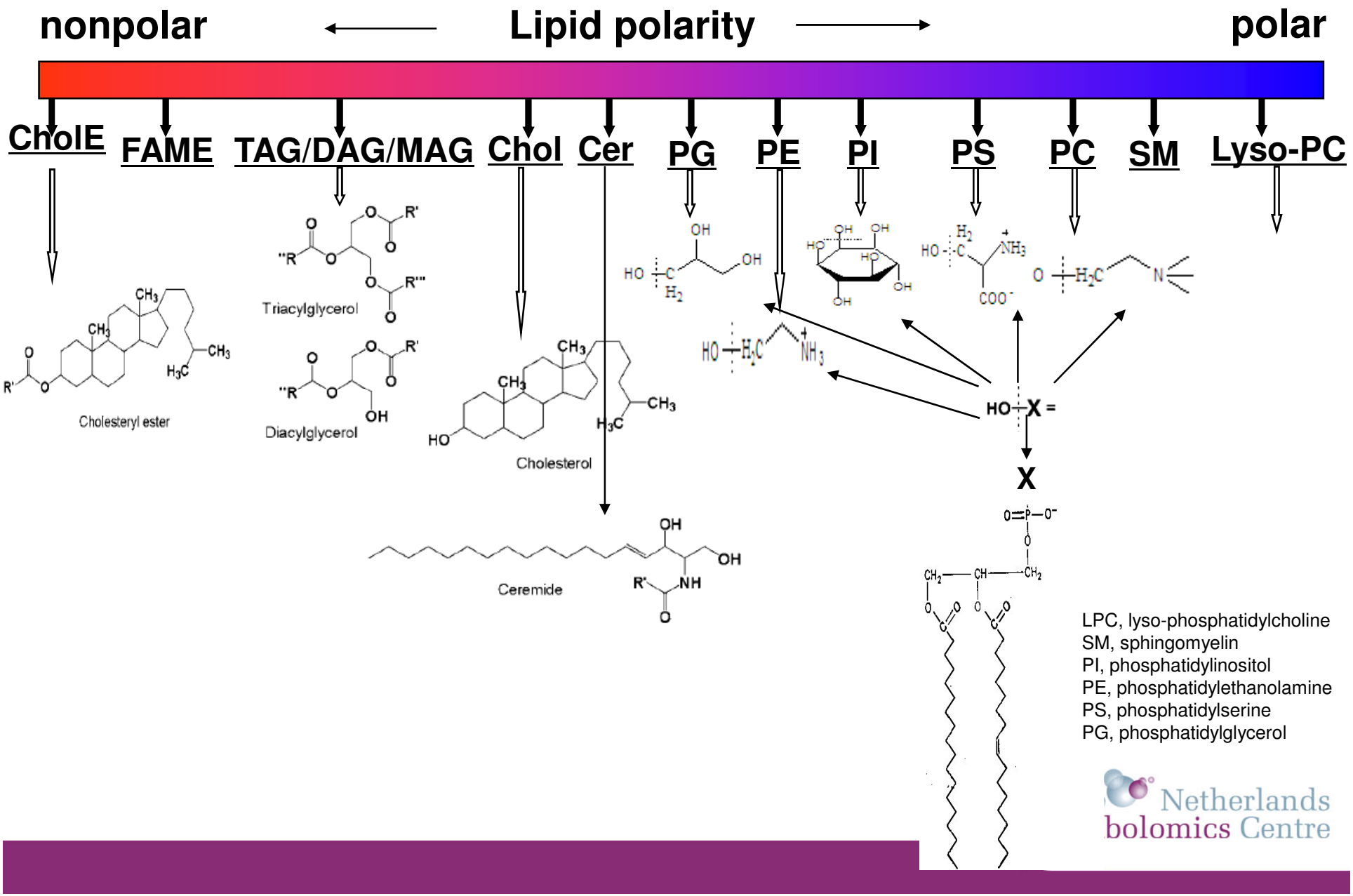
Profiling of lipids: various lipid classes



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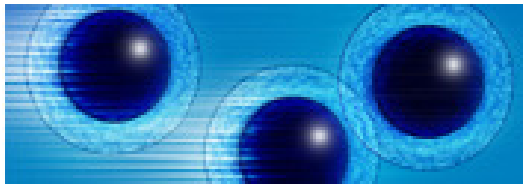
Profiling of lipids: various (LC-)MS strategies

- **Intact lipids**
  - RP stationary phases
  - Polar stationary phases ('straight phase')
  - Direct infusion
  - ESI-MS or APCI-MS
- **Eventually pre-fractionation**
- **Separation & hydrolysis/derivatization**
- **Isomers often not separated**
  (e.g. position of double bond)

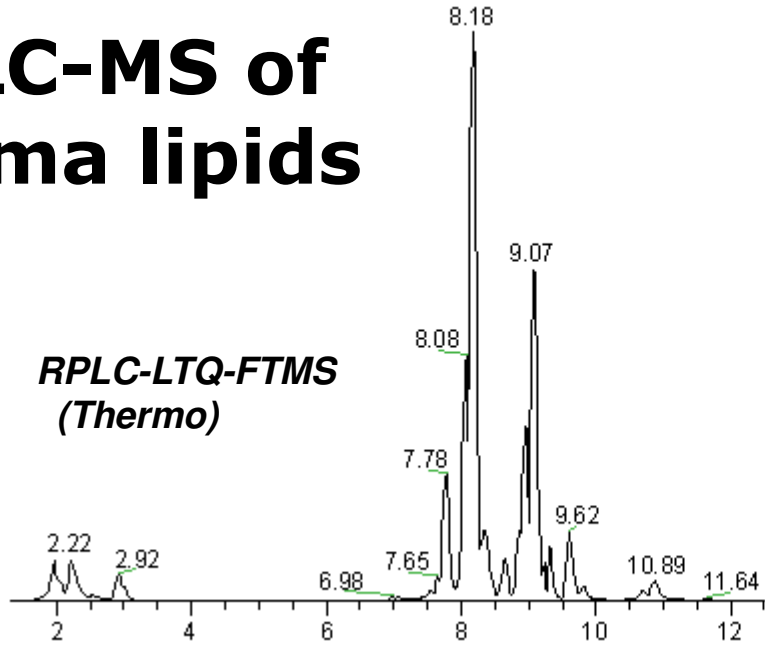
Development of RP-LC-MS of lipids

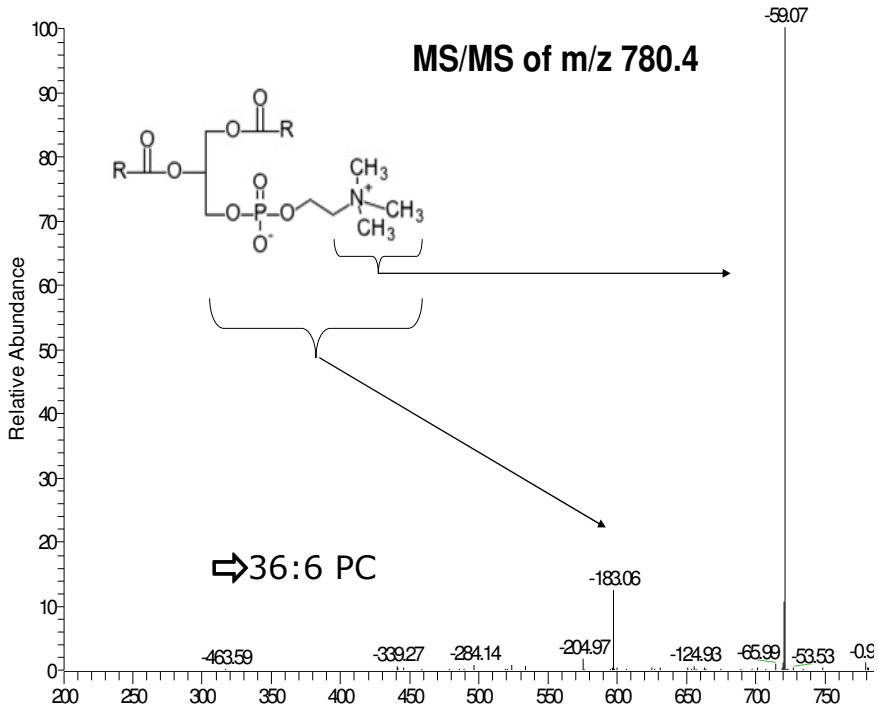
- **Various RP columns tested**
  - Fused core C8 column selected
    Ascentis Express C8: 2.1 x 150 mm (2.7 µm)



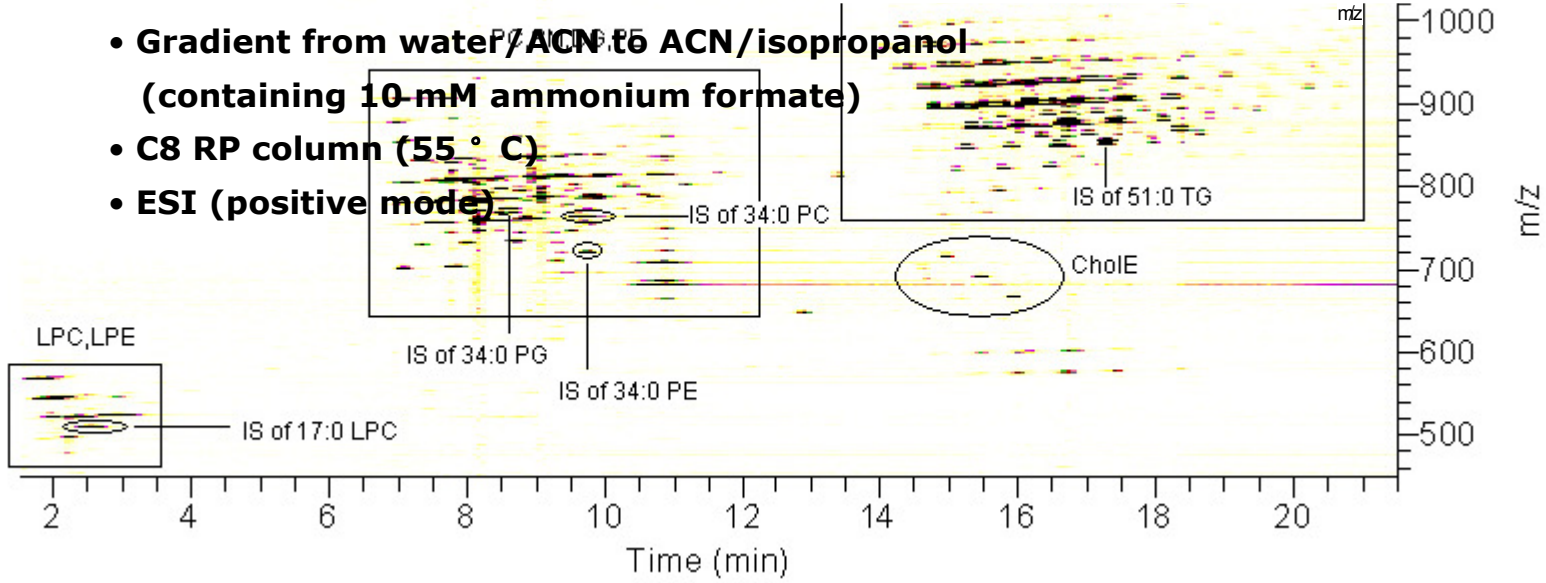
- **Column temperature: varied**
- **Gradient:**
  - Binary/ternary
  - Water, acetonitrile, isopropanol
  - Dichloromethane necessary?
- **Extraction:**
  - 2:1 DCM-MeOH, subsequent addition of water, centrifugation, aliquot of organic phase (modified from Bligh & Dyer)
  - 5 internal non-endogenous lipid standards added prior to extraction

RPLC-MS of plasma lipids





- **Gradient from water/ACN to ACN/isopropanol (containing 10 mM ammonium formate)**
- **C8 RP column (55 ° C)**
- **ESI (positive mode)**



⇨ **250 lipids identified & quantified using reconstructed ion chromatograms**

Hu et al., submitted

Contribution of genotype & environment to phenotype

Known:

differences in genotype
(+ environment)

↓

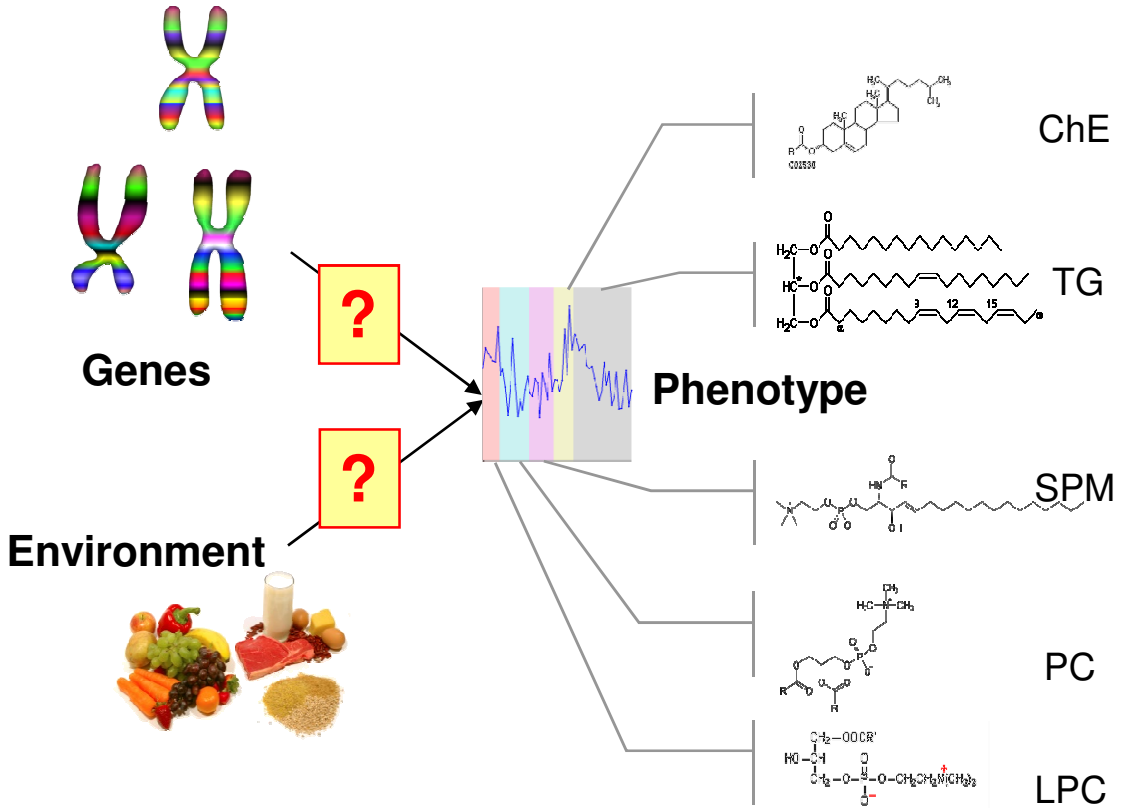
differences in phenotype
(metabolome)

This study:

similarities in genotype
(+ environment)

↓

similarities in phenotype
(metabolome)?



Lipid profiles by RPLC-MS

Differentiate between genes and environmental factors by studying twins with same genotype

Study population

- **23 twin pairs (21 monozygotic, 2 dizygotic); age 18.0 ± 0.2 y**
- **8 siblings (brothers/sisters); 17.4±4.3yr**
- **Nearly all living at home; all healthy**
- **Blood drawn after overnight fasting**
- **Questionnaires, e.g. recent subjective health**

- **Monozygotic twins share 100% of genes**

  → within-pair similarities in metabolite profiles?

- **Dizygotic twins & Non-twin siblings**

  → within-family similarities in metabolite profiles?

