Multiplex LC-MS/MS lysosphingolipids analysis in plasma for the screening of sphingolipidoses and Niemann-Pick type C disease

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Monique Piraud, Cécile Pagan, David Cheillan, Christine Vianey-Saban, Roseline Froissart.
Sphingolipidoses

The measurement of the primary accumulated sphingolipids in plasma is poorly informative.

Diagnosis based on enzymatic testing: Filipin staining test.

Lysosomal cholesterol trafficking and lipid storage disorder.
Sphingolipids/LysoSphingolipids in plasma

- Lysosphingolipids (LysoSL) = deacetylated form of the sphingolipids
- Role in pathophysiology?
- With the use of MS/MS Emerging biomarkers in plasma for screening of sphingolipidoses and Niemann-Pick type C

From Sun and Zhang, Advances in Gaucher Disease: Basic and Clinical Perspectives 260 pages, August 2013. Future Medicine Ltd.
Tandem Mass Spectrometry

Very sensitive, very specific technique

Allows the simultaneous measurement of numerous compounds all together, in complex mixtures, according to specific fragmentation of each molecule

« TRANSITION » = pair of (precursor ion)/(product ion)
**LC-MS/MS LysoSLs measurement**

- **LysoGlobotriaosylceramide** (LysoGb₃) **Fabry**
- **Lysogalactosyl-ceramide** (LysoGalCer) **Krabbe**
- **Lysoglucosyl-ceramide** (LysoGlcCer) **Gaucher**
- **Lysosphingomyelin** (LysoSM) **Niemann-Pick A/B**
- **LysoSM analogue 509** (LysoSM509) **Niemann-Pick A/B and C**

**Lysosulfatide** (LysoSulf) **Metachromatic leukodystrophy**

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**Diagnosis of sphingolipidoses: a new simultaneous measurement of lysosphingolipids by LC-MS/MS**


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**LC-MS/MS multiplex analysis of lysosphingolipids in plasma and amniotic fluid**

*Pettazzoni M et al. PLoS One. 2017*
**Method**

**Extraction/Purification**
- 200 µL of EDTA plasma + IS + MeOH
- SPE (Oasis MCX, 30 mg, 60 µm, Waters Corp)

**Liquid chromatography**
- C8 column (Uptisphère. Interchim©)
- Gradient of elution (Phase A: H2O 0.2% FA, Phase B ACN 0.2% FA)

**MS/MS— Multiple Reaction Monitoring Mode (MRM)**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>STANDARDS</th>
<th>Internal Standard **</th>
<th>MODE</th>
<th>TRANSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso Gb3</td>
<td>Lyso Gb₃</td>
<td>Glycinated lysoGb₃</td>
<td>Positive</td>
<td>786.5 &gt; 282.3</td>
</tr>
<tr>
<td>Lyso HexCer</td>
<td>Lyso GlcCer</td>
<td>Lyso GlcCer d5</td>
<td>Positive</td>
<td>462.3 &gt; 282.3</td>
</tr>
<tr>
<td>Lyso SM</td>
<td>Lyso SM d18:1</td>
<td>Lyso SM d17 :1</td>
<td>Positive</td>
<td>465.3 &gt; 184</td>
</tr>
<tr>
<td>Lyso SM 509</td>
<td>Lyso SM d18:1</td>
<td>Lyso SM d17 :1</td>
<td>Positive</td>
<td>509.3 &gt; 184</td>
</tr>
<tr>
<td>Lyso GM1</td>
<td>Lyso GM1</td>
<td>S1P d17 :1</td>
<td>Negative</td>
<td>1278.6 &gt; 290.1</td>
</tr>
<tr>
<td>Lyso GM2</td>
<td>/</td>
<td>S1P d17 :1</td>
<td>Negative</td>
<td>1116.6 &gt; 290.1*</td>
</tr>
</tbody>
</table>

* No standard; calculated from mass/structure
** Commercially available

**Positive mode**

LysoGLUCOSYLceramide and lysoGALACTOSYLceramide: not separated
Measured as lysohexosylceramide « LysoHexCer »

**Quantitative validation**
Except LysoGM1 and LysoGM2
LysoSL multiplex measurement:
Routine experience in Lyon
for the screening of sphingolipidoses and NPC
Fabry disease (X-linked)

