The general opinion is, that essential pre-requisites for the success of research projects are

- good experimental design
- precise, robust and reproducible analytical techniques (wet lab)
- sophisticated data analysis (dry lab) and interpretation
“….the quality of biospecimen is of paramount importance for the reliability and validity of research results and all downstream applications.” Fay Betsou (ISBER President 2013-2014)

⇒ pre-analytical phase!

Biobanking / Blood collection

Unfortunately, in general the interest of many investigators for the pre-analytical phase is only minor

In complex clinical studies blood collection is often entitled as the easy part
Typical questions of investigators (frequently asked after the end of their studies) are:

The centrifuge of our ward was highly frequented, consequently the processing of some blood samples was delayed. Is this a problem?

One third of the blood of our study was drawn by inexperienced medical students. Unfortunately this resulted in several more or less hemolytic samples. Can we still use the sample set?

Several biobank samples of my study have been thawed one or two times. Are they still suitable e.g. for metabolomics investigations?

The centrifuge was in another building. We could not process the blood samples before 2 h, is this a problem?

Rainer Lehmann, University Hospital Tübingen, Germany
Markers of biobank sample quality

➤ we applied metabolomics to investigated these preanalytical steps

Blood collection
- hemolysis

Transportation
- delayed processing

Storage
- Repetitive freeze/thaw cycles

Rainer Lehmann, University Hospital Tübingen, Germany

Metabolomics

TARGETED Metabolomics
- analysis of selected, well-known metabolites
- 20 - 300 metabolites per sample
- automation (commercial kits available)
- quantitative
- population based studies
- > 500 samples / week possible
- 2 weeks until final result

NON-TARGETED Metabolomics
- non-hypothesis driven, comprehensive approach
- > 2,000 metabolite-ion masses per sample
- identification of most important metabolites
- time consuming and labor intensive
- semi-quantitative
- well selected and characterized samples
- > 4 months until final result

Rainer Lehmann, University Hospital Tübingen, Germany
primary note for the coming slides: Scheme of data evaluation

Metabolomics data processing

- blood collection / hemolysis
- transportation
- storage

Hemolytic specimens occur frequently in clinical laboratories

- as high as 3.3% of all of the routine samples,
  that means e.g. around 100 – 130 samples / day in our lab

- they account for up to 40%–70% of all unsuitable specimens identified

G. Lippi et al. CCLM (2008)
Blood drawing

Hemolysis

Blood drawing → non-targeted metabolomics → classical parameters

n = 10

18% of all metabolite ion masses were significantly altered (p<0.05, FDR<0.1)

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Crucial point in non-targeted metabolomics

nontargeted Metabolomics

Bioinformatics → candidate list of features / ion masses

Elucidation of the metabolite identity

Data base searches / MS^n / confirmation by a standard compound

Blood drawing

Hemolysis

Identified metabolites (sign. changed):
- Lyso-PC 16:0
- Lyso-PC 18:0
- Lyso-PC 18:1
- Tryptophan
- Sphingosine 1-phosphate (S-1-P)
- N-acetylornithine
- C8-carnitine

Suggestions:
- Hemolytic samples should be excluded, at least from high resolution -omics research projects
- S-1-P, Trp, LPC C16:0,… should only carefully be nominated as biomarker candidates in research studies and must be thoroughly validated for robustness
- All biobank samples should be tested for hemolysis before storage
  → free Hb can easily be measured by a two wavelenght method

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Markers of biobank sample quality

- blood collection
- transportation / processing of blood
- storage

A 9 ml sample of blood from an adult subject contains

- \(5 \times 10^{10}\) red blood cells
- \(3 \times 10^9\) platelets
- \(8 \times 10^7\) leukocytes

„liquid tissue“
Experimental design

- 2h to 4 h exposure of blood to room temperature or ice water
  ➔ clinic internal handling / transportation

- 8h and 24 h exposure to room temperature ➔ external transportation

Transportation / processing of blood

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Transportation / processing of blood

Transport of whole blood
(EDTA-blood)

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Exposure of EDTA blood samples to room temperature

Transportation / processing of blood

Identified metabolites
- C2-carnitine
- C8-carnitine
- C12-carnitine
- N-acetylcarnitine
- Hypoxanthine
- Proline betaine
- Palmitic amide
- Sphingosine 1-phosphate
- Indole
- Tryptophan
- LPC 16:0
- LPC 18:0
- LPC 18:1
- LPC C20:3
- Oleamide
- L-Methionine
- Biliverdine
Identified metabolites

C2-carnitine
C8-carnitine
C12-carnitine
N-acetylornithine
Hypoxanthine
Proline betaine
Palmitic amide
Sphingosine 1-phosphate
Indole
Tryptophan
LPC 16:0
LPC 18:0
LPC 18:1
LPC C20:3
Oleamide
L-Methionine
Biliverdine

A targeted metabolomics approach which included several of these metabolites confirmed the findings


New (ongoing) studie

Goal:

- define confidence intervals for good and poor quality of blood samples in ca. 100 samples (samples were selected at random in a metabolic ward in our outpatient clinic)

- receive an impression of the number of changed metabolites
  
  → modern, more sensitive mass spec (triple TOF-MS), new analytical strategy

Analytical approaches

→ non-targeted metabolomics
→ targeted metabolomics

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**NON-targeted metabolomics**

positive mode detected ion masses: 3137
negative mode detected ion masses: 2910

**Significant changed metabolite ion masses**

→ 262 metabolite ion masses were significantly altered

<table>
<thead>
<tr>
<th></th>
<th>ESI +</th>
<th>ESI -</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>4 h</td>
</tr>
<tr>
<td></td>
<td>0.64 %</td>
<td>3.6 %</td>
</tr>
</tbody>
</table>

114 metabolite ions
148 metabolite ions

p < 0.05, FDR 0.05
n = 30
unpublished data

**NON-targeted metabolomics**

Potential markers of biobank sample quality

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unpublished data
NON-targeted metabolomics

Blood processing delayed by 4 h

ROC Curve

<table>
<thead>
<tr>
<th>Metabolite Ion Mass</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolite Ion Mass (A)</td>
<td>98.3</td>
<td>76.7</td>
</tr>
<tr>
<td>Metabolite Ion Mass (B)</td>
<td>91.7</td>
<td>98.9</td>
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<tr>
<td>Metabolite Ion Mass (C)</td>
<td>96.7</td>
<td>100</td>
</tr>
<tr>
<td>Metabolite Ion Mass (D)</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

n = 30

Unpublished data

Markers of biobank sample quality

- Clinical study
- Blood collection
- Centrifugation
- Plasma / Serum
- Sample preparation
- Analysis
- Data evaluation / bioinformatics
- Valid results

- Blood collection
- Transportation / processing of blood
- Storage
  - Repetitive freeze and thaw cycles

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Biobanking / stored samples

**Problem: the number of aliquots is limited**

- leads unavoidable to repetitive freeze and thaw cycles aiming to save sample material

we investigated EDTA plasma by non-targeted metabolomics:

controls (metabolomics sample pretreatment was performed at once after drawing blood)

1x frozen (= common biobank sample)
2x thawed / frozen
4 x thawed / frozen

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Biobanking / stored samples

- no significant alterations of metabolite ion masses between fresh controls and 1x frozen plasma samples were detected

- unexpectedly, only 4 masses changed significantly after 2x and 4x freeze/thaw cycles!

- we detected individual differences in the sample stability!

  instabilities of the metabolite pattern were detected in plasma samples of 2 out of 10 individuals, even in 1x frozen aliquots!

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and another aspect…. relevant for biobank samples intended for MS-driven –omics analyses

Additives included in the sample collectors may affect the ionization process during an LC-MS run thereby suppressing the ionization of metabolites or introduce interfering compounds.

Planning phase of studies

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Summary (1)

**Blood collection/storage** is the easy part of complex clinical studies, **BUT** may heavily affect the quality of biobank samples and consequently the outcome and success of research projects.

**SOPs** strictly regulate the exposure time of whole blood to room temperature or cooling (as well as the further processing), **BUT** this exposure time may vary from SOP to SOP, e.g. based on logistical problems and resulting compromises in the SOP. **and** the exposure time may be prolonged in occasional instances.

Summary (2)

The discovered pre-analytical biomarkers may help to identify:

- a) systematic inaccuracies that arise during processing of whole blood that affect the quality of biobank samples
- b) random errors leading to particular outliers in the sample set

**and**

Investigators should be careful in nominating metabolites identified to be sensitive to preanalytical alterations as biomarker candidates in their research studies.

Summary (3)
- Recommendations -

1.) the suitability of sample collectors should be tested before starting sample collection

2.) hemolytic samples must be excluded

3.) place blood immediately in ice water after drawing until further processing (for a fixed time; ideally not longer than 2 hours)
   - we prefer (EDTA-)plasma instead of serum as the favorable sample material
   - processing: centrifugation at 4°C, for 10 min, at 2000 × g

4.) the quality of biobank samples should be tested by using EDTA plasma aliquots before the samples are used in expensive and time consuming analytical projects

5.) handling of biobank samples:
   - samples should be thawed in ice water
   - non refrozen plasma aliquots of biobank samples are recommended
   - mix-up of samples exposed to different freeze and thaw cycles should be avoided

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Summary (4)
- Recommendations -

Poor sample quality does not necessarily mean that the biobank sample has to be discarded, but these samples should not be used for high resolution analyses, like metabolomics analysis

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Probenmaterial und Stabilität der Analytauf Blut</th>
<th>Stabilität in whole blood at room temperature</th>
<th>Stabilität in serum / plasma at room temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe</td>
<td>Albumin</td>
<td>Phosphat</td>
<td>Alk. Phosph.</td>
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<td>Organik</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Serum</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EDTA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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</table>

W. Guder et al., J. Lab. Med. 2002

Rainer Lehmann, University Hospital Tübingen, Germany

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