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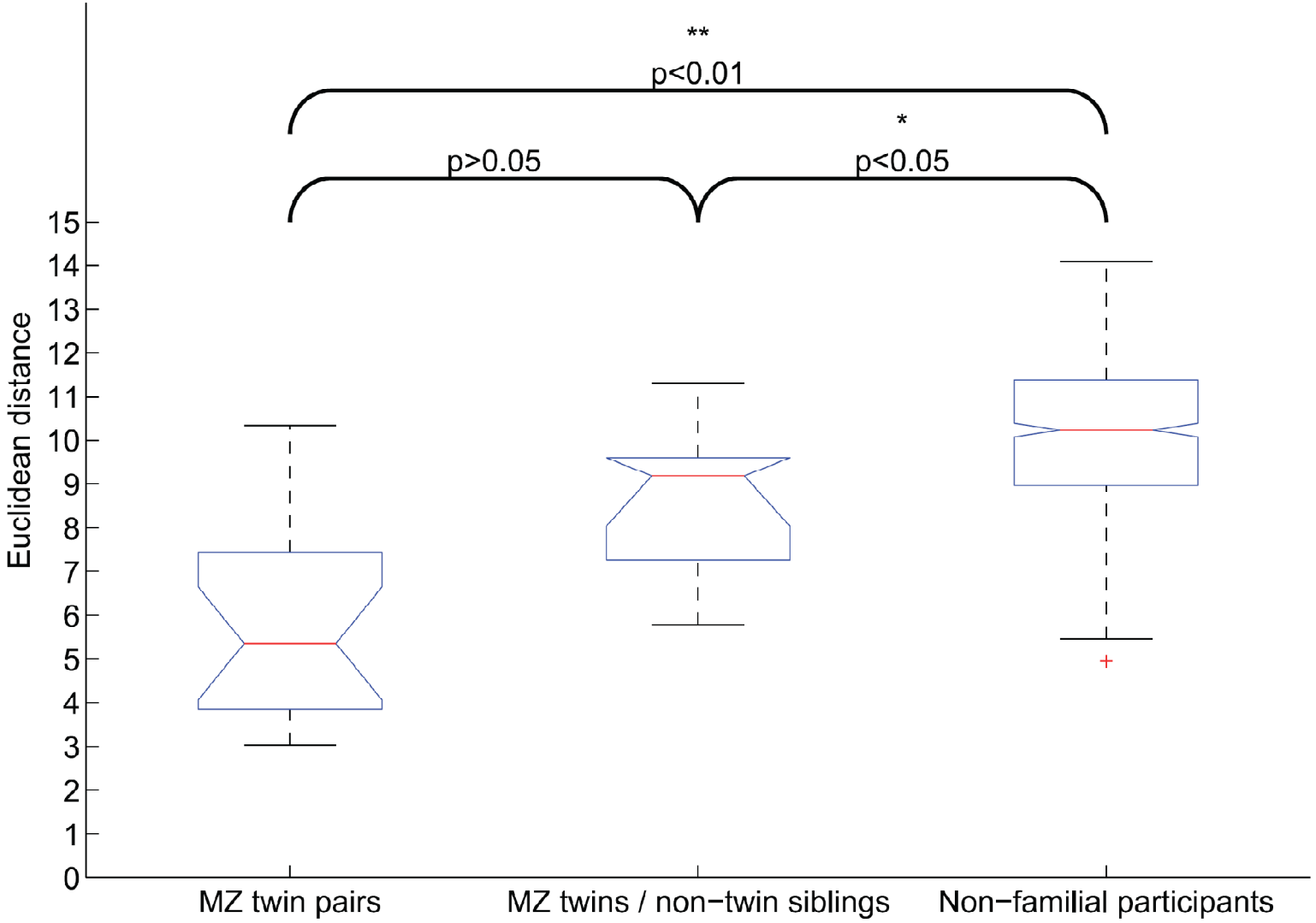
Results: Euclidian distances



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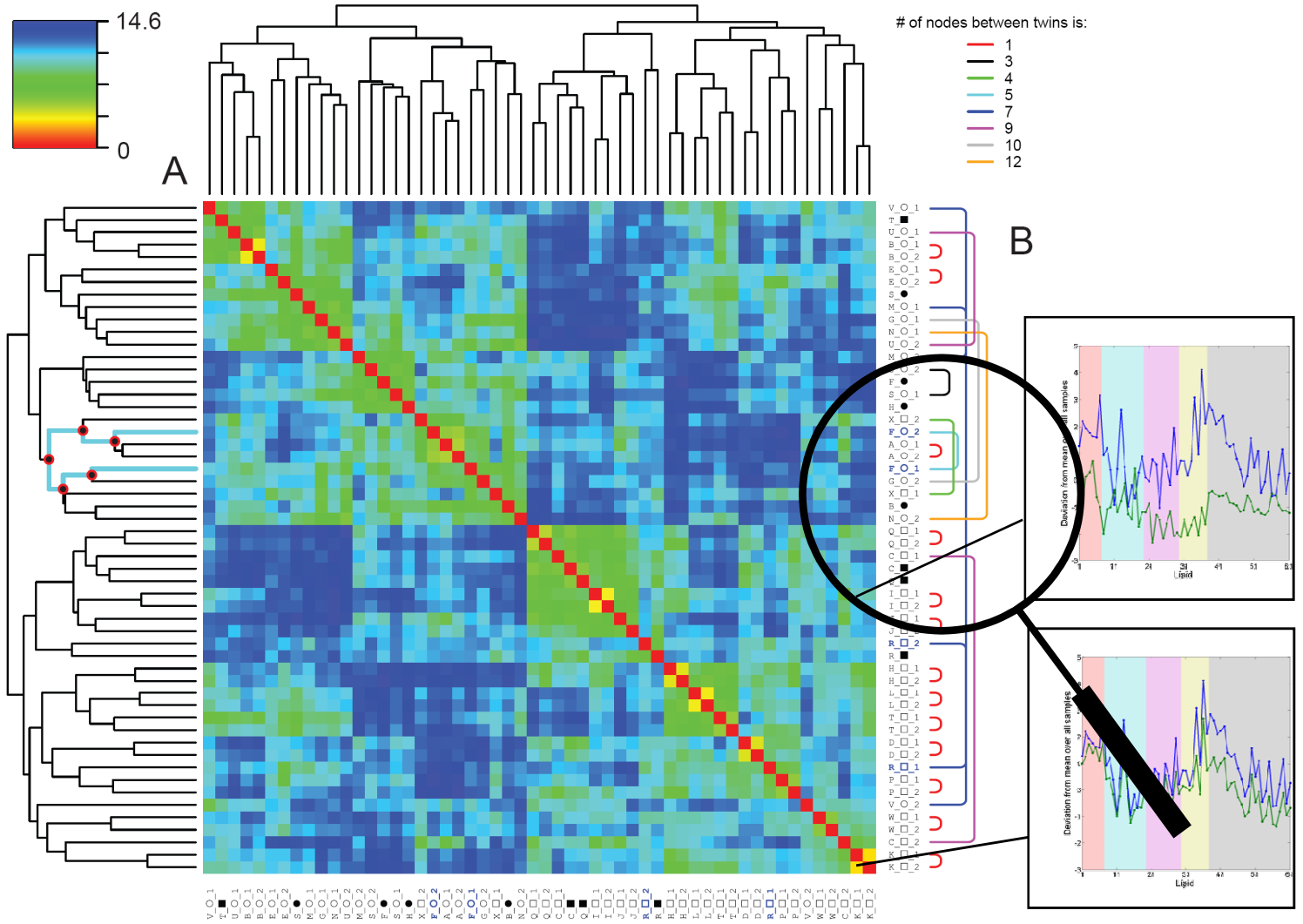
Hierarchical clustering of pairs of subjects

- **Similarity determined by correlation of lipid profiles**
- **Hierarchical clustering according to calculated similarities**



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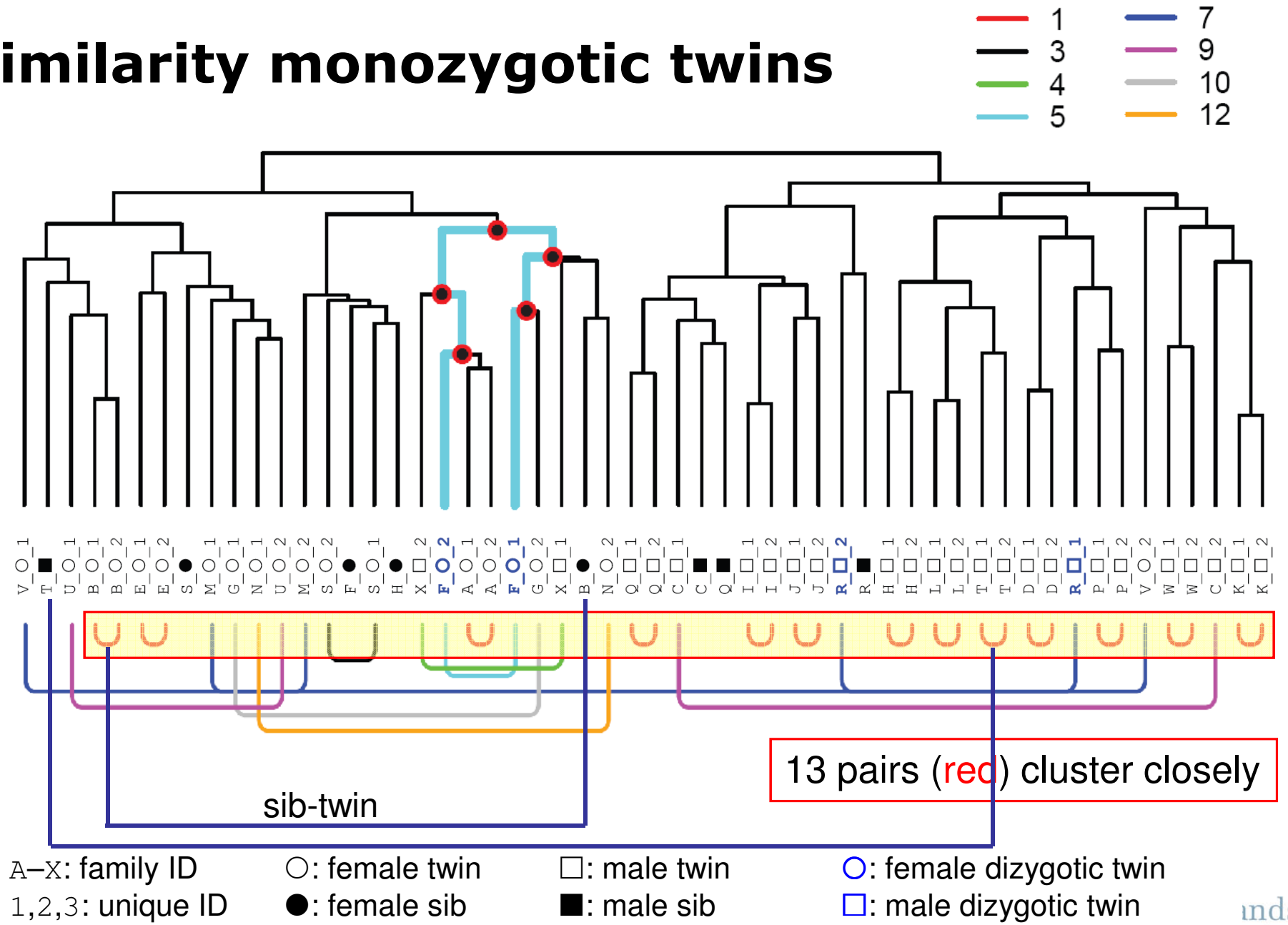
Similarity monozygotic twins



Intermediate conclusions

- **Males cluster with males, females with females**
- **13 of 21 monozygotic twin pairs cluster closely**
- **Most of them are male**
- **Dissimilarities are larger *(not all shown)***
  - between dizygotic twins,
  - among non-twin brothers/sisters,
  - and among nonfamilial individuals

Why are other monozygotic pairs dissimilar?

> genetic background indeed contributes to plasma lipid profile

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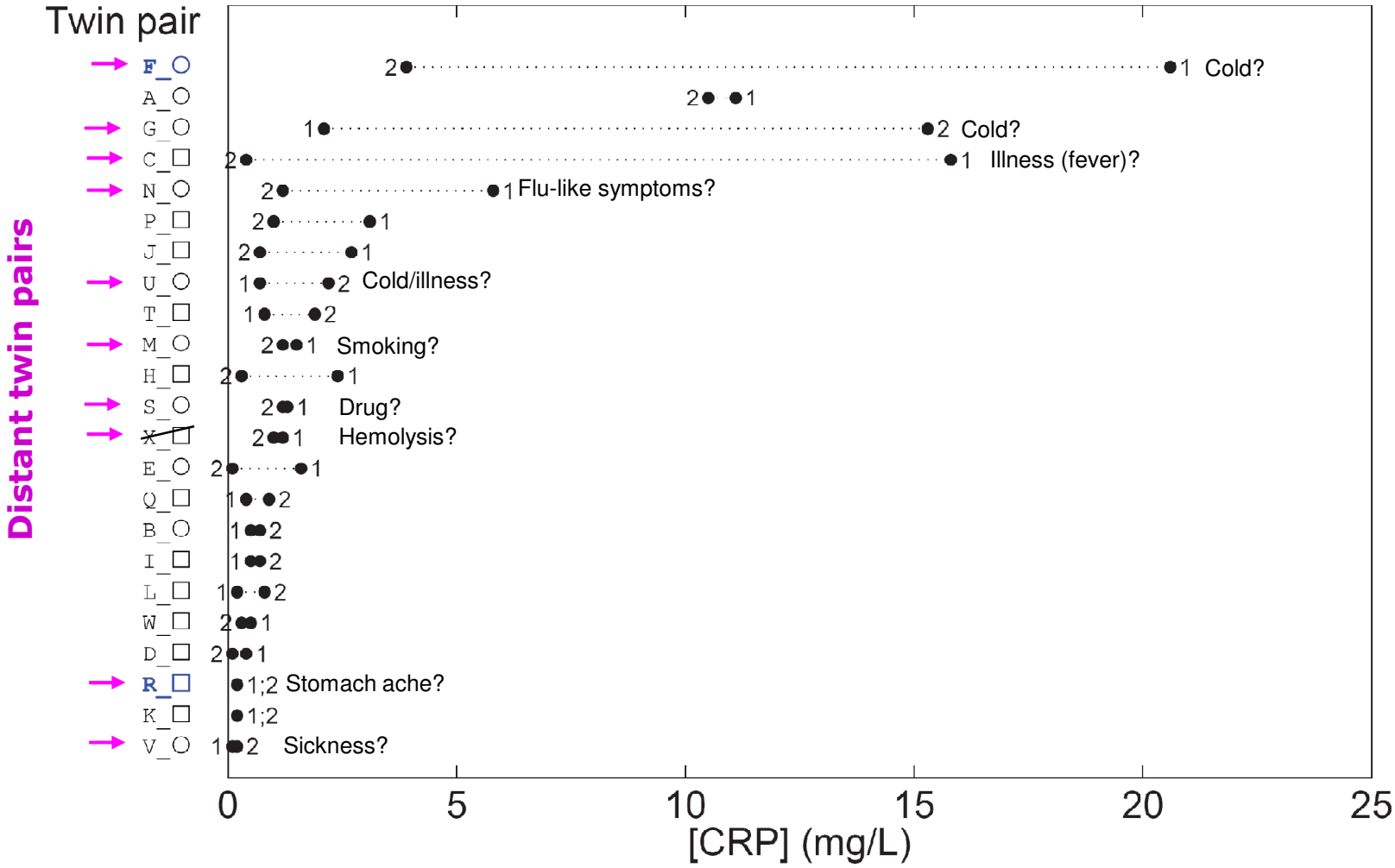
Inflammatory marker: C-reactive protein



Netherlands Metabolomics Centre

Draisma et al., OMICS (2008) 12: 17-31

Outline

- **Metabolomics**
  - Why, what, how
- **Netherlands Metabolomics Centre**
  - Aim & objectives
- **Profiling of Lipids**
  - Application to study twins
- **Profiling of amines**
  - Application to CSF and Plasma
- **Identification of unknown metabolites**
  - MS trees for structure elucidation
- **Applications**
  - Biomarker discovery of disease models
  - Network analysis of drug intervention
- **Conclusions & Acknowledgements**

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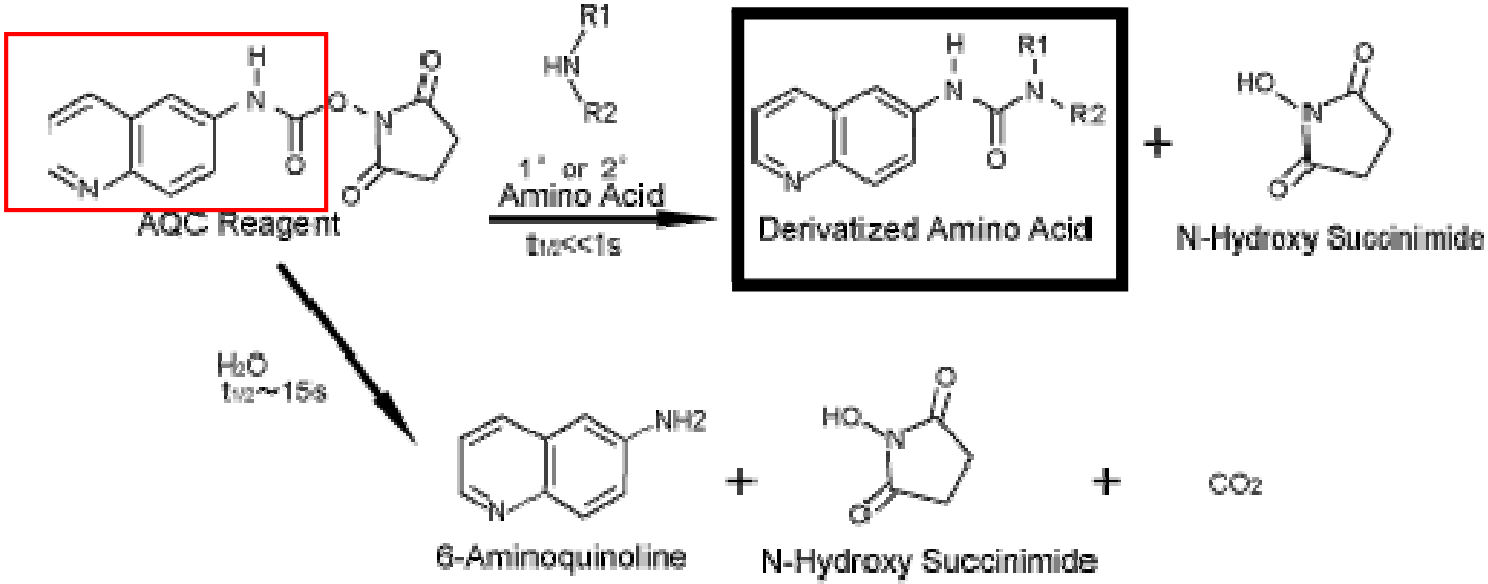
Amine profiling in CSF and Plasma by targeted derivatization

Derivatization reaction



Optimal conditions:

