Clinical Metabolomics by $^1$H-NMR Spectroscopy

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Transparency statement

Prof. Dr. Matthias Nauck

Honoraria for talks:
• Boehringer Ingelheim

Honoraria for consulting:
• Becton Dickinson

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• Siemens Healthineers, LVL, Bruker Biospin, Abbott, Radiometer, Becton Dickinson, Tosoh, ids, aerocom
Outline

• Metabolomics (short introduction)
• Pre- and analytical aspects
• Epidemiology and Biobanking
• Type 2 diabetes mellitus and markers of glucose metabolism
• Biological age
• Lipoprotein subfractions
• Fatty liver disease
Deep phenotyping can be defined as the precise and comprehensive analysis of phenotypic abnormalities in which the individual components of the phenotype are observed and described.
Metabolomics

- entirety of small molecules, typically less than 1kDa, in a biological sample like blood or urine but also in tissues or single cells

- Biomarker Discovery
- Pathway Discovery
- Pharmacometabolomics
- Translation of Biomarkers
- Newborn Screening
Metabolomics Techniques

Mass Spectrometry (MS)

Nuclear Magnetic Resonance (NMR) Spectroscopy
Metabolomics Techniques

Mass Spectrometry (MS)
- high sensitivity
- minimal sample volume
- destructive technique
- relatively high costs
- extensive sample preparation

Nuclear Magnetic Resonance (NMR) Spectroscopy
- highly reproducible
- minimal sample preparation
- non-destructive technique
- modest sensitivity
- higher sample volume
• **combination** of MS and NMR for the same sample using a automated pipetting roboter
$^1$H-NMR spectroscopy

$^1$H-NMR spectrum of a urine sample from the SHIP-TREND cohort obtained with a Bruker DRX-400 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Peak patterns of selected metabolites were annotated. TSP=3-trimethylsilyl-(2,2,3,3-D4)-1-propionate
**1H-NMR spectroscopy**

**1H-NMR spectrum of a urine sample** from the SHIP-TREND cohort obtained with a Bruker DRX-400 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Peak patterns of selected metabolites were annotated. TSP=3-trimethylsilyl-(2,2,3,3-D4)-1-propionate
1H-NMR Spectroscopy – Types of Data

2 Types of Data

**targeted**

Quantification of metabolites

Absolute concentrations of metabolites

**untargeted**

Bucket integration

Spectra are divided into small regions (buckets) with fixed width

e.g. ppm 0.5-9.5 with 0.018ppm width = 500 buckets
Quality assurance in the pre-analytical phase of human urine samples by $^1$H NMR spectroscopy

Kathrin Budde $^a,^1$, Ömer-Necmi Gök $^b,^1$, Maik Pietzner $^a$, Christine Meisinger $^c$, Michael Leitzmann $^d$, Matthias Nauck $^a,e$, Anna Köttgen $^b$, Nele Friedrich $^a,e,*$
$^1$H-NMR spectroscopy – Preanalytics Urine
Pre-analytical study design

Urine collection

Aliquots

Time

0 2h 4h 8h 12h 24h 48h 72h 1 week 1 mo

- Freeze at -80°C
- Centrifugation
- Buffer
- NMR measurement

Budde K. et al., Arch Biochem Biophys. 2016; 589
1H-NMR spectroscopy – Preanalytics Urine

- Pre-analytical aspects are **less strong than inter-individual differences**

- Freezing at **-80°C** seems to be sufficient

- Majority of metabolites was **stable up to 24h at 10°C**

Budde K. et al., Arch Biochem Biophys. 2016; 589
Glucose Duplicates; N=21653

Bias graph, relative

Bias graph, absolute
Duplicates after 5 years storage at -80°C

Glucose

\[ y = 0.1 + 1 \times x \]
Intercept 95%-CI: [-0.0318 - 0.1]
Slope 95%-CI: [1 - 1.0273]
Duplicates after 15 years storage at -80°C
Vitamin B12 – assay has changed
Study of Health in Pommerania - SHIP

