The Laboratory Diagnosis Of Peroxisomal Disease

Ries Duran, AMC, Amsterdam

Relevant Analyses for the Primary Diagnosis of Peroxisomal Disorders

Plasma	**Technique**
Very long-chain fatty acids	GC/MS, (ES-MS/MS)
Phytanic + pristanic acids	GC/MS
Bile acids (C_{27})	ES-MS/MS
Pipecolic acid	ES-MS/MS
Polyunsaturated fatty acids (Acylcarnitines)	GC
Acylcarnitines	ES-MSMS
Erythrocytes	
Plasmalogens	GC
Polyunsaturated fatty acids	GC
Urine	
Bile acids	ES-MS/MS
Dicarboxylic acids	GC/MS
Pipecolic acid	ES-MS/MS, (AAA)
Oxalic acid	IC
Glycolic acid	GC/MS/IC

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 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

↓

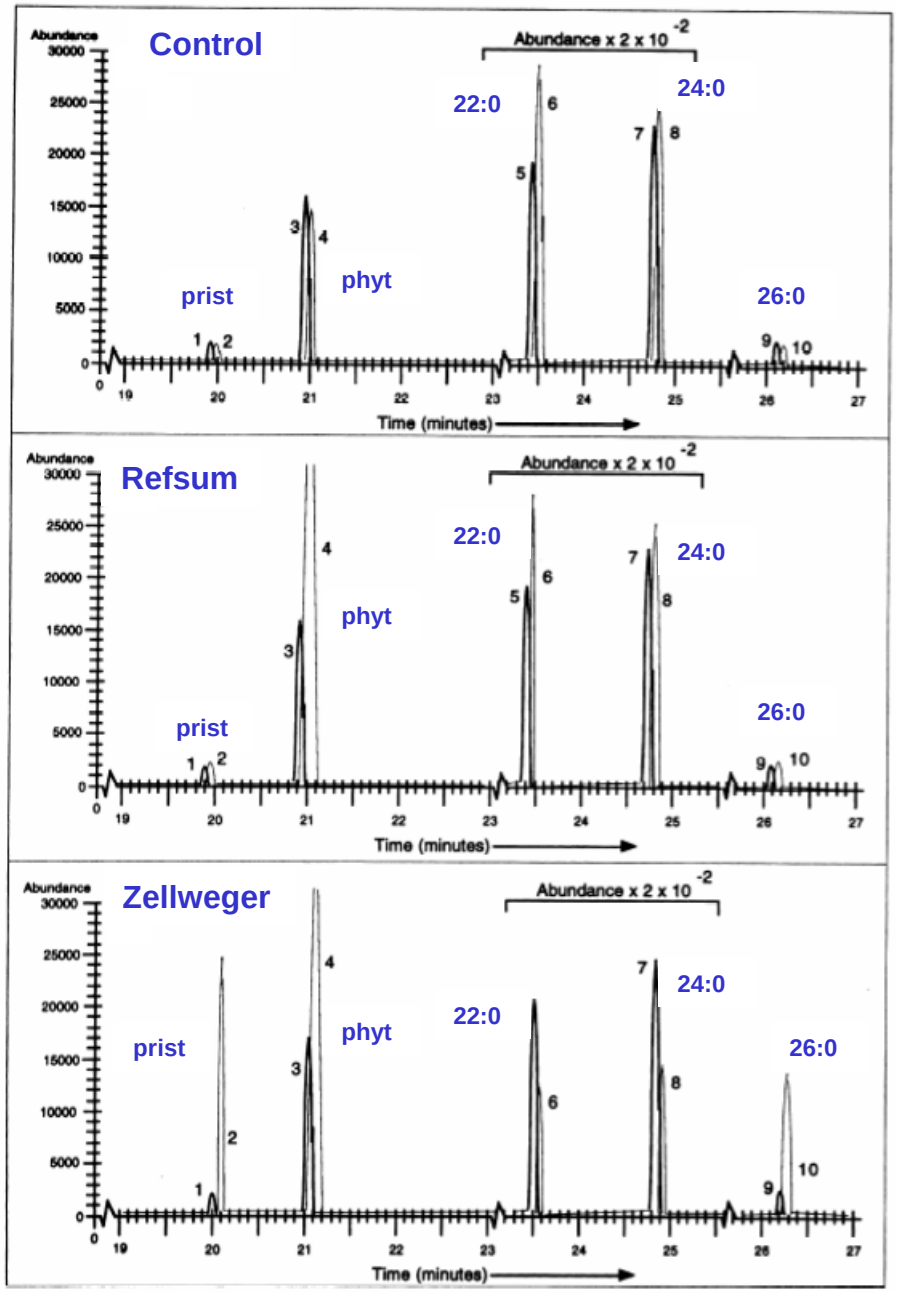
add – extract 4 ml hexane

↓

derivatise MTBSTFA

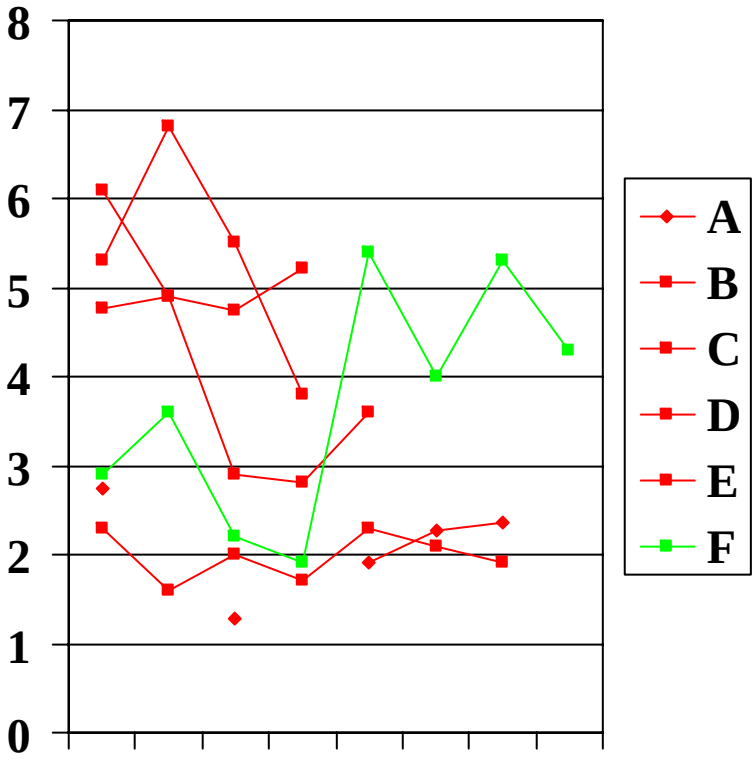
↓

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 C26 is 1.32 μmol/L; otherwise quite large variations occur and the most severely affected patients do not necessarily have the highest C26 values.