- pH > 7
- AA-Reagent Ratio > 1: 5
- Temp. 55°C
- MRM analysis -> Tag^+ ion (171)

adapted from Waters Accq-Tag Method

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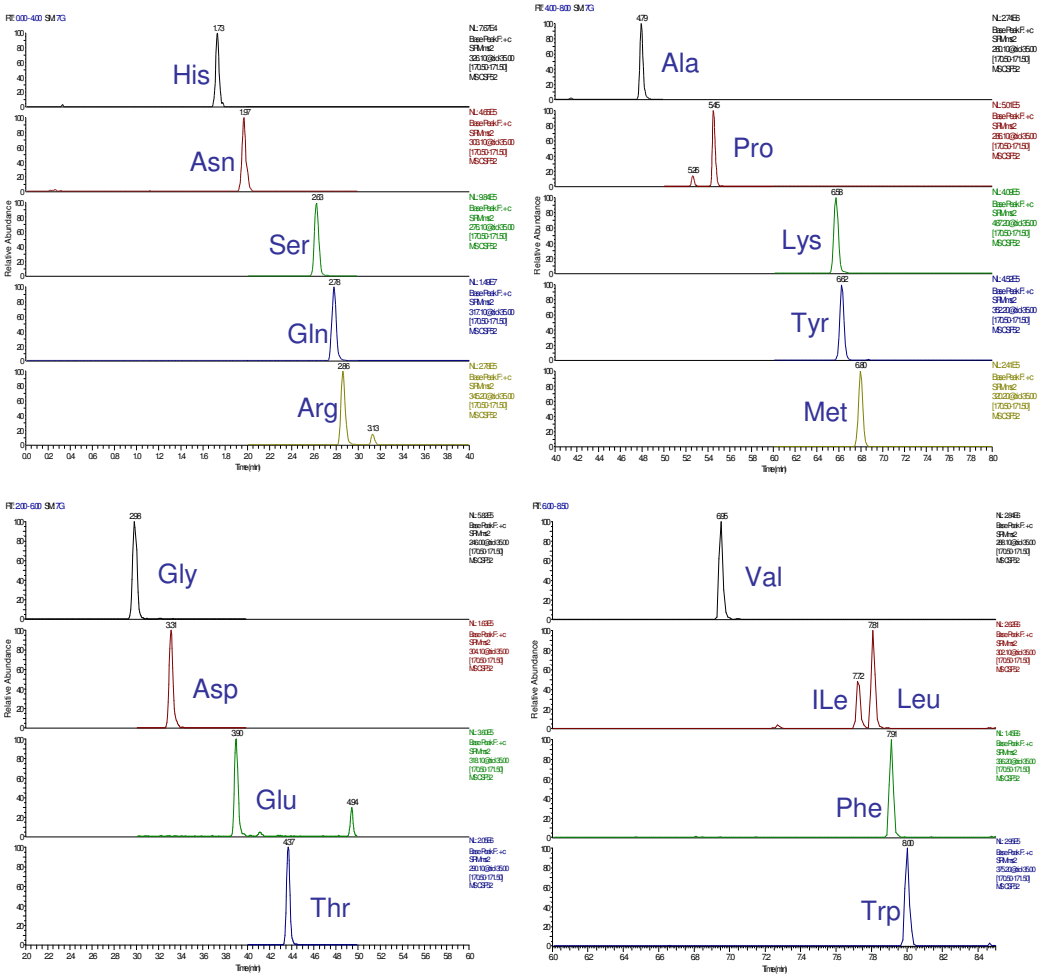
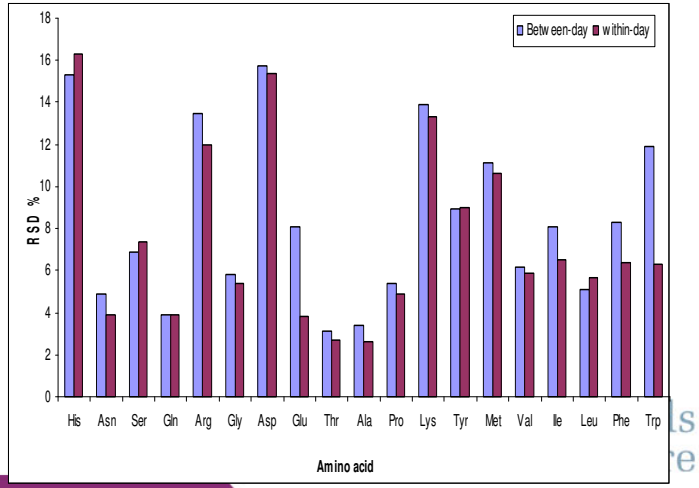
Amino Acids in CSF



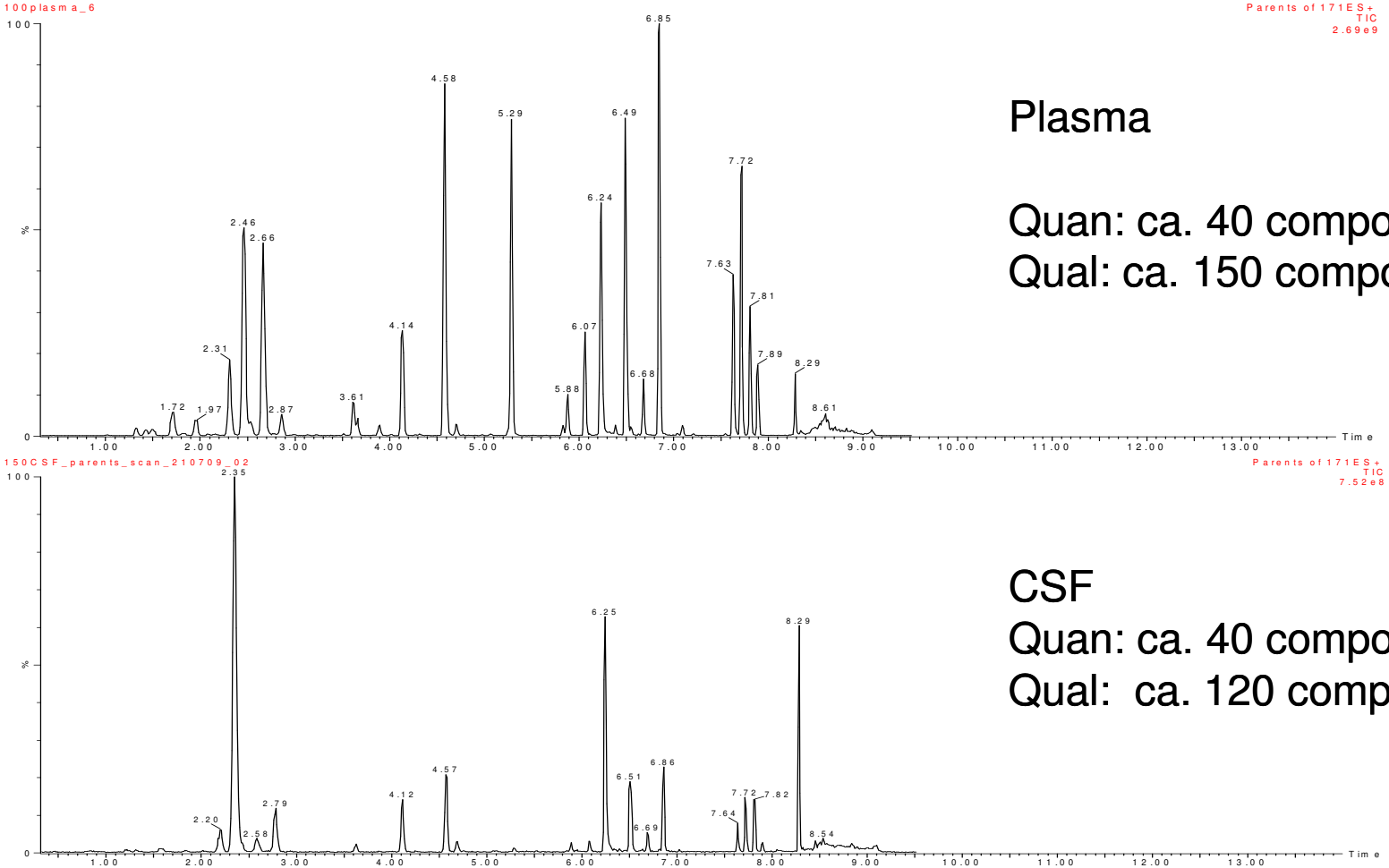
Linearity
- 4 orders of magnitude

Repeatibility
- majority < 10%

Recovery
- >90%



Amine profiling of Human Plasma and CSF



Plasma

Quan: ca. 40 compounds
Qual: ca. 150 compounds

CSF
Quan: ca. 40 compounds
Qual: ca. 120 compounds

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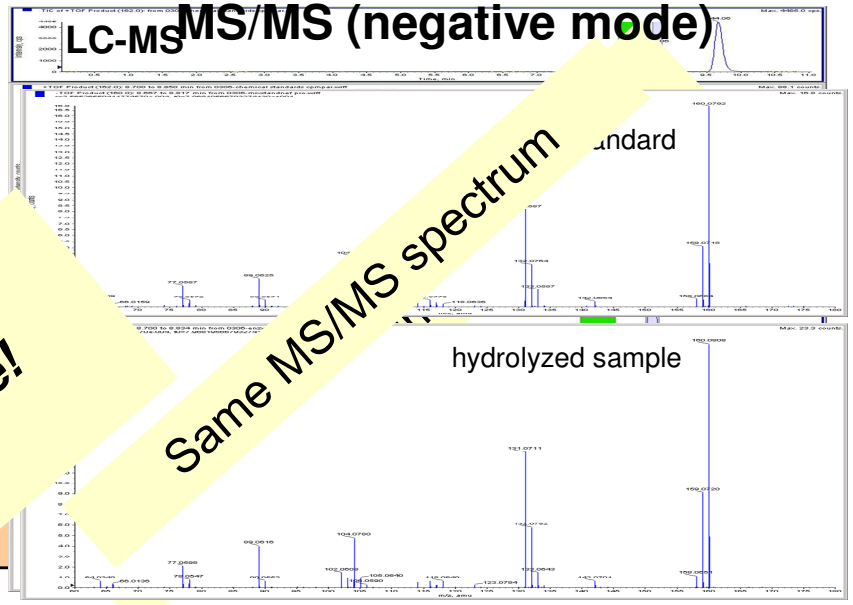
Identification of unknown potential biomarker



Same MS/MS spectrum

More efficient procedure desirable!

biomarker

High resolution FTMS

not in database
(Pubmed, KEGG
Dictionary of natural products)

loss of 176

glucuronic acid
conjugate

162.0550

Hydrolysis
(β-glucuronidase)

quinoline

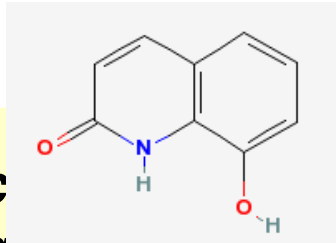
$C_9H_7NO_2$

MS/MS/MS

References of qui...s

neutral loss : 18(H_2O)

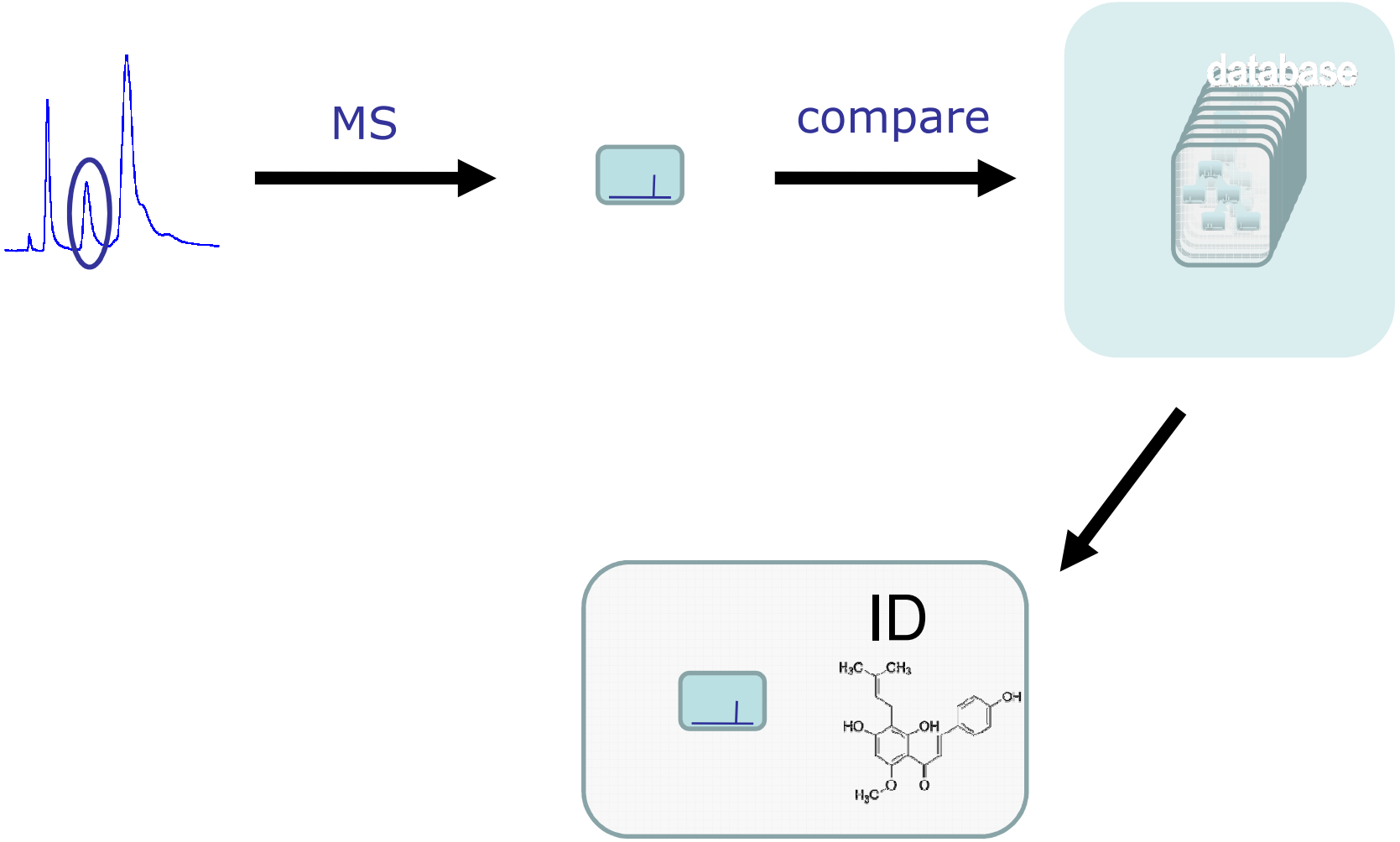
46(HCOOH), 28(CO), 27(...



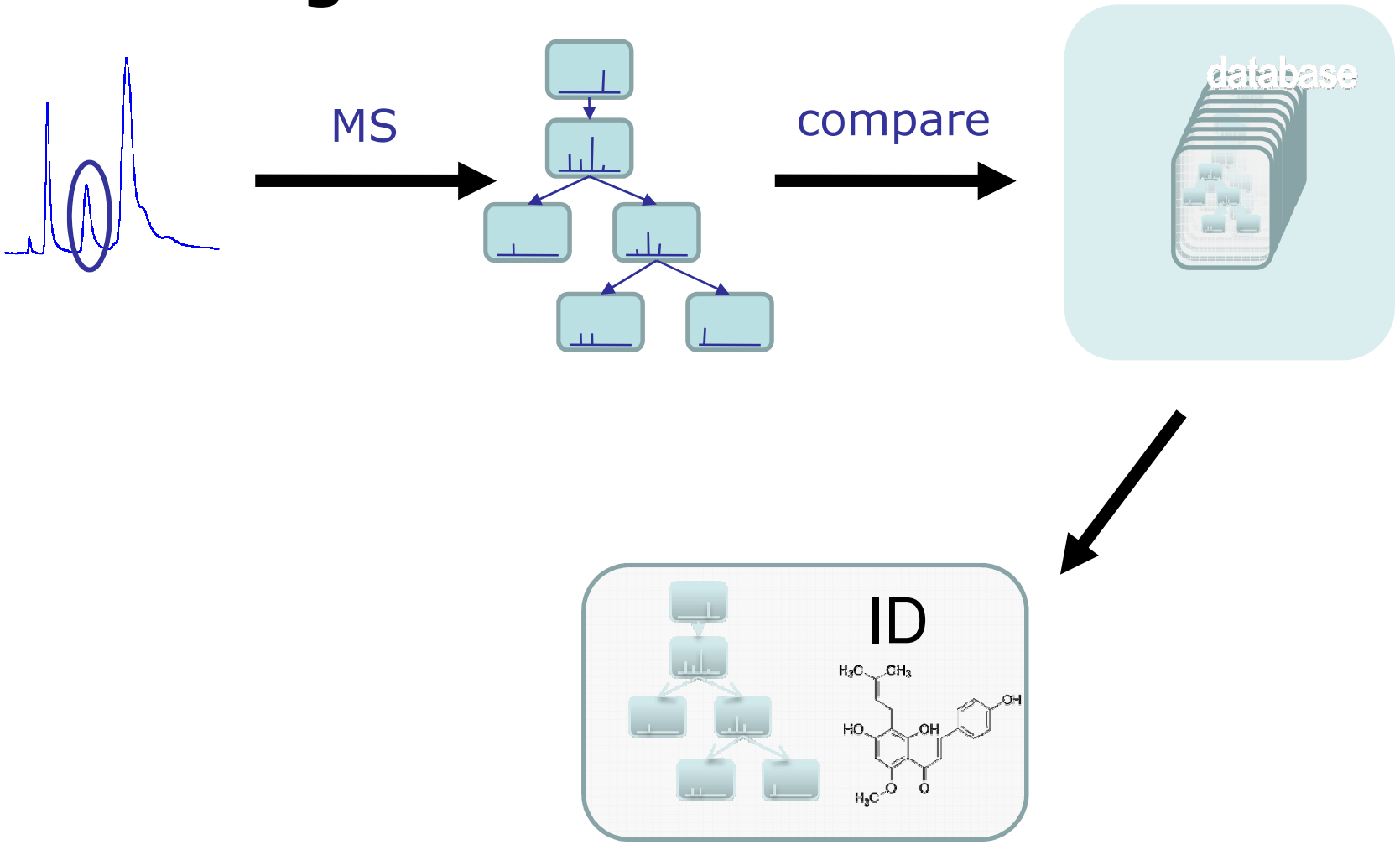
Identified as gluc...
conjugate of 2,8-q...
2,8-quinolinediol