N=4310

age: 20 - 79 years

1.000.000 aliquots

1997-2001 SHIP-0

2002-2006 SHIP-1

2008-2013 SHIP-2 SHIP-TREND

since 2014 SHIP-3

since 2015 SHIP-TREND 1
Research Biobank

BUILDING BETTER BIOBANKS

NATURE. 7 JUNE 2012, VOL 486,p.141-45

- High-quality biobanking = sample storage + QC-Process
- lack of appropriate QC-tools for sample collection, processing & storage
- research on pre-analytical and analytical storage effects on future biomarker measurements & multi-omics analyses

→ Insufficient provision of biomaterial information for publication (>50%)
German National Cohort

200,000 probands
18 study centers

Major diseases:
- CVD
- Diabetes mellitus
- Cancer
- Neurologic and psychiatric diseases
- Respiratory diseases
- Infectious diseases

Major exposures and risk factors:
- Body composition
- Physical activity
- Physical fitness
- Diet
- Smoking and alcohol consumption
- Psychosocial factors
- Socioeconomic status
- Sleep-related characteristics
- Chronic infections, immune factors, and microflora
- Occupational and environmental exposures
Table 4: Expected counts of incident cancer cases and incident non-cancer cases after 5, 10, 15 or 20 years of average follow-up, for the overall cohort (N = 200,000) or for the intensified sub-cohort (N ~ 60,000)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Expected cumulative incidence at study Level 1 (200,000 subjects)</th>
<th>Expected cumulative incidence at study Level 2 (~60,000 subjects)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average follow-up duration (years) (+ corresponding calendar date)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 years (2022)</td>
<td>10 years (2027)</td>
</tr>
<tr>
<td>Any cancer</td>
<td>5,100</td>
<td>13,000</td>
</tr>
<tr>
<td>Breast</td>
<td>780</td>
<td>1,800</td>
</tr>
<tr>
<td>Prostate</td>
<td>720</td>
<td>1,900</td>
</tr>
<tr>
<td>Colon, Rectum</td>
<td>670</td>
<td>1,800</td>
</tr>
<tr>
<td>Lung</td>
<td>560</td>
<td>1,400</td>
</tr>
<tr>
<td>Bladder</td>
<td>260</td>
<td>710</td>
</tr>
<tr>
<td>Kidney</td>
<td>190</td>
<td>500</td>
</tr>
<tr>
<td>non-Hodgkin L.</td>
<td>140</td>
<td>340</td>
</tr>
<tr>
<td>Pancreas</td>
<td>120</td>
<td>330</td>
</tr>
<tr>
<td>Corpus Uteri</td>
<td>120</td>
<td>320</td>
</tr>
<tr>
<td>Brain + CNS</td>
<td>90</td>
<td>200</td>
</tr>
<tr>
<td>Ovary</td>
<td>110</td>
<td>250</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>1,700</td>
<td>4,400</td>
</tr>
<tr>
<td>Stroke</td>
<td>1,600</td>
<td>4,300</td>
</tr>
<tr>
<td>Diabetes</td>
<td>5,800</td>
<td>13,000</td>
</tr>
<tr>
<td>Rheum. arthritis</td>
<td>250</td>
<td>590</td>
</tr>
<tr>
<td>COPD</td>
<td>2,300</td>
<td>5,800</td>
</tr>
<tr>
<td>Heart failure</td>
<td>1,600</td>
<td>4,600</td>
</tr>
<tr>
<td>Mortality</td>
<td>4,600</td>
<td>14,000</td>
</tr>
</tbody>
</table>

Calculations based on age-specific incidence from German Cancer Registries [23]

* 40,000 participants in the representative Level 2 study programme plus ~20,000 participants in the MRI study, who will additionally also have the Level 2 examinations
Biorepository of the National Cohort

Final Biorepository
- Fully Automated
- Tandem Solution
- 17+1 Tanks
- 280 m²x5m

- reorganization of the samples
- collection of follow up samples after 5 years
- picking of the samples for scientific purposes
Otherwise we compare…
Analysis Report
Bruker IVDr BioBank QC B.I.BioBankQC™ in Plasma/Serum

Sample ID: SHIPT1_20180717_2713890199_expno10.100000.10r
Measuring Date: 18-Jul-2018 02:34:43
Reporting Date: 18-Jul-2018 04:48:29, 6 page(s), Version 1.0.0
Quantification Method Version: BioBankQC PS 1.0.0

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RESEARCH USE ONLY: This is no clinical diagnostic analysis report. Must not be used for clinical (medical or IVD) diagnosis or for patient management! Additional concentration range information (95% range) provided numerically or graphically in this report must not be used for clinical diagnostic interpretation.

