The Laboratory Diagnosis Of Peroxisomal Disease

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### Relevant Analyses for the Primary Diagnosis of Peroxisomal Disorders

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<th><strong>Plasma</strong></th>
<th><strong>Technique</strong></th>
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<tr>
<td>Very long-chain fatty acids</td>
<td>GC/MS, (ES-MS/MS)</td>
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<tr>
<td>Phytanic + pristanic acids</td>
<td>GC/MS</td>
</tr>
<tr>
<td>Bile acids ($C_{27}$)</td>
<td>ES-MS/MS</td>
</tr>
<tr>
<td>Pipecolic acid</td>
<td>ES-MS/MS</td>
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<tr>
<td>Polyunsaturated fatty acids (Acylcarnitines)</td>
<td>GC</td>
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<td>Acylcarnitines</td>
<td>ES-MSMS</td>
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<th><strong>Erythrocytes</strong></th>
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<tr>
<td>Plasmalogenens</td>
<td>GC</td>
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<td>Polyunsaturated fatty acids</td>
<td>GC</td>
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<th><strong>Urine</strong></th>
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<tr>
<td>Bile acids</td>
<td>ES-MS/MS</td>
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<tr>
<td>Dicarboxylic acids</td>
<td>GC/MS</td>
</tr>
<tr>
<td>Pipecolic acid</td>
<td>ES-MS/MS, (AAA)</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>IC</td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>GC/MS/IC</td>
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Peroxisomal Parameters

CSF
- Pipecolic acid

Plasma
- VLCFA
- Phytanic acid
- Pristanic acid
- C_{27}-bile acids
- Pipecolic acid
- DHA
- Acylcarnitines

Plasmalogens
- DMA

Erythrocytes
- C_{27}-bile acids

Urine
- C_{27}-bile acids
- Pipecolic acid
- Dicarboxylic acids
- Oxalic acid

Bile
Analysis of very long-chain fatty acids and Phytanic / Pristanic acid

- plasma (100 μl)
- add $^2$H-internal standards (100 μl)
- add 2 ml 0.5 M HCl
  - 110º, 45 min
- add 2 ml 1.0 M NaOH
  - 110º, 45 min
- add – extract 4 ml hexane
- remove sterols – extract 4 ml 1 M KOH
- add – extract 4 ml hexane
- derivatise MTBSTFA
- analyse GC/MS (stable isotope dilution)
VLCFA, phytanic and pristanic acid by GC/MS
Are the VLCFA levels constant? 

\( C_{26} \) of Zellweger and X-ALD

The upper normal level of 
\( C_{26} \) is 1.32 \( \mu \text{mol/L} \); otherwise quite large variations occur and the most severely affected patients do not necessarily have the highest \( C_{26} \) values.
Electrospray ionization mass spectrometry of VLCFA (ESI-MS)

Procedure

• 100 µl plasma + 100 µl IS (²H₄-labeled C22:0, C24:0 and C26:0)
• hydrolysis with 1 ml acetonitrile / 37% HCl (4:1)
• incubate at 90ºC for 2 hours
• extract free fatty acids with hexane
• reconstitute in chloroform-methanol-water (50:45:5) + 0.01% ammonia
• analyze on mass spectrometer in negative ion mode
VLCFA analysis with ESI-MS

control

X-ALD
Peroxisomal Fatty Acid β-oxidation in Human Peroxisomes and its Deficiency

C26:0 Pristanic acid

Cholesterol

ATP

CoASH

Pristanic acid

CoASH

THCA

ALDP

AOX1

AOX2

DBP

pTH1

pTH2

ACoA oxidase def.

Racemase def.

D-bifunctional protein def.