HPLC-MS/MS

identification: assignment



identification: assignment with multistage MS



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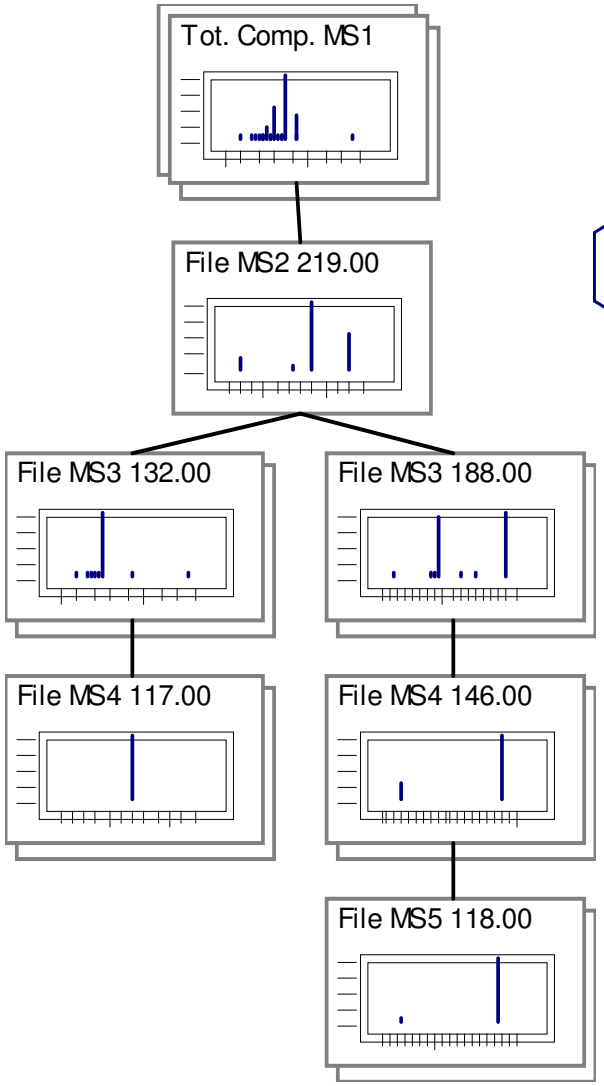
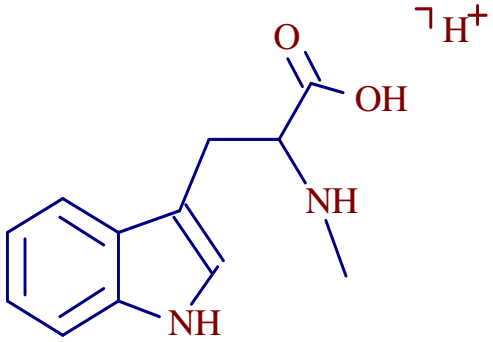
Mass spectral trees





Methyl-tryptophan

Idea: Parent ion results in same spectral sub-tree

=> Create database of sub-trees of substructures

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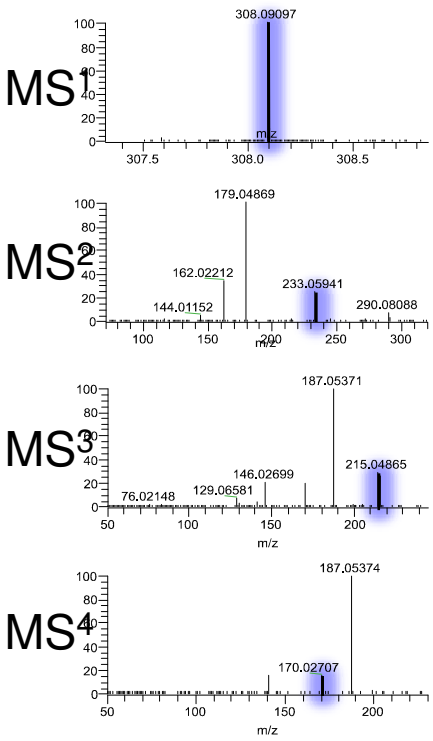
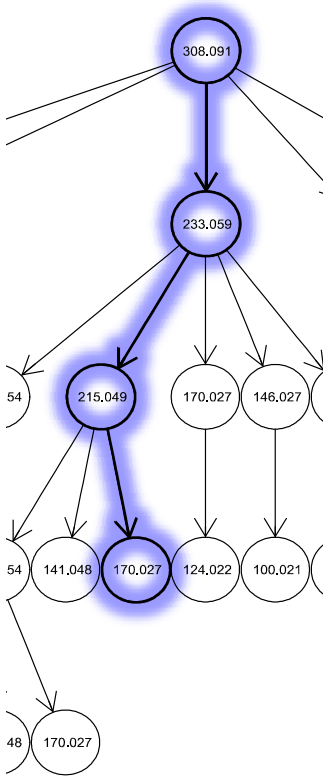
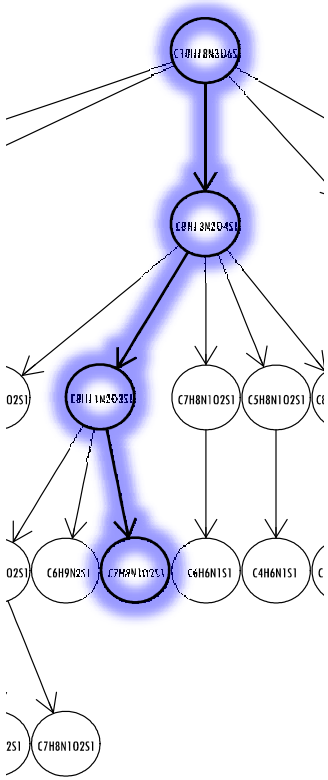
MS trees Fragmentation trees







MS^n experiment fragmentation tree (fragment masses) fragmentation tree (elemental compositions)

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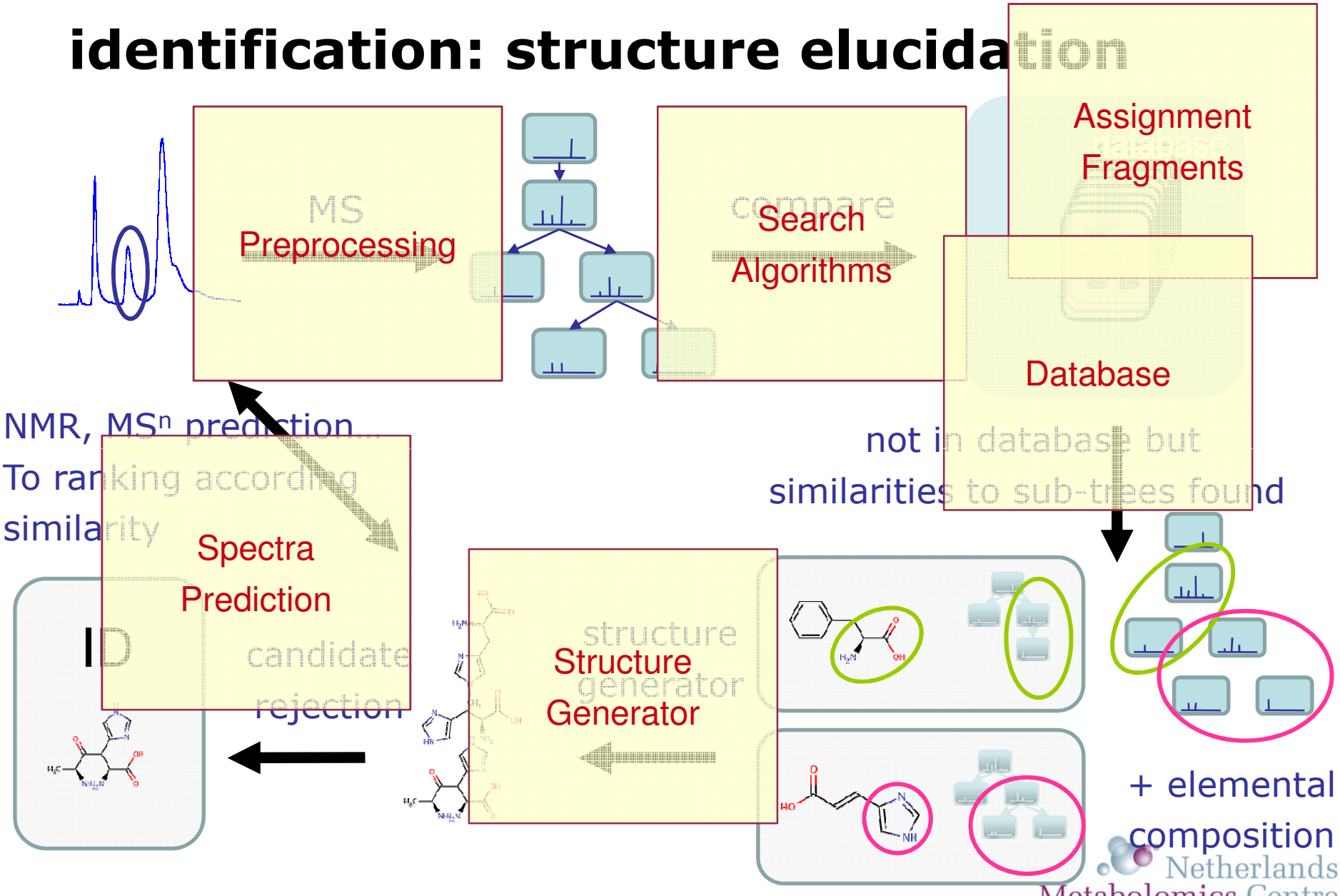
identification: structure elucidation



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Adding extra dimensions

Ion-Mobility (Q-)ToF

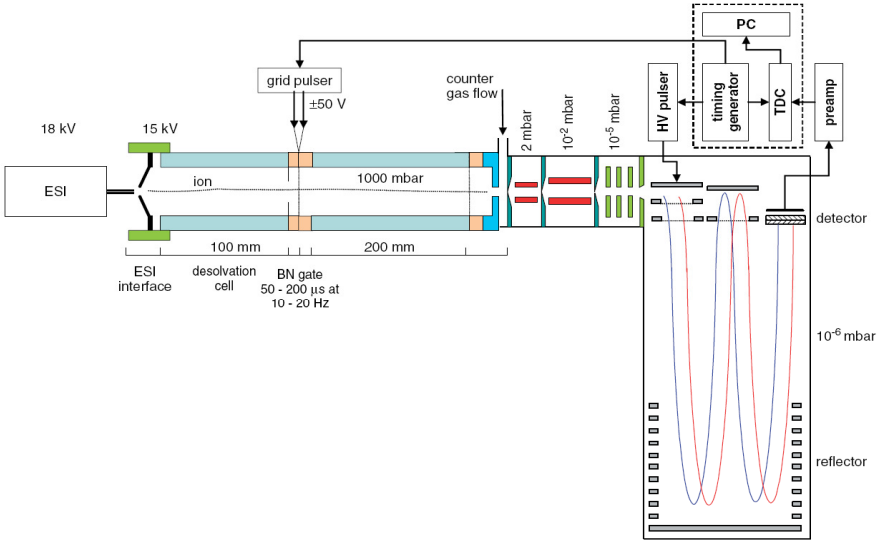
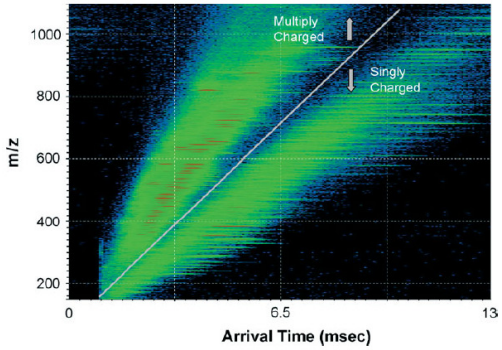


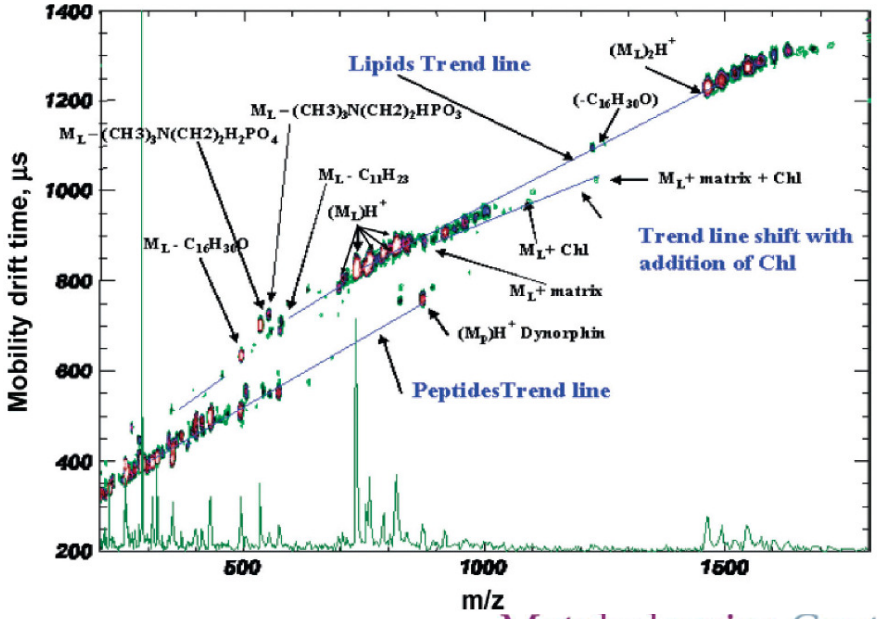
Figure 1. Schematic of an ambient-pressure IMS(tof)MS. Various components include (1) an electrospray ionization (ESI) source, (2) an ESI interface to the ion mobility spectrometer, (3) a desolvation chamber where the electrospray solvent is evaporated, (4) an ion gate which pulses packets of ions into the drift region, (5) the drift region where ions are separated according to their mobility, (6) a pinhole interface to vacuum, (7) transfer and focusing ion lenses to move the ions from high pressure to low pressure (8) a reflectron time-of-flight mass spectrometer. (Compliments of TofWerk, AG Thun, Switzerland).