Summary

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Flag</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMR Experiment Parameter Test</td>
<td>passed</td>
<td></td>
</tr>
<tr>
<td>NMR Experiment Quality Test</td>
<td>passed</td>
<td></td>
</tr>
<tr>
<td>NMR Preparation Quality Test</td>
<td>passed</td>
<td></td>
</tr>
<tr>
<td>Matrix Identity Test</td>
<td>EDTA plasma</td>
<td></td>
</tr>
<tr>
<td>Matrix Integrity Test</td>
<td>passed</td>
<td></td>
</tr>
<tr>
<td>Matrix Contamination Test</td>
<td>passed</td>
<td></td>
</tr>
</tbody>
</table>

3 Matrix Validation
3.1 Matrix Identity Test
The spectral fingerprint of the sample is consistent with EDTA plasma.

<table>
<thead>
<tr>
<th>Sample</th>
<th>K-EDTA mmol/L</th>
<th>Ca-EDTA mmol/L</th>
<th>Citric acid-B mmol/L</th>
<th>Flag</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA plasma</td>
<td>2.50 - 13.00</td>
<td>1.30 - 3.60</td>
<td>&lt;3.00</td>
<td>●</td>
</tr>
<tr>
<td>Citrate plasma</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>3.10 - 20.00</td>
<td>○</td>
</tr>
<tr>
<td>Heparin plasma</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;3.00</td>
<td>○</td>
</tr>
<tr>
<td>Serum</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;3.00</td>
<td>○</td>
</tr>
</tbody>
</table>
$^1$H-NMR spectroscopy – Type 2 Diabetes

IDF DIABETES ATLAS Sixth edition 2014 update
Transverse NMR spectroscopy – Type 2 Diabetes

**Inter99**
- Longitudinal changes in fasting glucose, HbA1c and HOMA index (5-year Follow-up)

**SHIP**
- 137 incident diabetes cases (5-year Follow-up)
Sex differences in urine metabolites related with risk of diabetes using NMR spectroscopy: results of the study of health in pomerania

Nele Friedrich¹,² · Kathrin Budde¹ · Karsten Suhre³,⁴ · Uwe Völker²,⁵ · Ulrich John⁶ · Stephan B. Felix²,⁷ · Heyo K. Kroemer⁸ · Hans J. Grabe⁹ · Henry Völzke¹⁰ · Matthias Nauck¹,² · Henri Wallaschofski¹,²

• 2709 participants (1353 men and 1356 women)
• 137 incident diabetes cases (5-year Follow-up)
• 56 quantified urinary metabolites (¹H-NMR spectroscopy), 903 metabolite ratios
• Sex-specific logistic regression adjusted for age and waist circumference
Incident Type 2 Diabetes Mellitus

Confirmation of previous findings

Association of TMAO and trigonelline with incident diabetes among women

Friedrich N. et al., Metabolomics 2015
Incident Type 2 Diabetes Mellitus

Friedrich N. et al., Metabolomics 2015
Changes in Glycemic Markers

- up to know mostly case-control designs focused on the classification of incident diabetic cases

- gradual changes in glycemic control could be addressed using changes in continuous markers like fasting plasma glucose, glycated hemoglobin (HbA1c) or insulin resistance (HOMA-IR)

- predict those changes we are applying urine metabolite measurements
Changes in Glycemic Markers

2 study populations based on Inter99:

- I) whole population (n = 3986)
- II) subjects without diabetes, HbA1c < 5.7% and eGFR > 60ml/min (n = 1347)

urine metabolome assessed by $^1$H-NMR spectroscopy (400MHz)

- 17 metabolites
- 500 buckets

Friedrich N. et al., Diabetes & Metabolism 2017
Changes in Glycemic Markers

- correlation of urine metabolites between baseline and 5-year follow-up
- More than the half of metabolites with Spearmen Cor. > 0.4