Mutations on GLA gene
Classical forms / Variant forms with specific variant mutations on GLA gene

**Heterozygote females**
- *Normal* $\alpha$ Gal A: 30% cases
- *Normal* (U)Gb$_3$:
  - 18% cases (classical forms)
  - most of cases (variant forms)

**FEMALES DIAGNOSIS**
- $\alpha$ Gal A activity
- (U) Gb$_3$
- GLA gene
# Fabry disease: Plasma LysoGb₃

## Results

**LYON, France**

<table>
<thead>
<tr>
<th></th>
<th>Males Classical form</th>
<th>Males Variant form*</th>
<th>Females Classical form</th>
<th>Females Variant form**</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>77</td>
<td>15</td>
<td>10</td>
<td>44</td>
<td>228</td>
</tr>
<tr>
<td>LysoGb₃ (nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>91</td>
<td>5.8</td>
<td>5.7</td>
<td>1.4</td>
<td>&lt; 0.6 (99th perc.)</td>
</tr>
<tr>
<td>Range</td>
<td>30 - 173</td>
<td>1.8 - 11.1</td>
<td>1.1 - 31.2</td>
<td>0.3 - 4</td>
<td>0 - 1.1</td>
</tr>
</tbody>
</table>

Normal in 4/8 cases

* Variant GLA mutations in males
  - p.Ile232Thr
  - p.Met296Val
  - p.Arg301Gln
  - p.Arg363His
  - p.As215Ser (n=2)
  - p.Ile198Thr
  - p.Phe113Leu (n=3)

** Variant GLA mutations in females
  - p.Arg363His (n=3)
  - p.As215Ser
  - p.Phe113Leu (n=4)
Fabry disease: Correlation \((U)Gb_3 / (P) LysoGb_3\)

**MALES**

- Controls (n=44)
- Male classical form (n=12)
- Male variant form (n=8)

**FEMALES**

- Controls (n=44)
- Female classical form (n=38)
- Female variant form (n=2)

**Results**

LYON, France

Plasma LysoGb\(_3\) is a much more sensitive biomarker of screening than urinary Gb\(_3\)
## Gaucher disease: LysoHexCer

### Results LYON, France

<table>
<thead>
<tr>
<th></th>
<th>GAUCHER</th>
<th>Saposin C deficiency</th>
<th>KRABBE (infantile)</th>
<th>KRABBE (juvenile / adult)</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>n = 27</td>
<td>n = 1</td>
<td>n = 8</td>
<td>n = 4</td>
<td>n = 228</td>
</tr>
<tr>
<td>LysoHexCer (nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>160</td>
<td>76</td>
<td>13</td>
<td>1.2</td>
<td>&lt; 3.3 (99th perc.)</td>
</tr>
<tr>
<td>Range</td>
<td>5 - 427</td>
<td>-</td>
<td>9 - 22</td>
<td>0.9 – 1.4</td>
<td>0.1-3.5</td>
</tr>
</tbody>
</table>

**Notes:**
- Highly elevated in GD, large range of values, can be very moderately elevated in some cases
- Elevated in case of saposin C deficiency
- In GD: Correlation with chitotriosidase and CCL18 (= indirect and non specific biomarkers)
- *Treated by SRT*
Gaucher disease: LysoHexCer/GBA Genotype correlation

Results of the French cohort, untreated Gaucher patients

Lower LysoHexCer values in patients homozygous for N370S mutation of GBA gene
Gaucher disease: LysoHexCer in Amniotic Fluid (AF)

In case of non-immune hydrops fœtalis (NIHF)
New powerful biomarker in AF of fetal GD

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Gaucher AF</th>
<th>NIHF Controls AF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LysoHexCer</strong> (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>&lt; 0.7</td>
<td>460 **     **</td>
<td>&lt; 0.7</td>
</tr>
<tr>
<td>Range</td>
<td>68 - 996</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


*** p<0.001
### Krabbe disease: LysoHexCer

**Results**

**LYON, France**

<table>
<thead>
<tr>
<th>Condition</th>
<th>GAUCHER</th>
<th>KRABBE (infantile)</th>
<th>KRABBE (juvenile / adult)</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysoHexCer (nmol/L) Mean</td>
<td>n = 27</td>
<td>160</td>
<td>13</td>
<td>1.2</td>
</tr>
<tr>
<td>LysoHexCer (nmol/L) Range</td>
<td>5 - 427</td>
<td>9 - 22</td>
<td>0.9 - 1.4</td>
<td>0.1-3.5</td>
</tr>
</tbody>
</table>