- Ions pulsed into region filled with 'inert' gas (near atm. pressure)
- Drift time is proportional to mobility
- Separation based on mobility
  - -size, shape and weight
- Low field strength (200 V/cm)

- 'Low' sensitivity

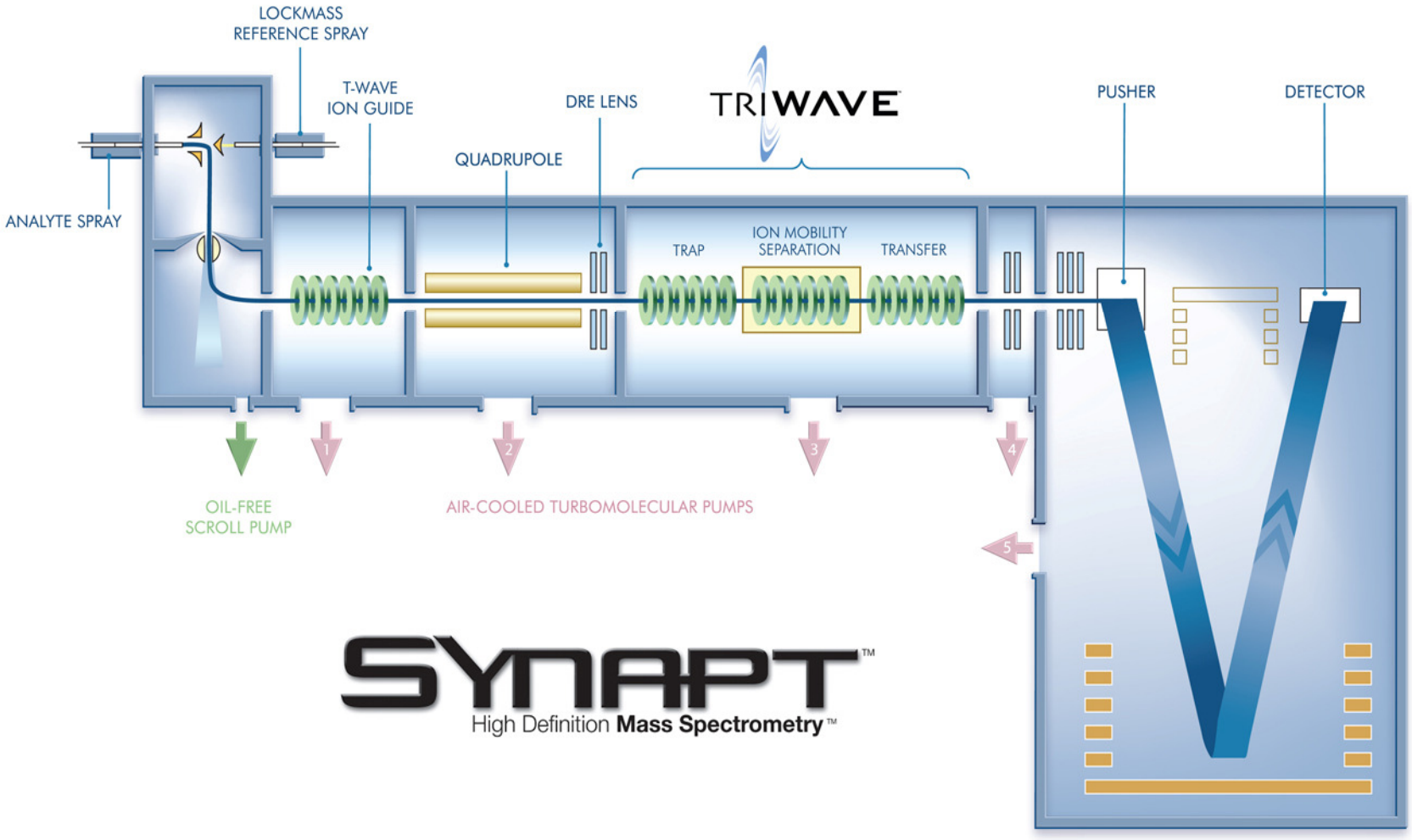
+ extra dimension for separation of (structural) isomers





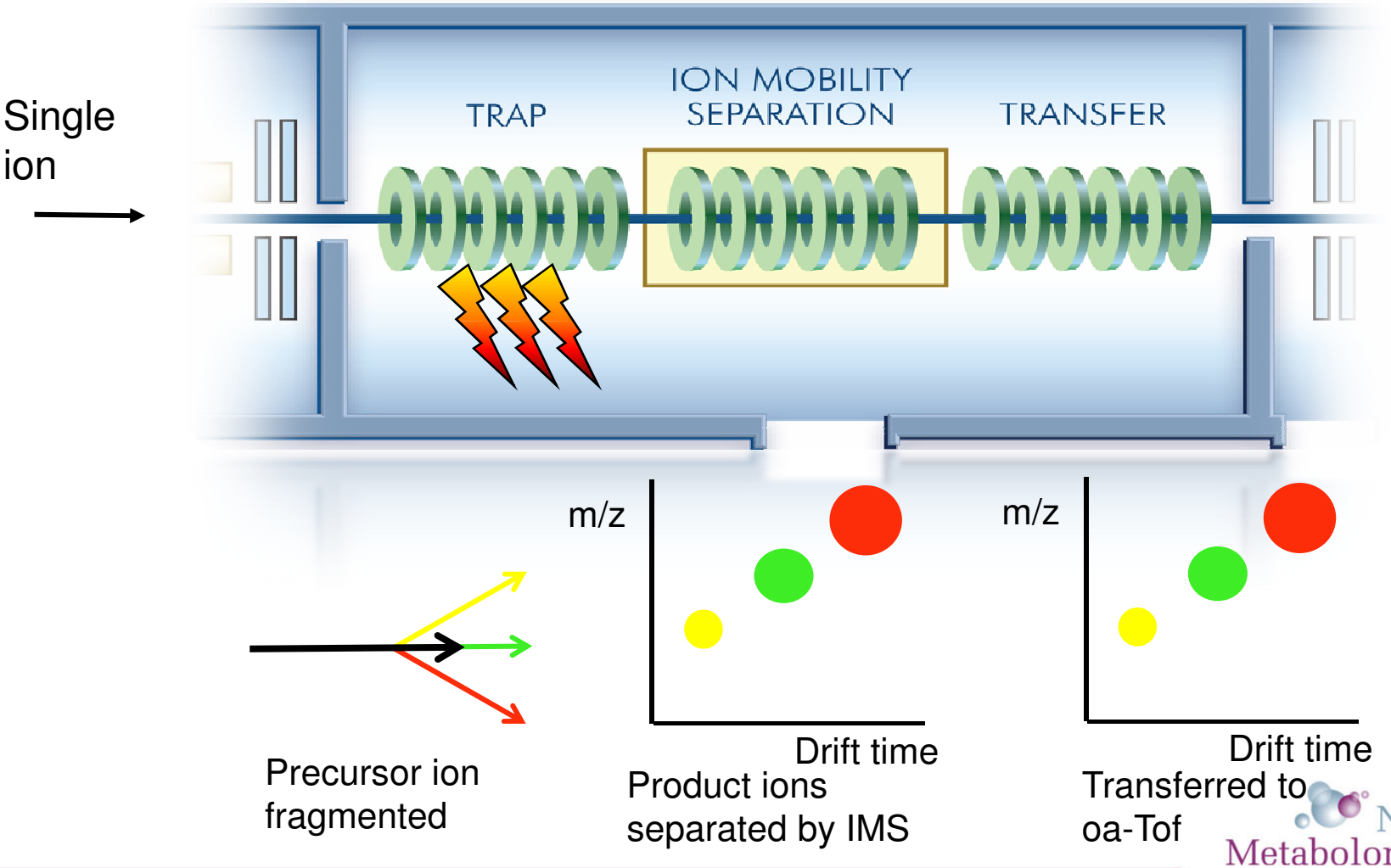
High Efficiency Travelling Wave IMS/MS^n ...

Waters TriWave is the enabling technology



Structural Elucidation of Metabolites

…Trap fragmentation (MS/MS)



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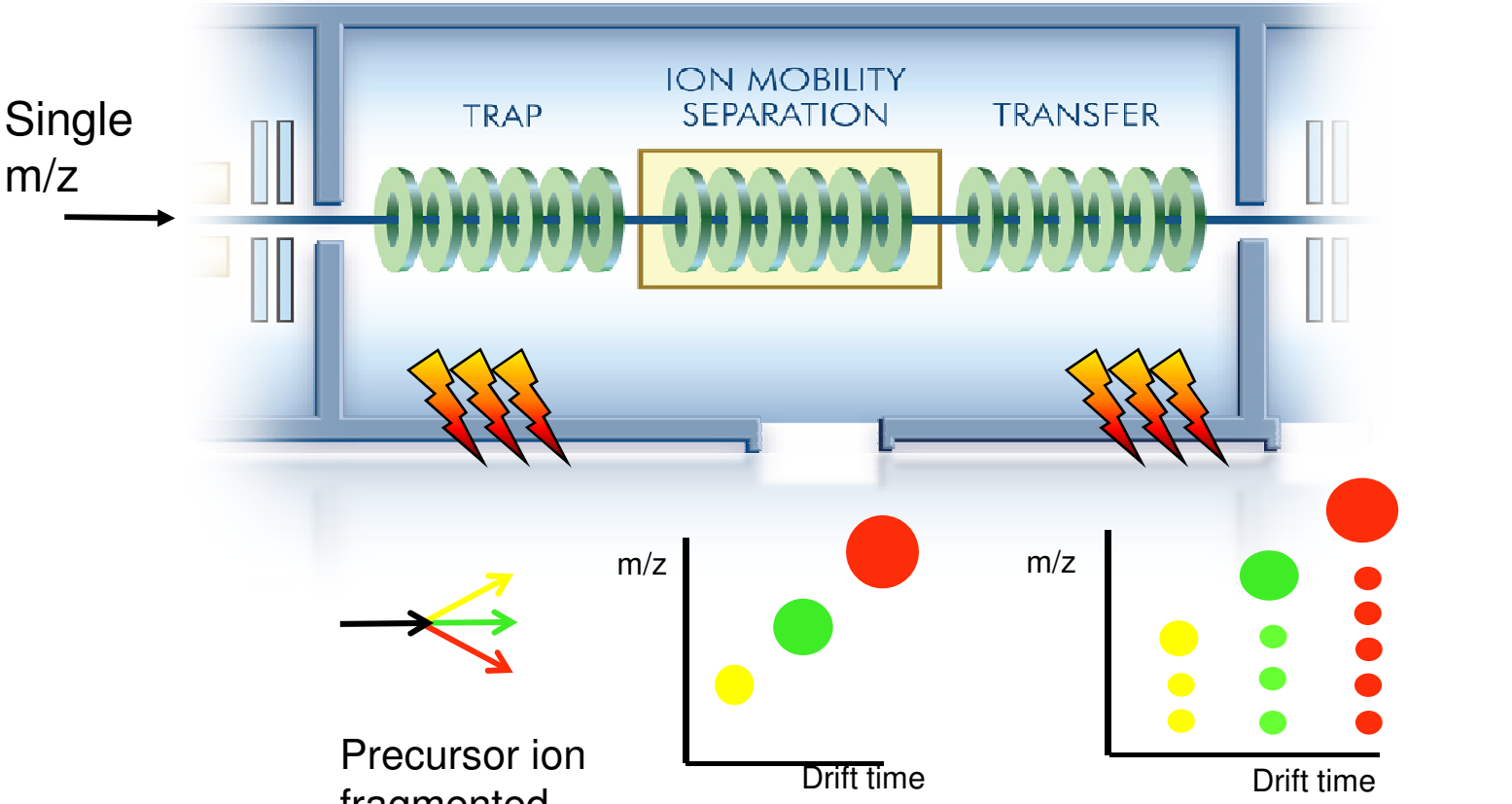
Structural Elucidation of Metabolites

…Time Aligned Parallel (TAP) Fragmentation



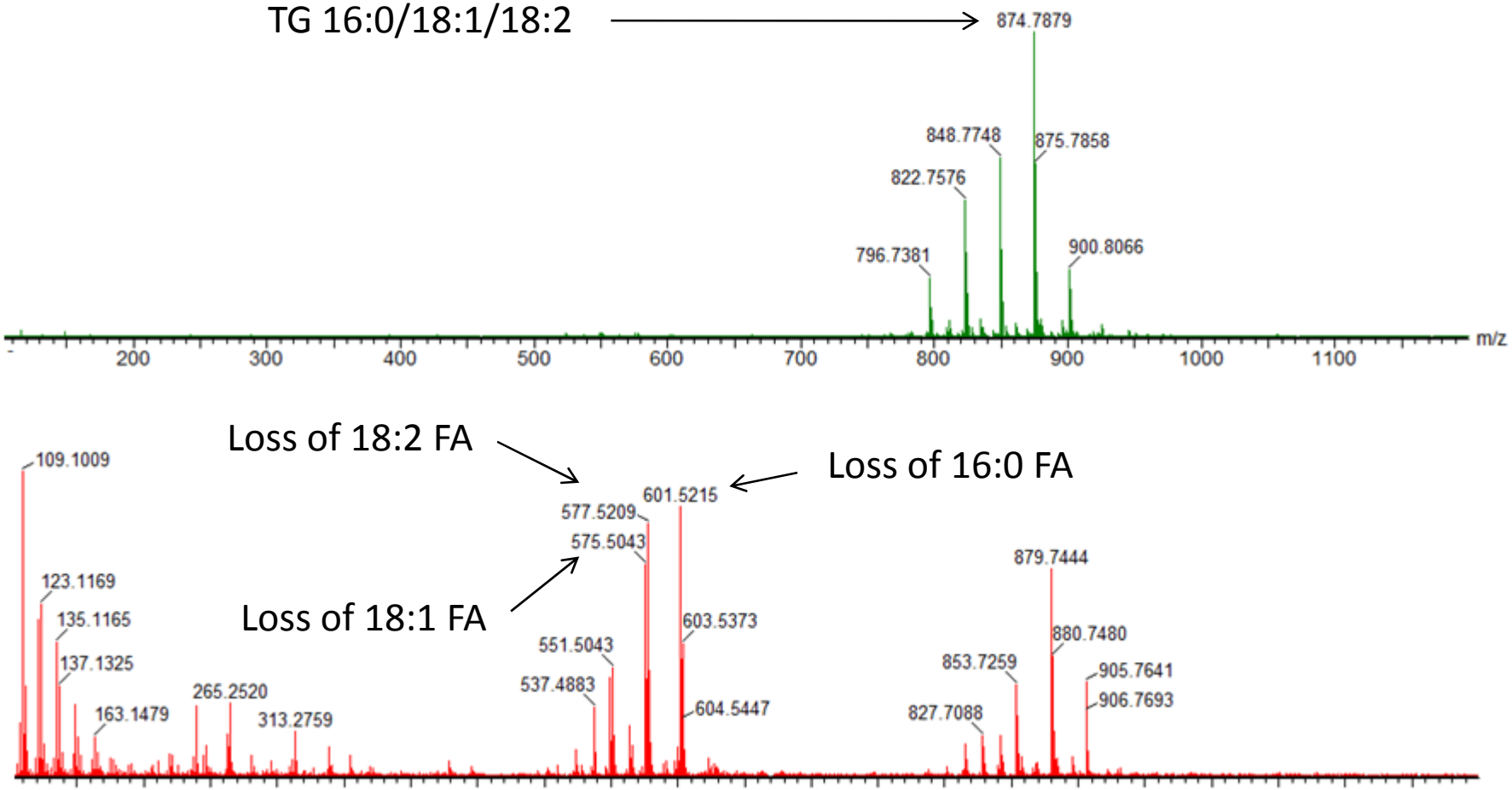
Precursor ion fragmented

Product ions separated by IMS

Precursor and products share same drift time

Metabolomics Centre

Low and high energy fragmentation spectra for TG 16:0/18:1/18:2



J. Castro-Perez, *et al.* , submitted

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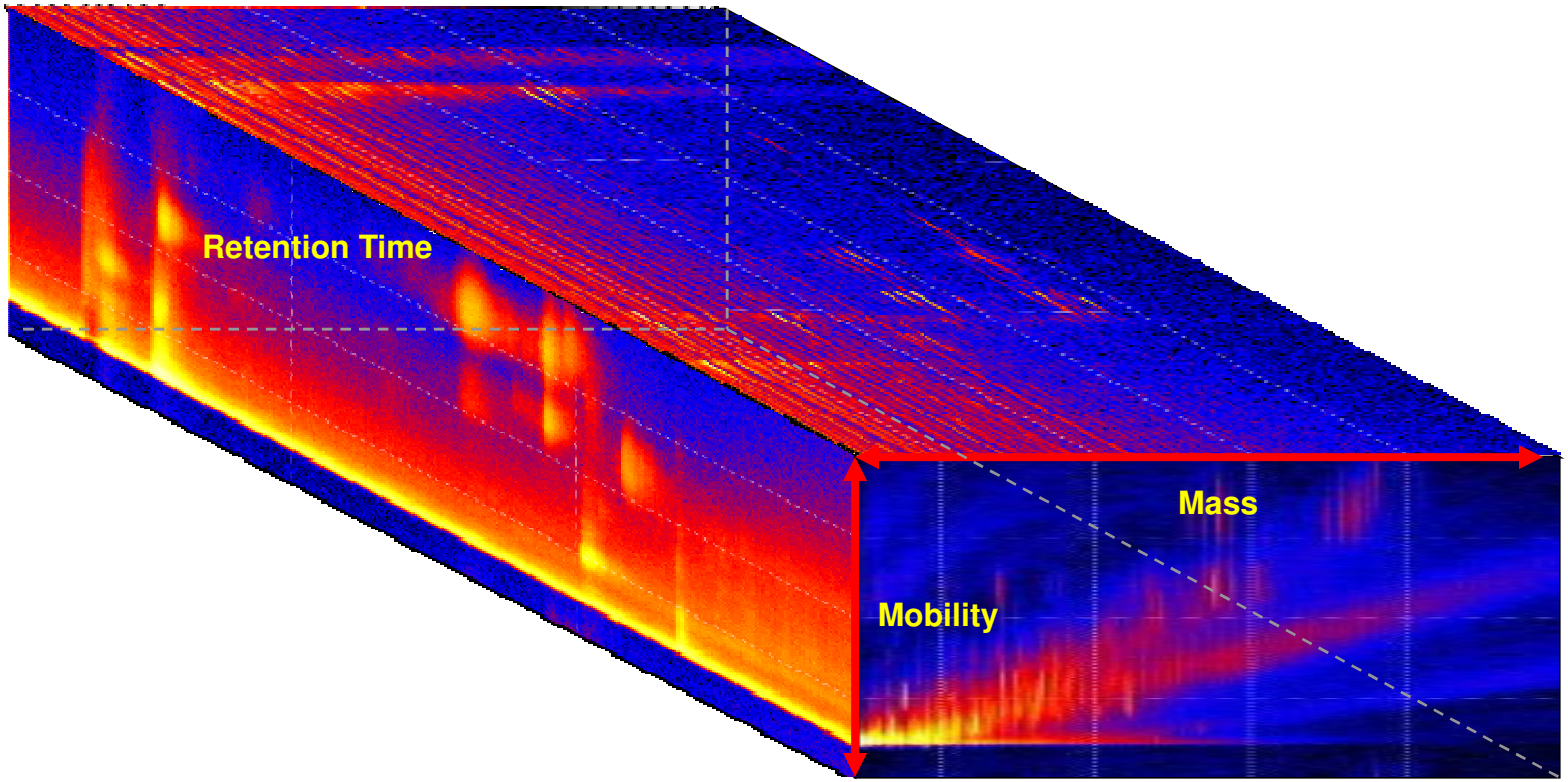
3D Data Orientation (LC + IMS + MS)