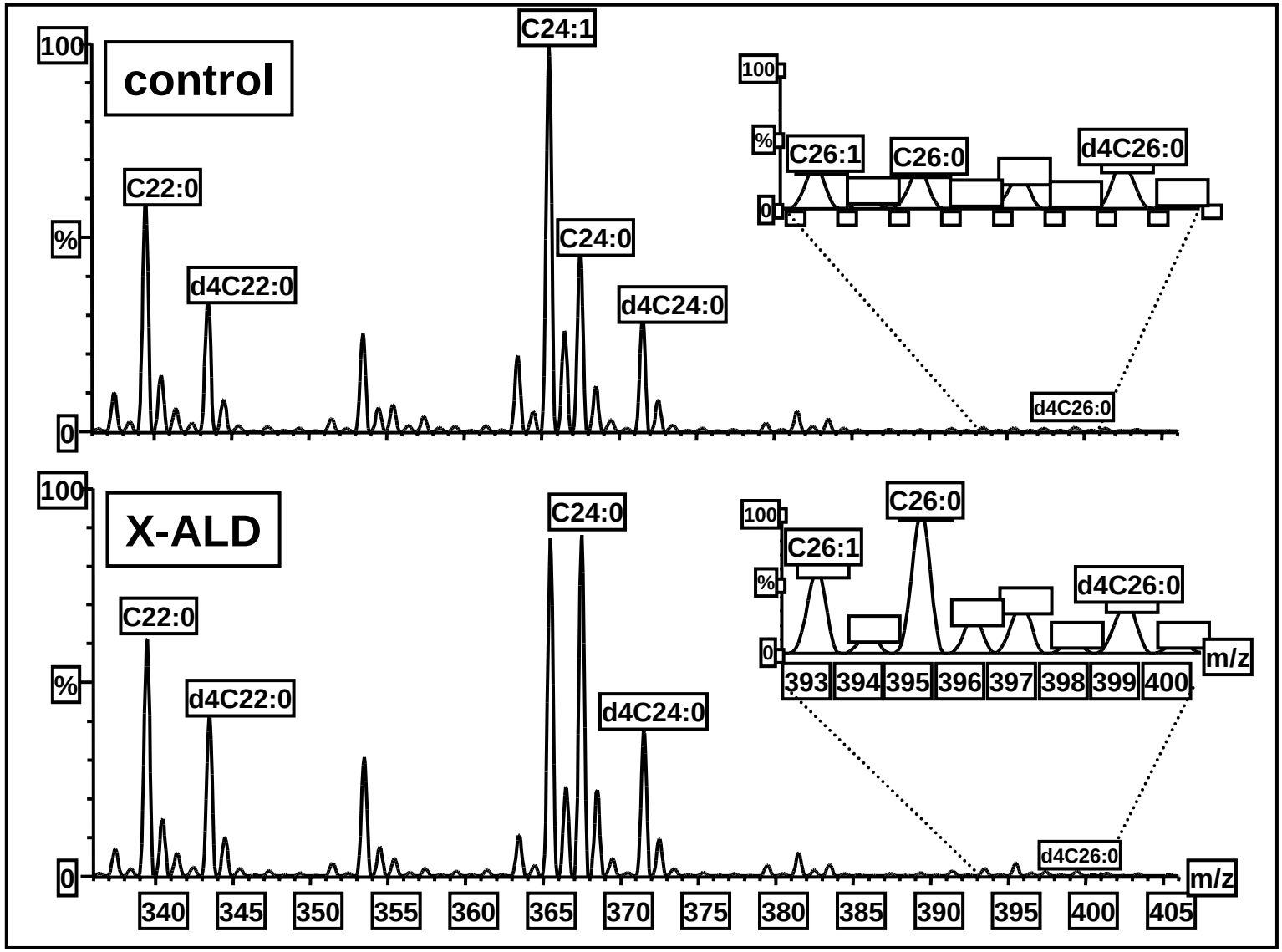
Electrospray ionization mass spectrometry of VLCFA (ESI-MS)