**Description**

- **Moderate increase** in infantile KD
- **In normal range** in juvenile and adult forms
- Saposin A deficiency? To be evaluated

**Graph**

- LysoHexCer = Psychosine

**Legend**

- Controls (n=228)
- FD male Classical (n=15)
- FD female Classical (n=44)
- FD female Variant (n=10)
- Saposin C deficiency (n=8)
- GD (n=27)
- Krabbe disease: LysoHexCer
- Infantile KD (n=8)
- NPC < 10 (n=25)
- NPC > 10 (n=26)
- GM1/2 (n=20)
- LysoHexCer = Psychosine
Niemann-Pick diseases

Niemann-Pick type A/B
Sphingomyelinase deficiency
• Tedious enzyme activity measurement

Niemann-Pick type C
Lysosomal cholesterol trafficking and lipid storage disorder
• Filipin staining test on cultured fibroblasts
• Genetic testing NPC1 or NPC2 gene

Chitotriosidase
Moderate but inconstant elevation
6% of patients are homozygous for 24 bp duplication in CHIT1 gene and 30 – 40% heterozygotes

Oxysterols plasma (2010)
Cholestane-3β,5α,6β-triol (and 7-ketocholesterol)

LysoSM (2014)

LysoSM « 509 » = LSM + 44 (2015)
## Results LYON, France

<table>
<thead>
<tr>
<th></th>
<th>NPA/B</th>
<th>NPC* &lt; 10 y</th>
<th>NPC* &gt; 10 y</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LysoSM (nmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>16.5</td>
<td>0.7</td>
<td>1.0</td>
<td>&lt; 1.9 (99&lt;sup&gt;th&lt;/sup&gt; perc.)</td>
</tr>
<tr>
<td>Range</td>
<td>2.4 - 69.6</td>
<td>0.2 - 2.1</td>
<td>0.2 - 3.5</td>
<td>0.1-2.0</td>
</tr>
<tr>
<td><strong>LysoSM509 (MoM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>214</td>
<td>149</td>
<td>63</td>
<td>&lt; 4.3 (99&lt;sup&gt;th&lt;/sup&gt; perc.)</td>
</tr>
<tr>
<td>Range</td>
<td>98-515</td>
<td>34 - 390</td>
<td>7 - 144</td>
<td>0-9.1</td>
</tr>
</tbody>
</table>

* Some patients are treated
GM1 & GM2 gangliosidoses: LysoGM1/GM2 in plasma

**IS: d17 Sphingosine-1P**

**LysoGM1**
- Controls
- GM1 gangliosidosis
  - LysoGM1 1278.6 > 290.1
  - n = 5/6

**LysoGM2**
- Controls
- GM2 gangliosidosis
  - LysoGM2 1116.6 > 290.1
- Sandhoff 7/9
- Tay-Sachs 3/4

In spite of poor recovery results (lack of specific IS)  
**Good specificity**
- Not detected in controls
- Abnormal presence of LysoGM1 and LysoGM2
  - in GM1 gangliosidosis (5/6 cases)
  - in Sandhoff diseases (7/9 cases)
  - in Tay-Sachs diseases (3/4 cases)

Lack of sensitivity for mild and adult forms
Conclusion

Our LysoSLs multiplex assay: 6 lysoSLs including LysoGM1 and LysoGM2

- **Efficient and rapid biochemical screening tool**
  - Small plasma sample volume (pediatrics +++)
  - Better screening of FD males with variant form, and females
  - More sensitive screening of NPC than oxysterols
  - Differential screening of NPC, NPA/B and GD in the same run
  - Specific screening of fetal GD
  - Useful for the monitoring of patients (FD, GD)

- **Positive results to be confirmed by enzymatic measurement, and molecular studies**
- **Adults and mild phenotype: possibly normal screening**

- **Other analogues of LysoSL** (mass variation on the sphingosine moiety):
  could be interesting biomarkers to be evaluated?
(C Auray-Blais, Sherbrooke, Canada)
Acknowledgments

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