Metabolic Fingerprint:
- Alanine
- Citric acid
- Creatinine
- Formic acid
- Glycine
- N,N-dimethylglycine
- Succinic acid
- Trigonelline
Changes in Glycemic Markers

Friedrich N. et al., Diabetes & Metabolism 2017

08.11.2018
Matthias Nauck: Clinical Metabolomics by 1H-NMR Spectroscopy
Changes in Glycemic Markers

markers of shifts in the gut microbiome

Friedrich N. et al., Diabetes & Metabolism 2017
Changes in Glycemic Markers

Friedrich N. et al., Diabetes & Metabolism 2017
Biological Age

Measuring Biological Age via Metabonomics: The Metabolic Age Score

Johannes Hertel, Nele Friedrich, Katharina Wittfeld, Maik Pietzner, Kathrin Budde, Sandra Van der Auwera, Tobias Lohmann, Alexander Teumer, Henry Völzke, Matthias Nauck, and Hans Jürgen Grabe

• based on 59 urine metabolites in the SHIP-0 cohort
• by design statistical independent of chronological age

Hertel J et al., J Proteome Res. 2016
Bruker IVD Quantification in Urine

Analysis Report

Sample ID: 20180607_Urin2_115_expno10.100000.10r
Measuring Date: 08-Jun-2018 02:12:11
Reporting Date: 08-Jun-2018 02:19:12, 4 page(s), Version 1.0.0
Quantification Method Version: Quant-UR B.1.0.0

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Contents
1. Creatinine
2. Amines and derivatives
3. Amino acids and derivatives
4. Benzene and substituted derivatives
5. Carboxylic acids
6. Fatty acids and derivatives
7. Keto acids and derivatives
8. Purine, Pyridine and Pyrimidine derivatives
9. Sugars and derivatives
Biological Age

Diabetic participants have a higher biological age

Hertel J et al., J Proteome Res. 2016
Age trajectory

- Principle component analysis of the urine metabolome in SHIP (N=4300)
- Color coding for age
- Distinct shift within age groups
Age trajectory

- Establishment of age-specific reference intervals for the metabolome
- Development of analytical tools for determination and analysis of these data
Conclusion Type 2 Diabetes

• (Sub)clinical changes in glycemic control have a urinary fingerprint

• those relate to shifts in the gut microbiome (e.g. TMAO), nutritional behavior (e.g. trigonelline) and one-carbon metabolism (e.g. betaine)

• biological age score is able to summarize complex metabolic interactions
Greifswald Approach to Individualized Medicine

The objective is to use state-of-the-art diagnostics and novel therapeutic interventions that take into account the specific requirements and characteristics of the individual patient in order to optimize the effectiveness of treatments, avoid adverse reactions and reduce health care costs.
Translation into clinical practise
GANI_MED cohorts

Heart failure
Renal Failure
Stroke
Metabolic Syndrome
COPD/Lung Cancer
Sepsis

Clinical examination
Imaging
Biomarkers (incl. OMICs)

Matthias Nauck: Clinical Metabolomics by 1H-NMR Spectroscopy
Lipoprotein Subfractions

The structural components of lipoproteins (A) and their relation to diameter and density (B)

Ridker P. Lancet. 2014; 384..
LDL-subfractions

Large LDL particles

small, dense LDL

Baumstark et al. Biochim Biophys Aacta 1990;1037:48-57
Lipoprotein Subfractions

- laboratory intensive determination by ultra centrifugation and separate measurement of each density fragment

  **1 week**

- such analyses were done for more than 200 participants at the Institute of Clinical Chemistry and Laboratory Medicine in Greifswald

- $^1$H-NMR spectra from the same samples were obtained

- in cooperation with Bruker BioSpin an algorithm was trained to predict laboratory data from NMR-spectra (B.I.LISA™)

  **30 minutes**
## Lipoprotein Subfractions

<table>
<thead>
<tr>
<th></th>
<th>Triglyceride</th>
<th>Cholesterol</th>
<th>Free Cholesterol</th>
<th>Phospho-lipids</th>
<th>Apo-A1</th>
<th>Apo-A2</th>
<th>Apo-B</th>
<th>LDL-particle</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLASMA</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VLDL</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VLDL-1 to -6</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
<td>✓</td>
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</tr>
<tr>
<td>IDL</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
<td>✓</td>
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</tr>
<tr>
<td>LDL</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>LDL-1 to -6</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
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<tr>
<td>HDL</td>
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<td>HDL-1 to -4</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
</tr>
</tbody>
</table>
\textbf{Bruker IVDr Lipoprotein Subclass Analysis}