SCPα def.
Fatty acid oxidation in peroxisomes
A neonate with extreme hypotonia was subjected to selective screening of peroxisomal disease at the age of one week. VLCFA were strikingly abnormal, whereas pristanic acid and C27-bile acids increased with age. The pattern was consistent with D-BP deficiency, including normal plasmalogenes.
Peroxisomal Parameters

CSF
- Pipecolic acid

Plasma
- VLCFA
- Phytanic acid
- Pristanic acid
- C_{27}-bile acids
- Pipecolic acid
- DHA
- Acylcarnitines
- Plasmalogens DMA

Erythrocytes
- C_{27}-bile acids

Urine
- C_{27}-bile acids
- Pipecolic acid
- Dicarboxylic acids
- Oxalic acid

Bile
Fatty acid oxidation in peroxisomes
Peroxisome Biogenesis Defect
Bile Acids in urine (tandem-MS)
Analysis of Plasma C_{27}-bile acids

- Plasma (50 μl) + I.S. (²H)
- Deproteinise (acetonitril)
- Evaporate → 100 μl H₂O / CH₃OH → MS/MS

HPLC / MS/MS

- RP-C₈
- 50 x 1.0 mm
- A. Formate buffer pH 8.1 + CH₃OH
- B. Acetonitril / 10% H₂O

MRM
- Tau-conj.
- C_{29}-dic.
- Gly-conj.
- Free acids
LC-MS/MS of plasma bile acids

C29 Bile dicarboxylic acid

Tauro-THCA

Int. St. Tauro-CA

DHCA (free)

Int. St. CA (free)
Peroxisomal Parameters

CSF
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Plasma
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- C_{27}-bile acids
- Pipecolic acid
- DHA
- Acylcarnitines
- Plasmalogens
- DMA
- C_{27}-bile acids

Urine
- C_{27}-bile acids
- Pipecolic acid
- Dicarboxylic acids
- Oxalic acid

Erythrocytes

Bile
D.M. Danks, P. Tippett, C. Adams, P. Campbell

Cerebro-hepato-renal syndrome of Zellweger. A report of eight cases with comments upon the incidence, the liver lesion, and a fault in pipecolic acid metabolism.

Journal of Pediatrics 86 (1975), 382-387
There is a wide variation of plasma Pipecolic acid in Zellweger

The upper normal level of plasma pipecolic acid is approx. 5 μmol/L. PBD patients generally range from 15 μmol/L upwards, although mild patients may be as low as 8 μmol/L. A steady increase with age accompanies a bad clinical evolution.
Is there a relationship between phytanic and pipecolic acid?

Two PBD-patients showed a steady increase of pipecolic acid over a three-year-period. Only one patient had a concomitant phytanic acid increase.

Phytanic acid is of minor importance in most mild PBD-patients.
Pipecolic acid may be increased in

- Peroxisome biogenesis defects
- Vitamin B₆-responsive convulsions (antiquitin defects)
- Hyperlysinemias
- Hyperprolinemia type 2
- Unexplained conditions

but NOT in

- Isolated peroxisomal enzyme defects
Peroxisomal Parameters

**CSF**
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- C_{27}-bile acids
- Pipecolic acid
- DHA
- Acylcarnitines

**Urine**
- C_{27}-bile acids
- Pipecolic acid
- Dicarboxylic acids
- Oxalic acid

**Erythrocytes**
- Plasmalogens
- DMA

**Bile**
- C_{27}-bile acids
Erythrocyte

Plasmalogens (= ether phospholipids)

\[ \text{CH}_3\text{DH}/\text{HCl} \]

80ºC; 4hr

Fatty acid methyl esters

+ dimethylacetals

extraction

Capillary GC
## The PEX 7 spectrum

<table>
<thead>
<tr>
<th>Clinical presentation</th>
<th>Biochemical presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phytanic</td>
</tr>
<tr>
<td>Refsum</td>
<td>↑</td>
</tr>
<tr>
<td>MR + cataract</td>
<td>↑</td>
</tr>
<tr>
<td>Severe RCDP</td>
<td>↑</td>
</tr>
</tbody>
</table>
# Early increase of Plasma Phytanic acid in RCDP-patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Phytanic acid (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 day</td>
<td>0.7</td>
</tr>
<tr>
<td>2</td>
<td>3 days</td>
<td>5.8</td>
</tr>
<tr>
<td>3</td>
<td>1 week</td>
<td>4.4</td>
</tr>
<tr>
<td>4</td>
<td>2 weeks</td>
<td>9.9</td>
</tr>
<tr>
<td>5</td>
<td>2 weeks</td>
<td>13.2</td>
</tr>
<tr>
<td>6</td>
<td>3 weeks</td>
<td>9.3</td>
</tr>
</tbody>
</table>

| Controls * | 0-4 months | 0.04-5.3 |

Peroxisomal Parameters

- **CSF**
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  - Phytanic acid
  - Pristanic acid
  - C_{27}-bile acids
  - Pipecolic acid
  - DHA
  - Acylcarnitines
  - Plasmalogens
  - DMA