…presentation of multi dimensional data



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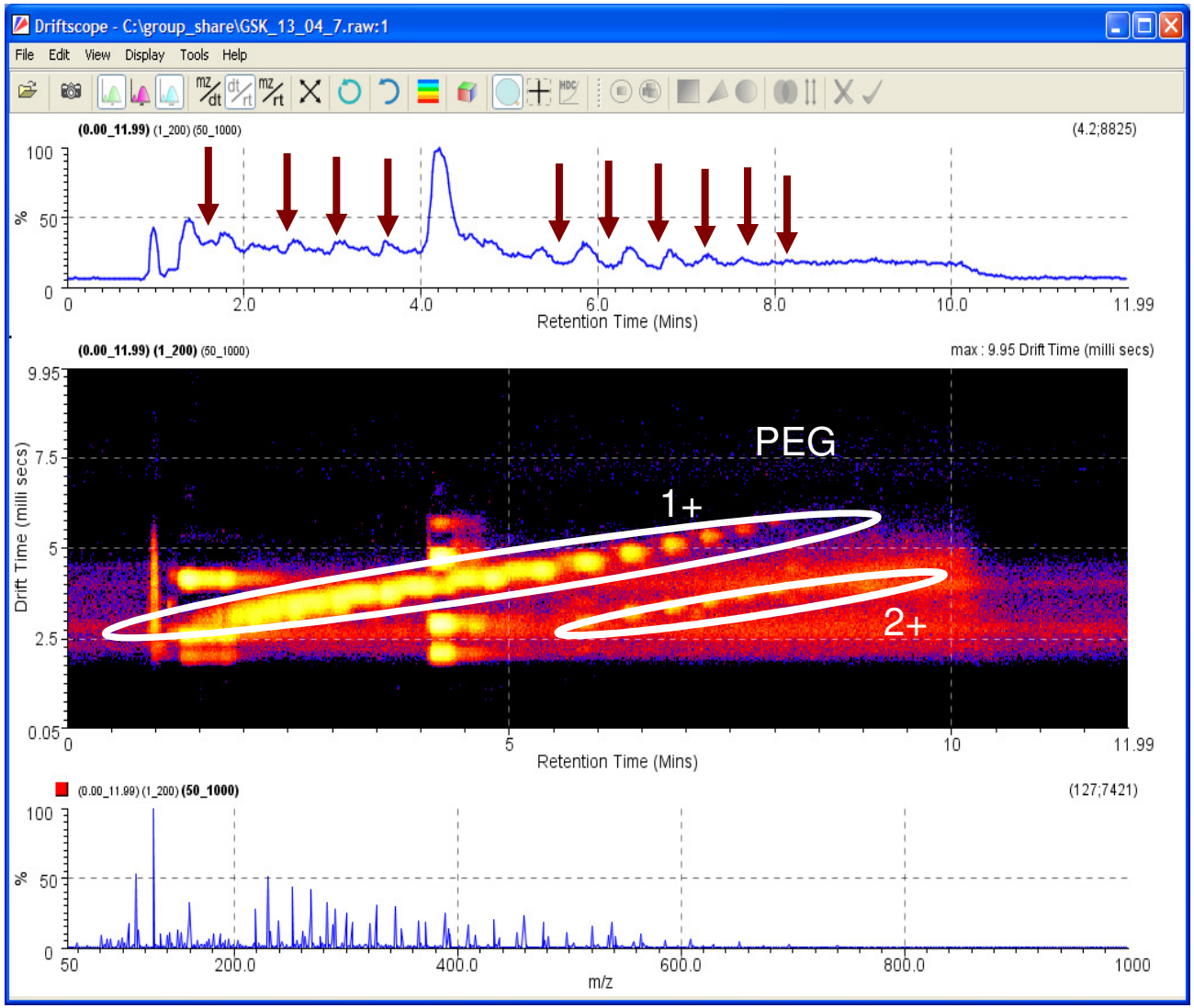
DriftScope™ Informatics

…Visualize & Manipulate multidimensional datasets



2D
Retention Time
& Intensity

3D
Drift Time,
Retention Time
& Intensity

2D
M/Z & Intensity

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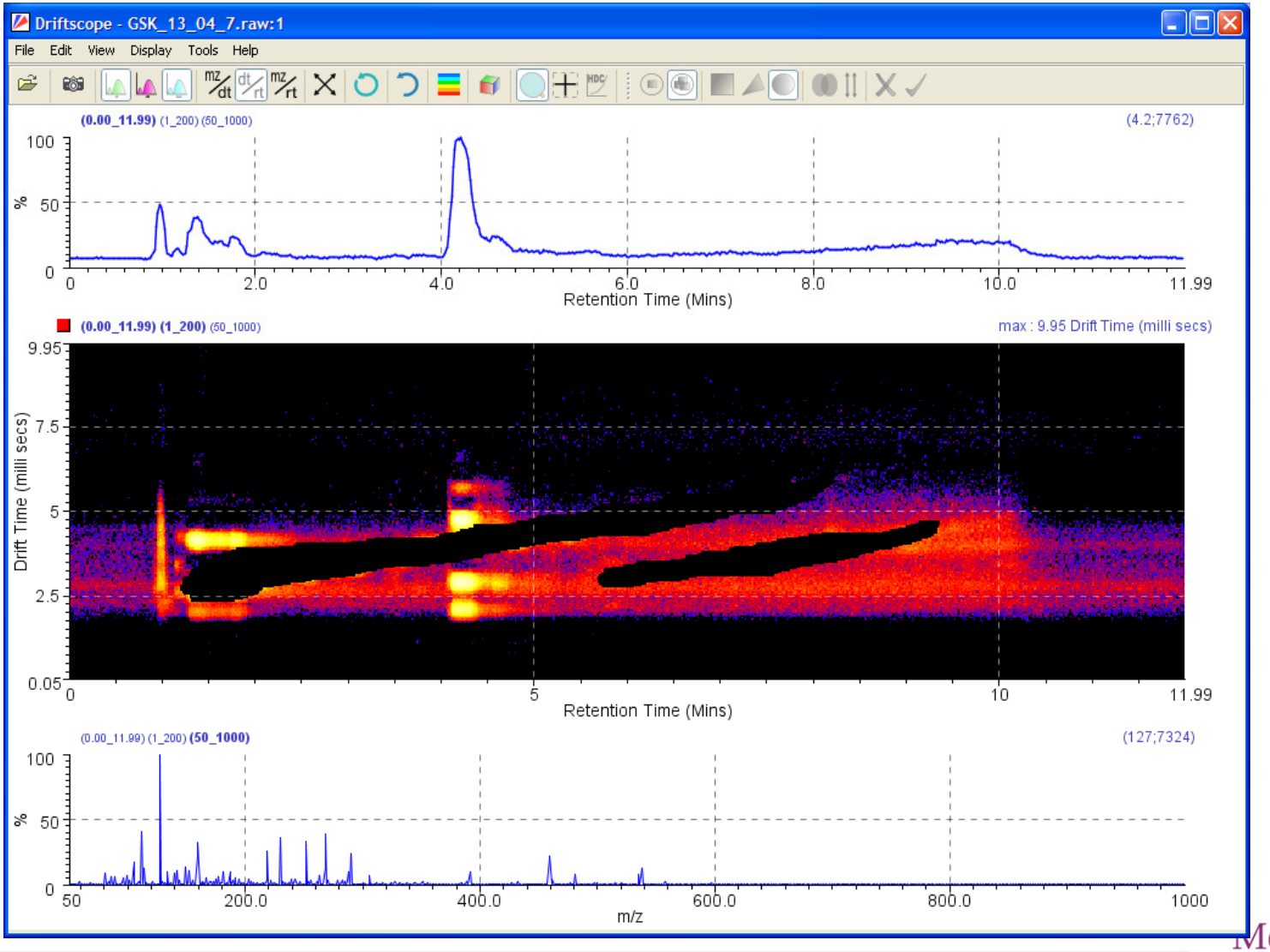
DriftScope™ Informatics

…Remove Background, Reduce Complexity



COMBIVIR TABLET

Formulated with Macrogol (major component being PEG-400)

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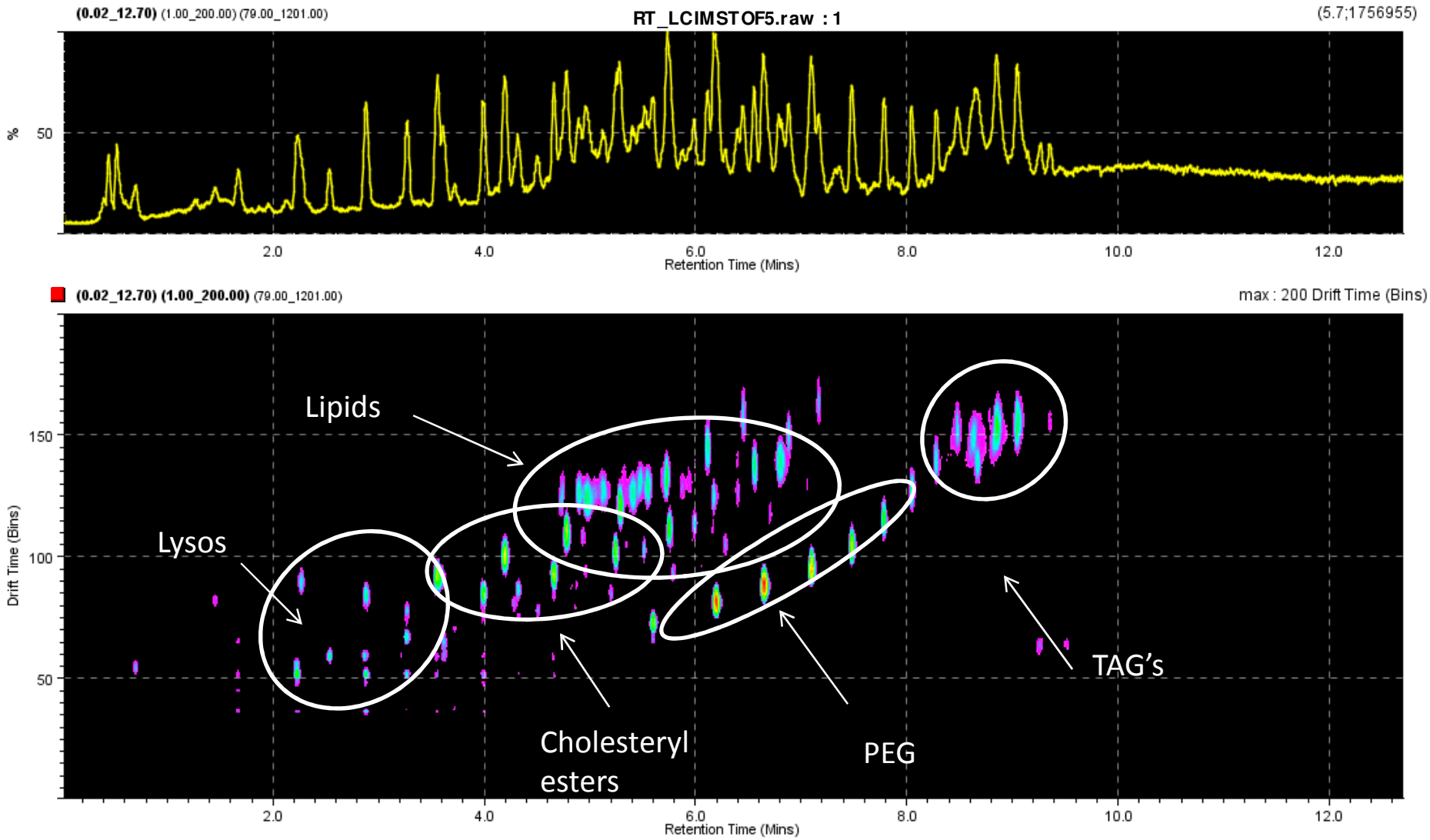
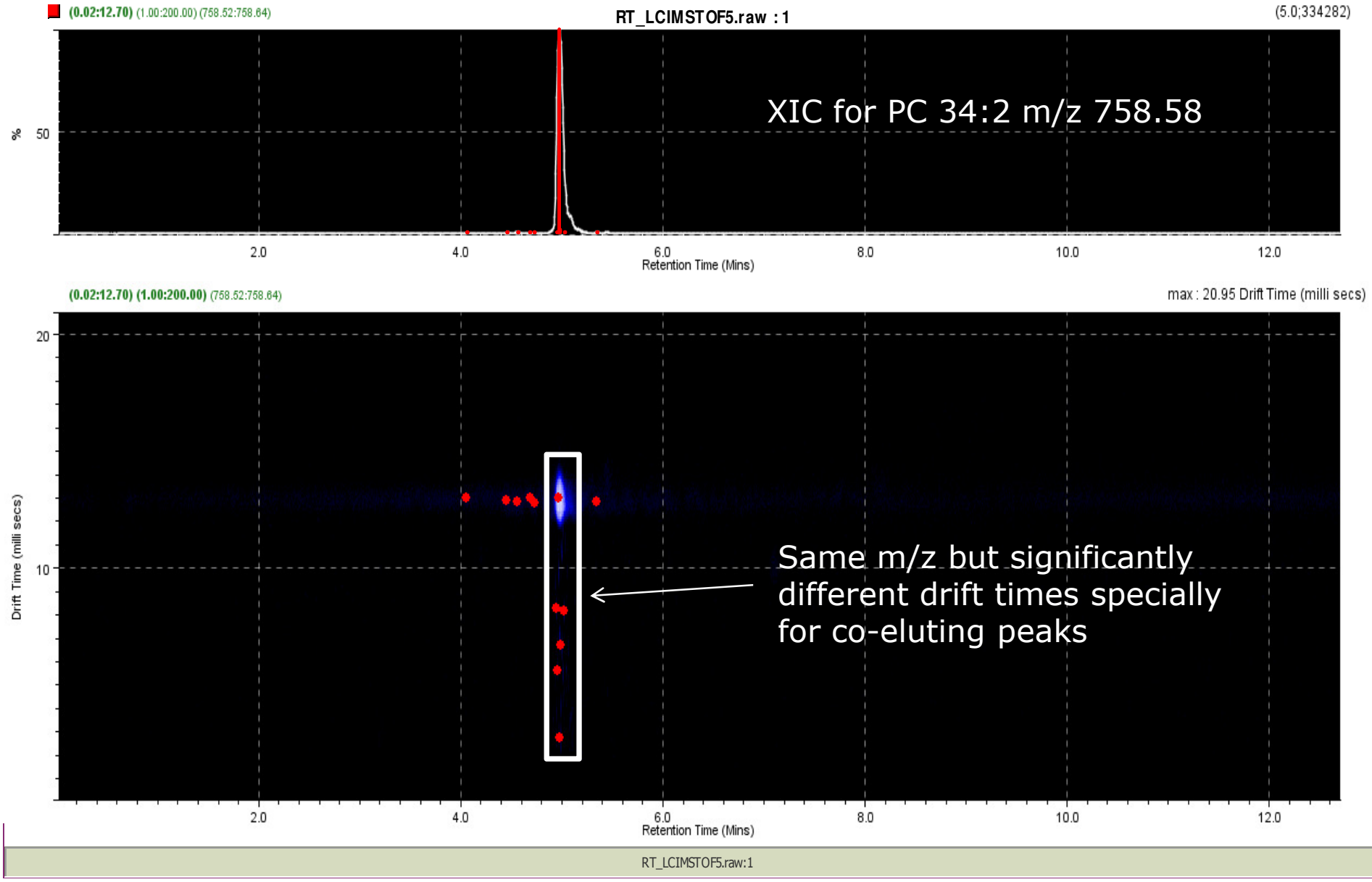
Christine Eckers *et al., Rapid Commun Mass Spectrom.* 21(7) (2007) 1255-63.