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\textbf{Analysis Report}

**Bruker IVDr Lipoprotein Subclass Analysis B.I.LISA™**

Sample ID: SHIPT1_20180717_2713890199_expno10.100000.11r

Measuring Date: 18-Jul-2018 02:24:42
Reporting Date: 18-Jul-2018 04:47:58, 8 page(s), Version 1.0.0
Model Version: PL-5009-01/001

\textbf{Disclaimer}

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\textbf{Main Parameters}

<table>
<thead>
<tr>
<th>Key</th>
<th>Parameter</th>
<th>Value</th>
<th>Unit</th>
<th>95% Range of Model</th>
<th>Graphics $^{(*)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPTG</td>
<td>TG</td>
<td>122</td>
<td>mg/dL</td>
<td>53 - 490</td>
<td></td>
</tr>
<tr>
<td>TPCH</td>
<td>Chol</td>
<td>185</td>
<td>mg/dL</td>
<td>140 - 341</td>
<td></td>
</tr>
<tr>
<td>LDCH</td>
<td>LDL-Chol</td>
<td>114</td>
<td>mg/dL</td>
<td>55 - 227</td>
<td></td>
</tr>
<tr>
<td>HDCH</td>
<td>HDL-Chol</td>
<td>36</td>
<td>mg/dL</td>
<td>35 - 96</td>
<td></td>
</tr>
<tr>
<td>TPA1</td>
<td>Apo-A1</td>
<td>117</td>
<td>mg/dL</td>
<td>112 - 217</td>
<td></td>
</tr>
<tr>
<td>TPA2</td>
<td>Apo-A2</td>
<td>28</td>
<td>mg/dL</td>
<td>24 - 48</td>
<td></td>
</tr>
<tr>
<td>TPAB</td>
<td>Apo-B100</td>
<td>94</td>
<td>mg/dL</td>
<td>48 - 160</td>
<td></td>
</tr>
</tbody>
</table>

$^{(*)}$ Grey horizontal boxes represent 95\% range of model, black vertical lines represent sample value.
Lipoprotein Subfractions

Concentration found for actual sample

Triglycerides distribution (concentrations in mg/dL together with 95% range of model)
Fatty Liver Disease

- overnutrition
- insulin resistance (?)

healthy liver

NAFLD

- lipotoxicity / oxidative stress
- inflammatory stimuli
- insulin resistance (!)

- TG accumulation
- VLDL secretion
- de novo lipogenesis

NASH

- hepatocyte damage
- inflammation
- fibrosis

risk factor for
- type 2 diabetes mellitus (T2DM)
- cardiovascular disease (CVD)
- chronic kidney disease (CKD)

Cohen JC et al. Science 2011
Adams LA et al. Gut 2017
Lipoprotein Subfractions and Fatty Liver

- associations analyses among 769 SHIP-Trend participants with MRI quantified liver fat

Pietzner M et al., JCEM (2018)
Lipoprotein Subfractions and Fatty Liver

- *no association* with (total) LDL-cholesterol

Pietzner M et al., JCEM (2018)
Lipoprotein Subfractions and Fatty Liver

- no association with (total) LDL-cholesterol
- inverse association with large-dense LDL

Pietzner M et al., JCEM (2018)
Lipoprotein Subfractions and Fatty Liver

- **no association** with (total) LDL-cholesterol
- **inverse association** with large LDL
- **positive association** with small-dense LDL

Pietzner M et al., JCEM (2018)
Closing Remarks

- $^1$H-NMR is a robust, fast and highly reproducible spectroscopic technique to compile molecular fingerprints of diseases.

- The highly informative content of NMR-spectra allows the development of life trajectories with a single technique.

- $^1$H-NMR strongly advances lipoprotein-based diagnostics and risk stratification and its translation to daily clinical practice.

- Minimal sample preparation in combination with highly reproducible measurements allow for a world wide compilation of comparable Metabolomics data.
Thank you very much for your attention!