Procedure

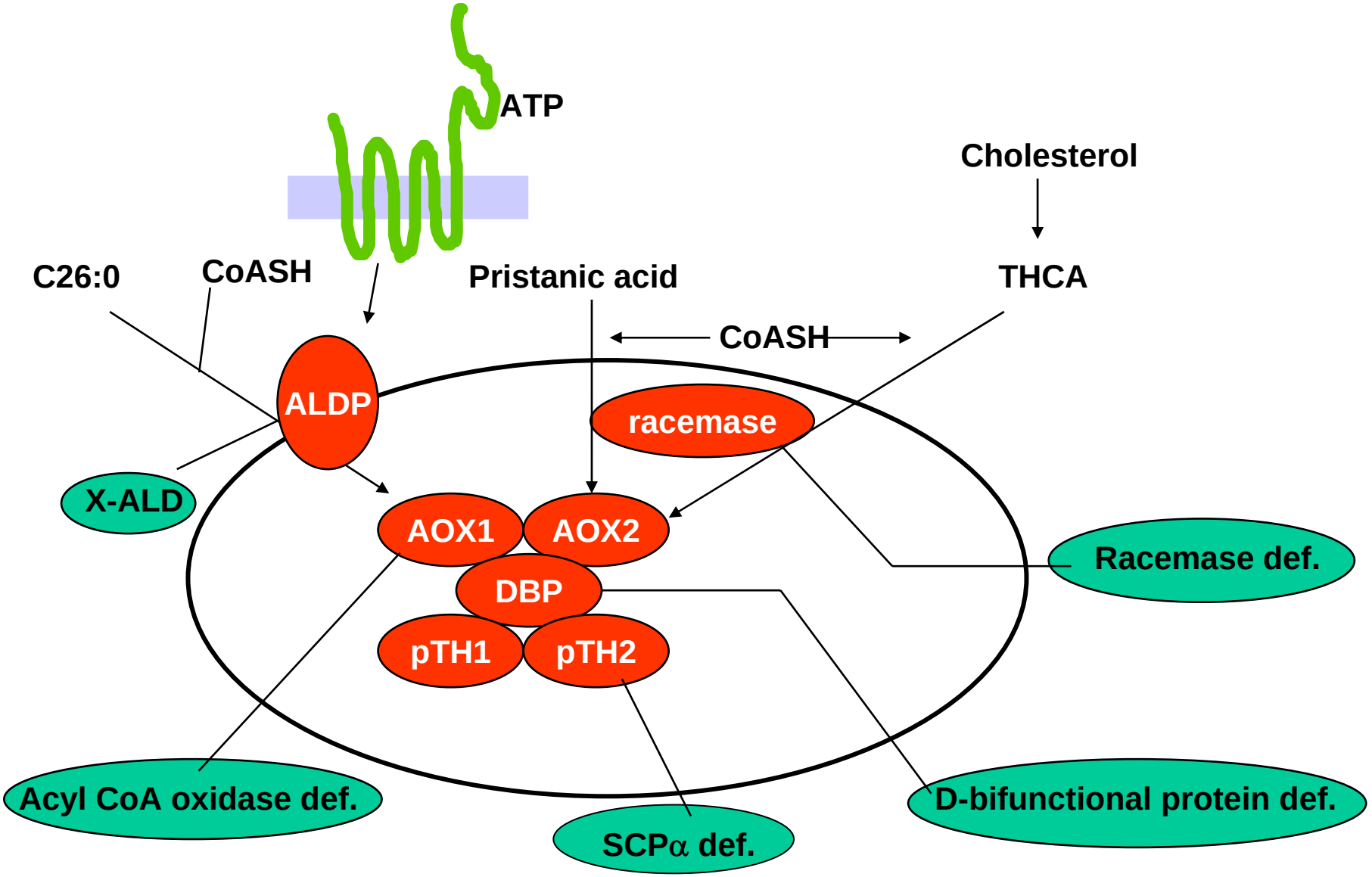
- 100 µl plasma + 100 µl IS ($^2H_4$-labeled C22:0, C24:0 and C26:0)
- hydrolysis with 1 ml acetonitrile / 37% HCl (4:1)
- incubate at 90ºC for 2 hours
- extract free fatty acids with hexane
- reconstitute in chloroform-methanol-water (50:45:5) + 0.01% ammonia
- analyze on mass spectrometer in negative ion mode