UPLC/IMS/TOF Lipid analysis

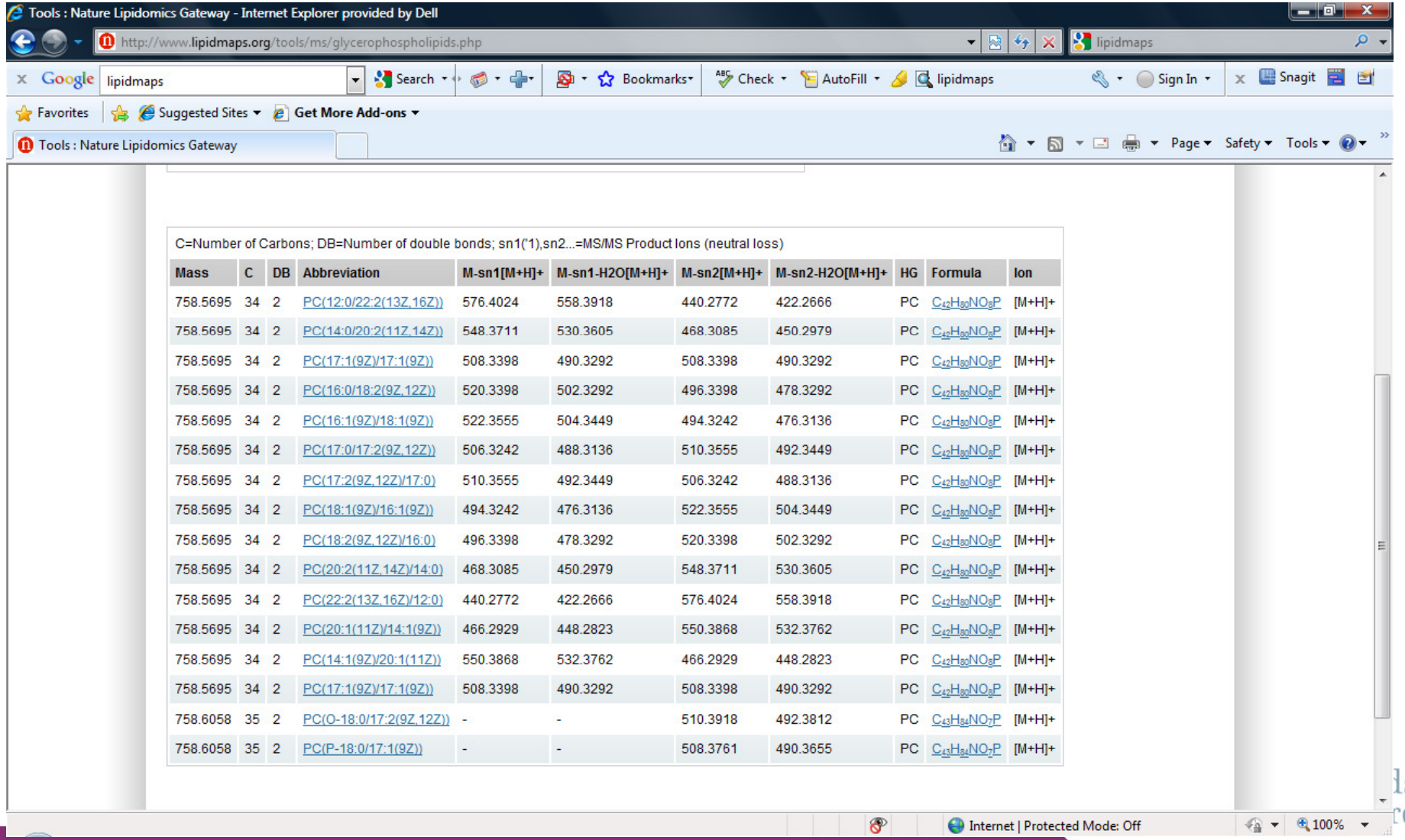


An extra dimension with IMS

PC 34:2 (many possible combinations)



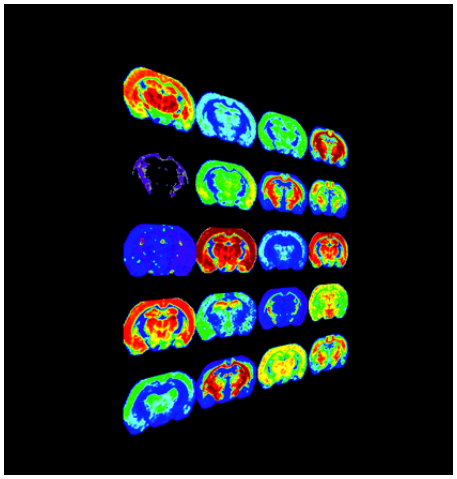
Possible lipids with this mass see below – there are 16 possible entries all with the same elemental composition



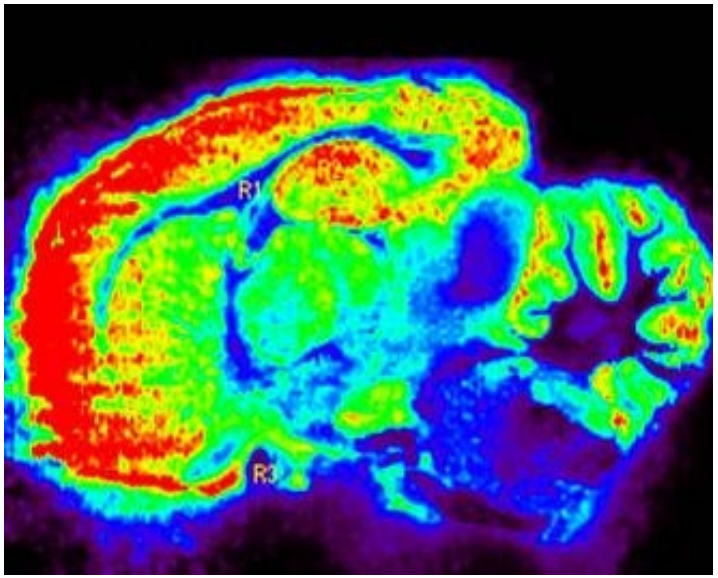
C=Number of Carbons; DB=Number of double bonds; sn1('1),sn2...=MS/MS Product Ions (neutral loss)

Mass	C	DB	Abbreviation	M-sn1[M+H]+	M-sn1-H2O[M+H]+	M-sn2[M+H]+	M-sn2-H2O[M+H]+	HG	Formula	Ion
758.5695	34	2	PC(12:0/22:2(13Z,16Z))	576.4024	558.3918	440.2772	422.2666	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(14:0/20:2(11Z,14Z))	548.3711	530.3605	468.3085	450.2979	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(17:1(9Z)/17:1(9Z))	508.3398	490.3292	508.3398	490.3292	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(16:0/18:2(9Z,12Z))	520.3398	502.3292	496.3398	478.3292	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(16:1(9Z)/18:1(9Z))	522.3555	504.3449	494.3242	476.3136	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(17:0/17:2(9Z,12Z))	506.3242	488.3136	510.3555	492.3449	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(17:2(9Z,12Z)/17:0)	510.3555	492.3449	506.3242	488.3136	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(18:1(9Z)/16:1(9Z))	494.3242	476.3136	522.3555	504.3449	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(18:2(9Z,12Z)/16:0)	496.3398	478.3292	520.3398	502.3292	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(20:2(11Z,14Z)/14:0)	468.3085	450.2979	548.3711	530.3605	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(22:2(13Z,16Z)/12:0)	440.2772	422.2666	576.4024	558.3918	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(20:1(11Z)/14:1(9Z))	466.2929	448.2823	550.3868	532.3762	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(14:1(9Z)/20:1(11Z))	550.3868	532.3762	466.2929	448.2823	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.5695	34	2	PC(17:1(9Z)/17:1(9Z))	508.3398	490.3292	508.3398	490.3292	PC	$C_{42}H_{80}NO_8P$	[M+H]+
758.6058	35	2	PC(O-18:0/17:2(9Z,12Z))	-	-	510.3918	492.3812	PC	$C_{43}H_{84}NO_7P$	[M+H]+
758.6058	35	2	PC(P-18:0/17:1(9Z))	-	-	508.3761	490.3655	PC	$C_{43}H_{84}NO_7P$	[M+H]+

Imaging Mass Spectrometry



PNAS cover nov 2008
Protein images of rat brain sections



Bunch, University of Birmingham.

Lipid distribution in a rat brain

Netherlands Metabolomics Centre

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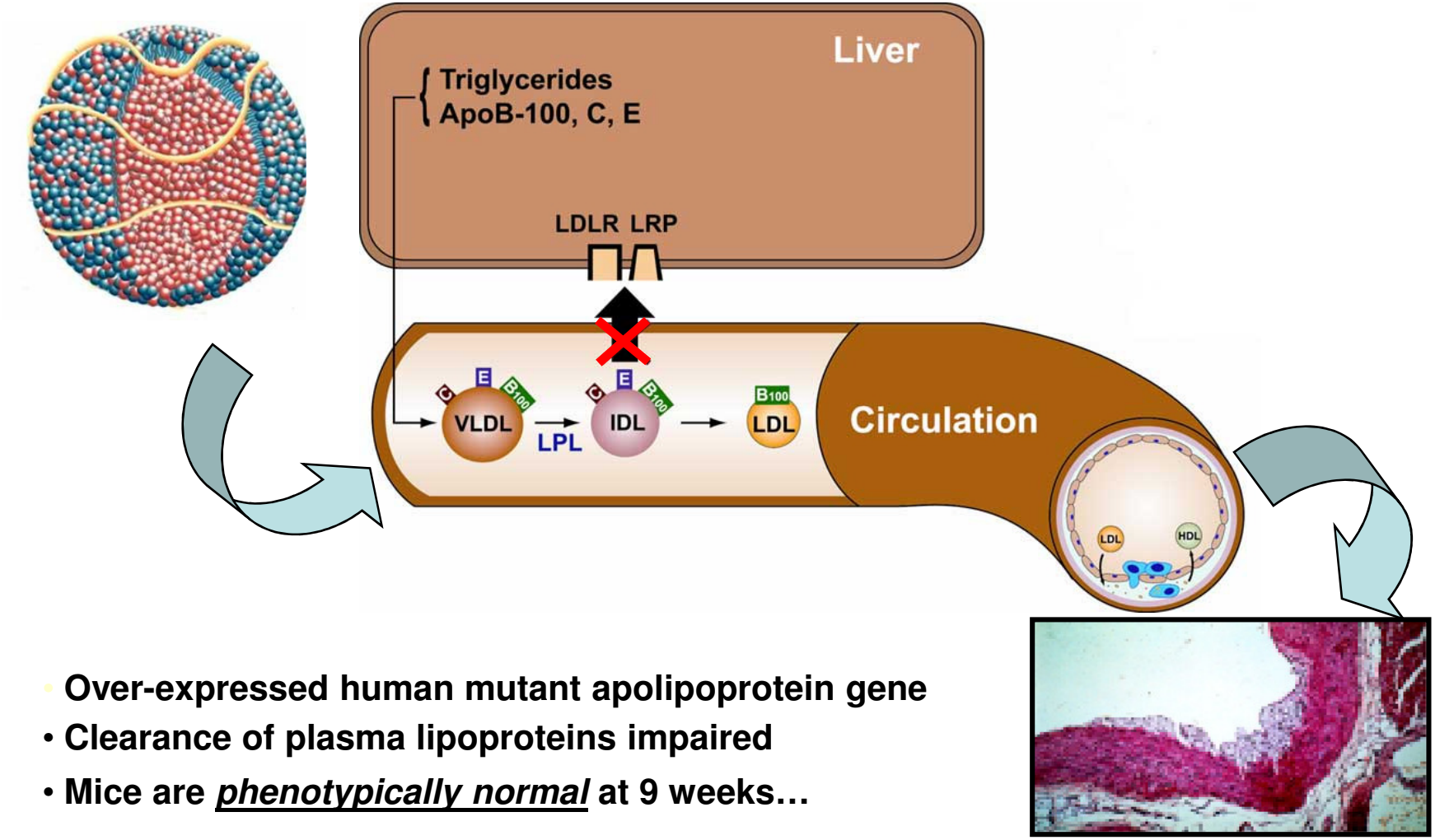
Early Biomarkers of Disease

ApoE3 Leiden Transgenic Mouse Model



- Over-expressed human mutant apolipoprotein gene
- Clearance of plasma lipoproteins impaired
- Mice are ***phenotypically normal*** at 9 weeks…

Atherosclerotic Changes In *Mature* Mice (25 wks)

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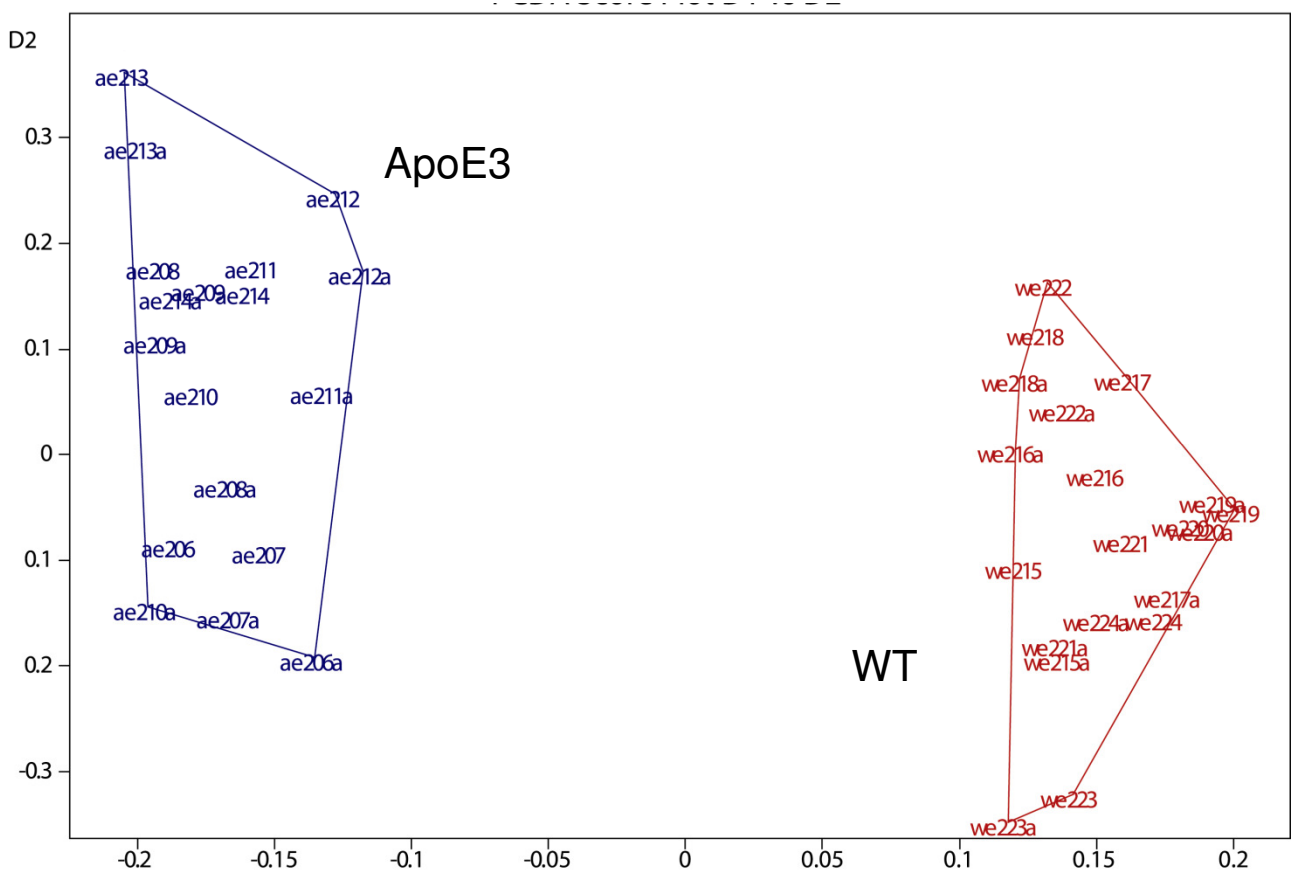
Clish et al., Omics 8(1), 2004, 3-13

Metabolomic Analysis
- Plasma Lipid Analysis



- Mouse Plasma
- Metabolite (lipid)
- LC-MS

•**Clustering Reveals Discrete Differences**
(at 9 week age; pre-atherosclerotic)

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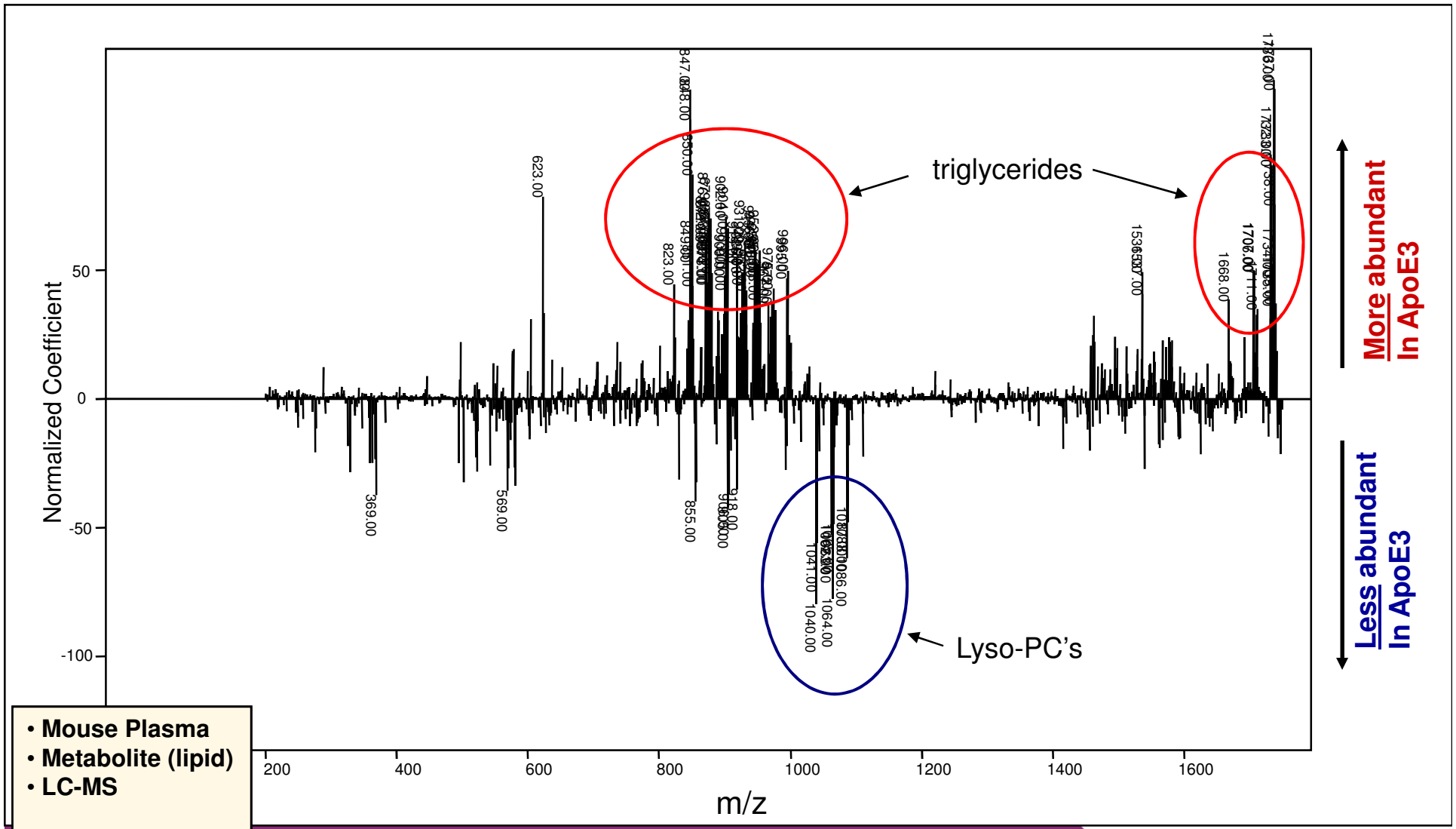
Clish et al., Omics 8(1), 2004, 3-13