- **Erythrocytes**
  - C_{27}-bile acids

- **Urine**
  - C_{27}-bile acids
  - Pipecolic acid
  - Dicarboxylic acids
  - Oxalic acid

- **Bile**
Analysis of Acylcarnitines

plasma (50 μl)

add internal standards (²H-labelled)

deproteinise / extract with acetonitril

make butyl esters (butanol /acetyl chloride)

electrospray MS/MS (parents of 85)
Peroxisome Biogenesis Defect
Acylcarnitines in plasma (tandem-MS)
Peroxisomal Parameters

- **CSF**
  - Pipecolic acid

- **Plasma**
  - VLCFA
  - Phytanic acid
  - Pristanic acid
  - C$_{27}$-bile acids
  - Pipecolic acid
  - DHA
  - Acylcarnitines

- **Urine**
  - C$_{27}$-bile acids
  - Pipecolic acid
  - Dicarboxylic acids
  - Oxalic acid

- **Erythrocytes**
  - Plasmalogens
  - DMA

- **Bile**
  - C$_{27}$-bile acids
Analysis of urine Organic acids

Urine (1-5 ml); acidified

Extraction (ethyl acetate)

Derivatisation (trimethylsilyl-)

Gas chromatography / mass spectrometry
Urine Organic acids in Zellweger

1. $\omega$-Oxidation
   - C6-dicarboxylic
   - C8, C8:1-dicarboxylic
   - C10, C10:1-dicarboxylic
   - 3-OH-C10-dicarboxylic
   - 2-OH-C10-dicarboxylic
   - C7-dicarboxylic
   - C9-dicarboxylic

2. Other
   - 3,6-epoxy-C14-dicarboxylic
   - 4-OH-phenyllactic
   - 2-OH-isovaleric
## Characteristic Biochemical Genetic Findings in the Various Disorders of Peroxisomal Function

<table>
<thead>
<tr>
<th>Disorder</th>
<th>C\textsubscript{24} + C\textsubscript{26} fatty acid</th>
<th>C\textsubscript{27} bile acid</th>
<th>Phytanic acid</th>
<th>Pristanic acid</th>
<th>Pipecolic acid</th>
<th>Plasmalogens (ery’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBD severe</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>PBD mild</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>n</td>
</tr>
<tr>
<td>RCDP</td>
<td>n</td>
<td>n</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>n</td>
</tr>
<tr>
<td>Bifunctional protein</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>n</td>
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<tr>
<td>X-ALD male</td>
<td>↑</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>X-ALD female</td>
<td>n- ↑</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Refsum</td>
<td>n</td>
<td>n</td>
<td>↑↑</td>
<td>↓</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Racemase</td>
<td>n</td>
<td>↑</td>
<td>n- ↑</td>
<td>↑</td>
<td>n</td>
<td>n</td>
</tr>
</tbody>
</table>
Follow-up studies for the confirmation of peroxisomal disease

1. Fibroblast studies
   - $\beta$-oxidation of VLCFA / pristanic acid
   - $\alpha$-oxidation of phytanic acid
   - Catalase staining
   - Plasmalogen biosynthesis
   - Individual enzymes (DBP, AMACR, etc.)
Follow-up studies for the confirmation of peroxisomal disease

2. Molecular genetic studies
   - Complementation groups (PEX 1, PEX 2, etc.)
   - mutation screening of the PEX gene(s)
   - mutation screening related to individual enzymes
Conclusion

1. The assay of VLCFA alone is not sufficient
2. Never analyse phytanic acid without pristanic acid
3. Plasma bile acids have a diagnostic significance (urine less so)
4. Pipecolic acid is only increased in PBD-patients
5. Pipecolic acid may be increased in non-peroxisomal patients
6. The value of urine organic acids and plasma acylcarnitines remains to be established
7. One patient had normal plasma parameters, but fibro’s were diagnostic.