Metabolomic Analysis
- Plasma Lipid Analysis

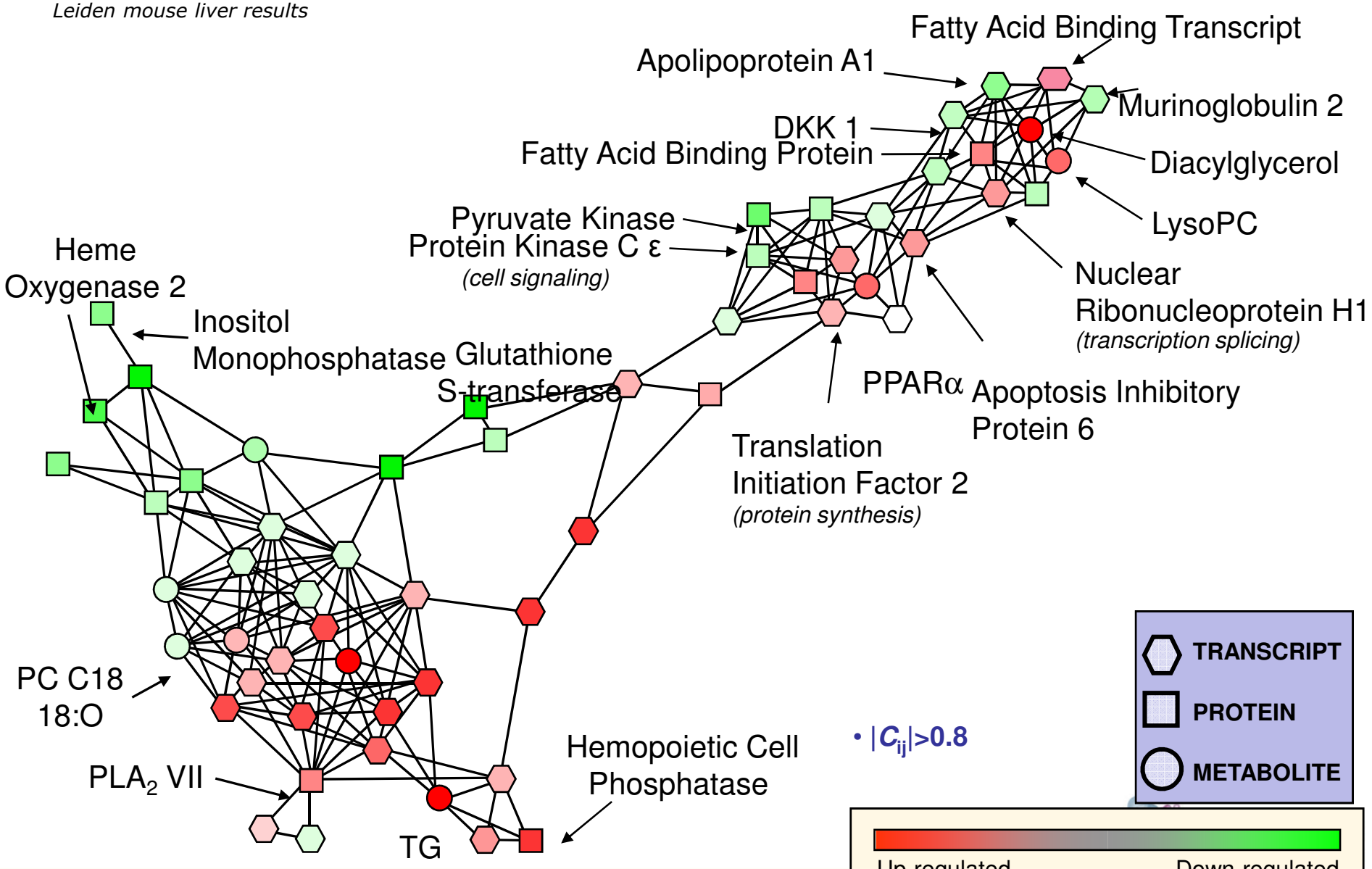


- **Mouse Plasma**
- **Metabolite (lipid)**
- **LC-MS**

Clish et al., Omics 8(1), 2004, 3-13

Early Biomarkers of Disease: Atherosclerosis

Correlation Network based on ApoE3
Leiden mouse liver results

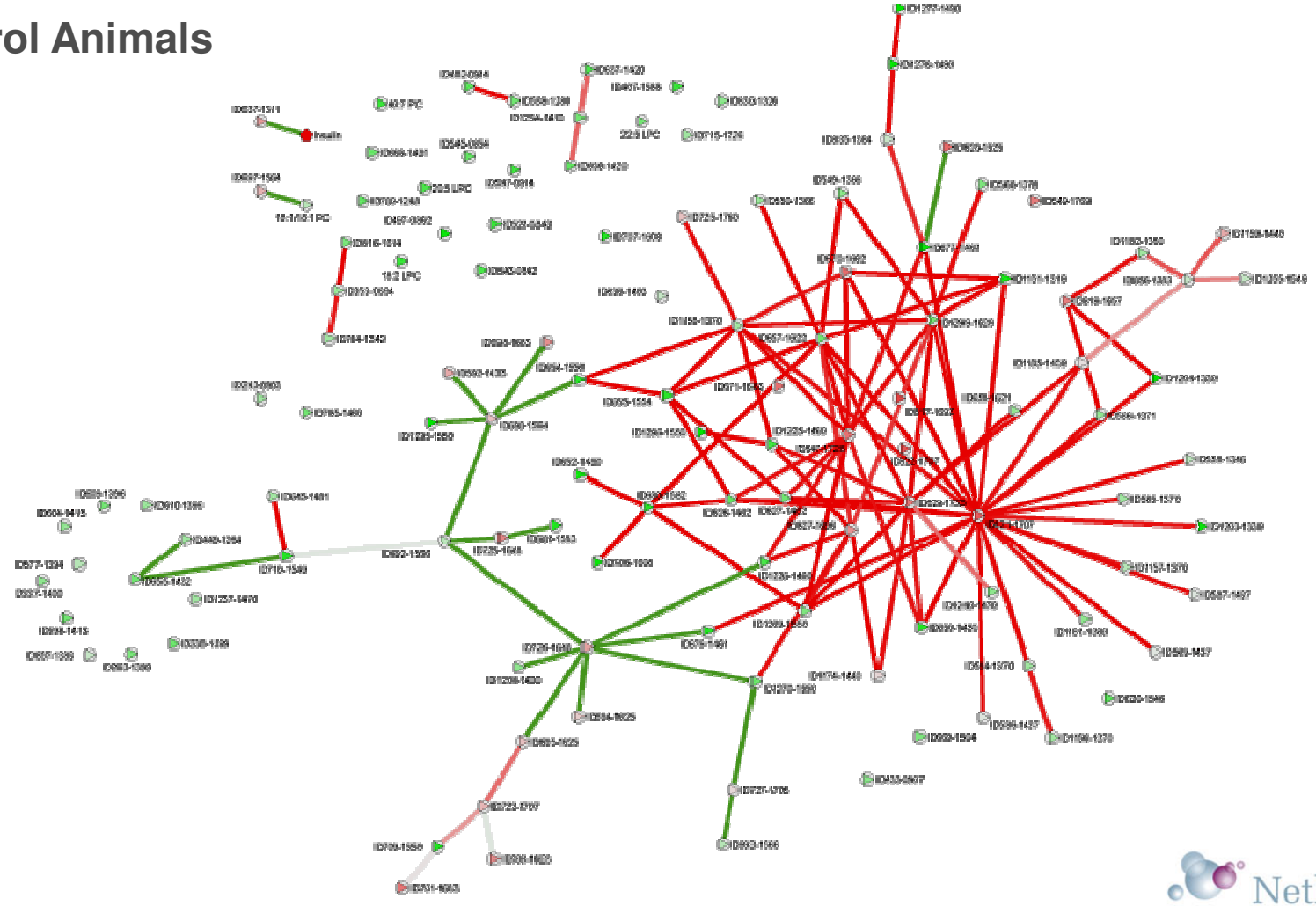


Clish et al., Omics 8(1), 2004, 3-13

Correlation Network in for Animal Model of T2DM

Compartment: fat tissue

Control Animals



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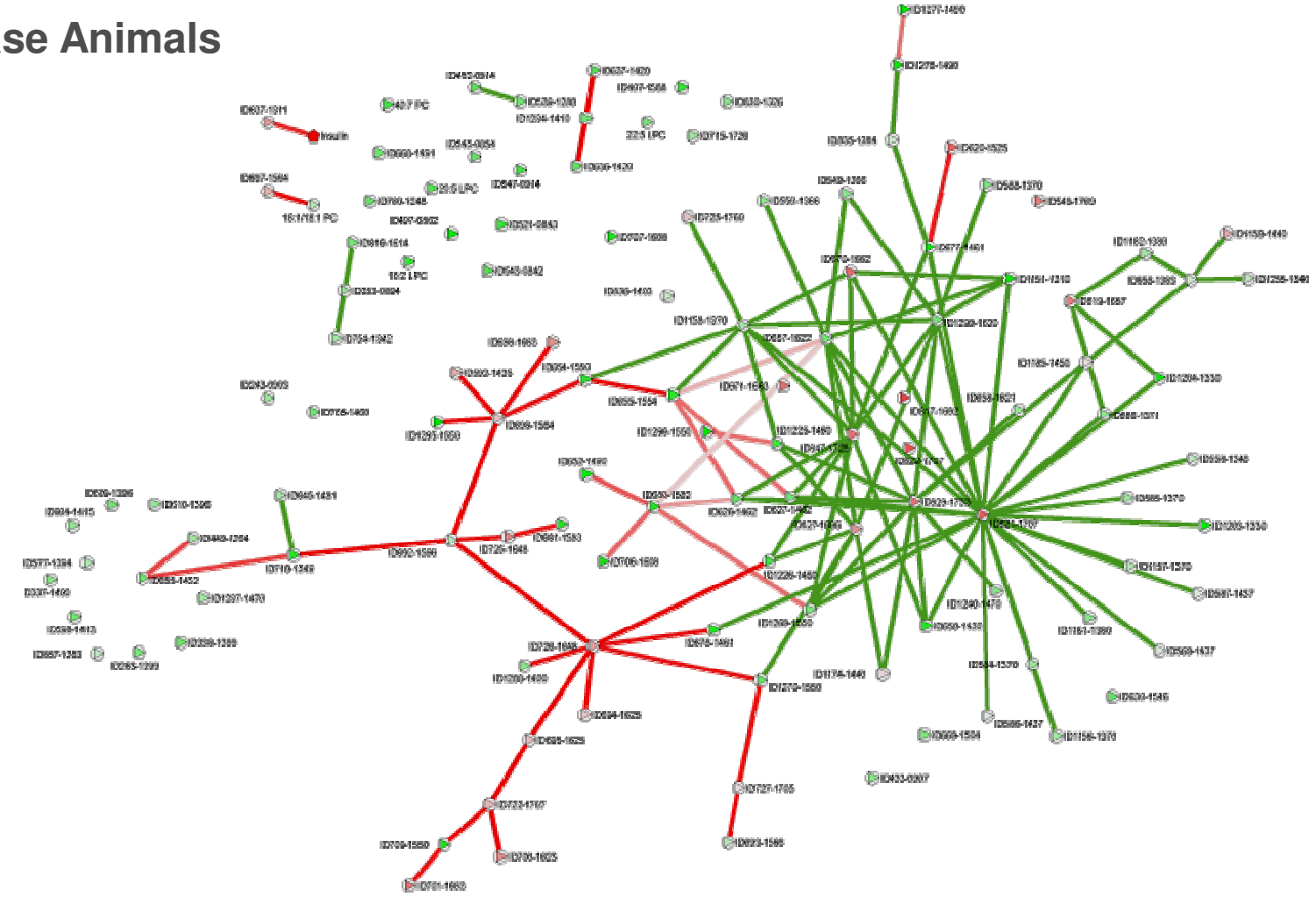
Correlation Network in for Animal Model of T2DM

Compartment: fat tissue

Disease Animals

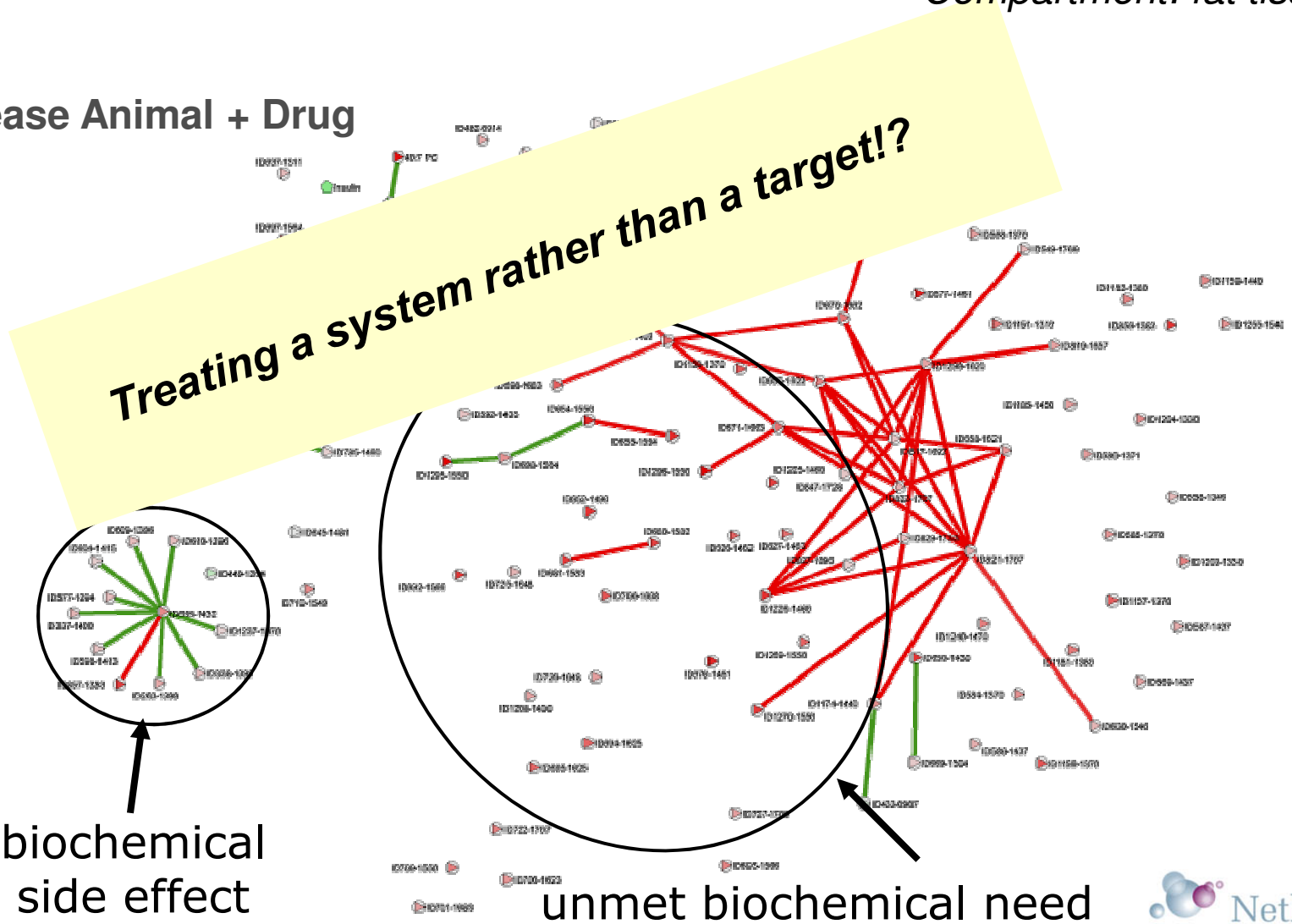


Van der Greef et al. J Proteome Res. 2007 6(4):1540-1559

Correlation Network in for Animal Model of T2DM

Compartment: fat tissue

Disease Animal + Drug



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Van der Greef et al. J Proteome Res. 2007 6(4):1540-1559

Conclusions

• Tools like GC-MS, LC-MS , NMR will **all** deliver reliable and usefull results

• Undesirable need to improve coverage, enhance detection and identification possibilities with current techniques

•Integration of tools is essential in System biology research

• Essential to Systems Biology approach is
  - •the right DoE
  - •a robust/standardized analytical method
  - •being able to ‘statistically sound’ correlate data

• Metabolomics is an integral part of Systems Biology
  - • understand the underlying biochemical questions
  - • describe the pathological phenomena
  - • etc.

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