Valianpour et al, Mol Genet Metab, 2003

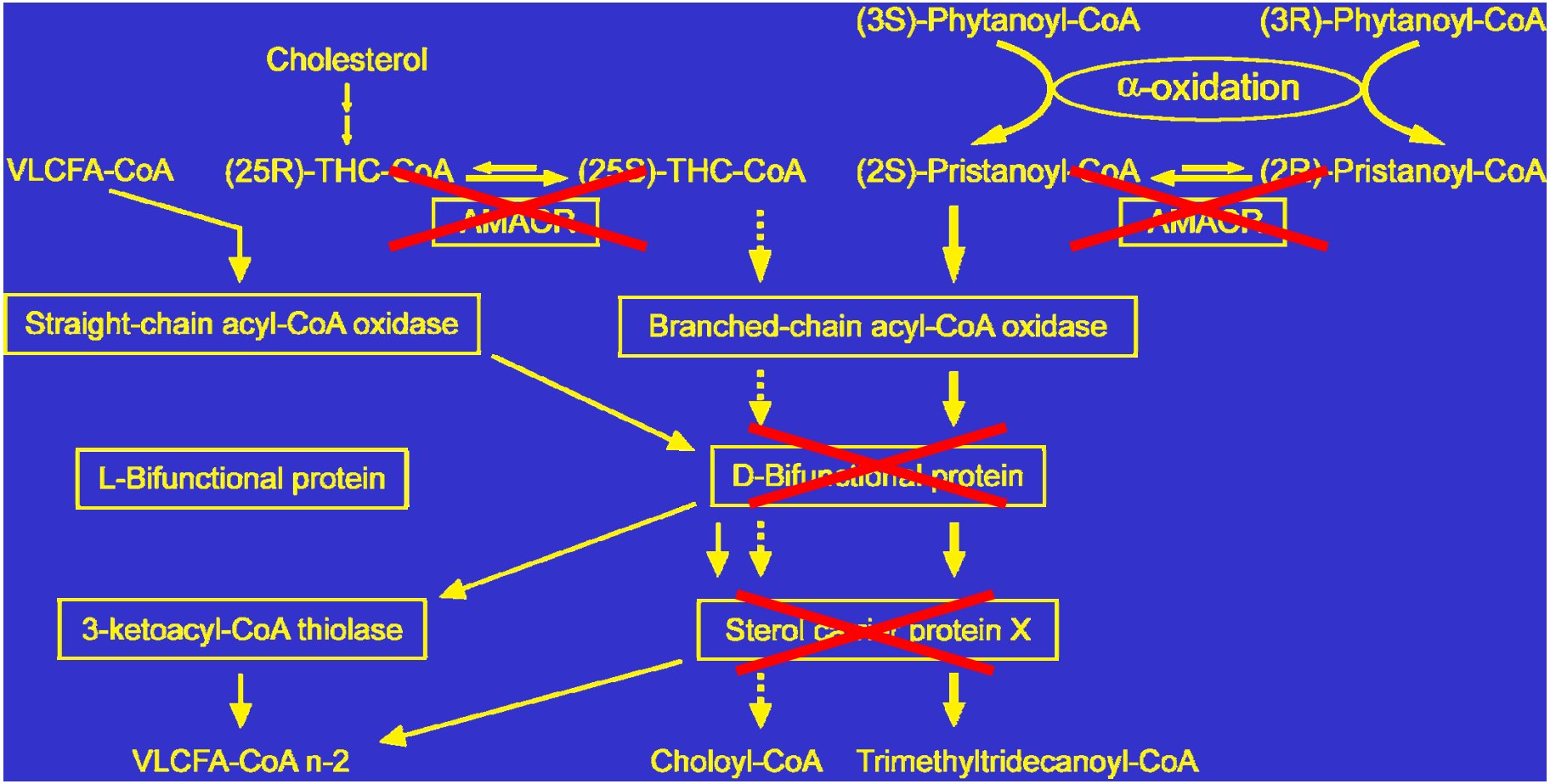
VLCFA analysis with ESI-MS



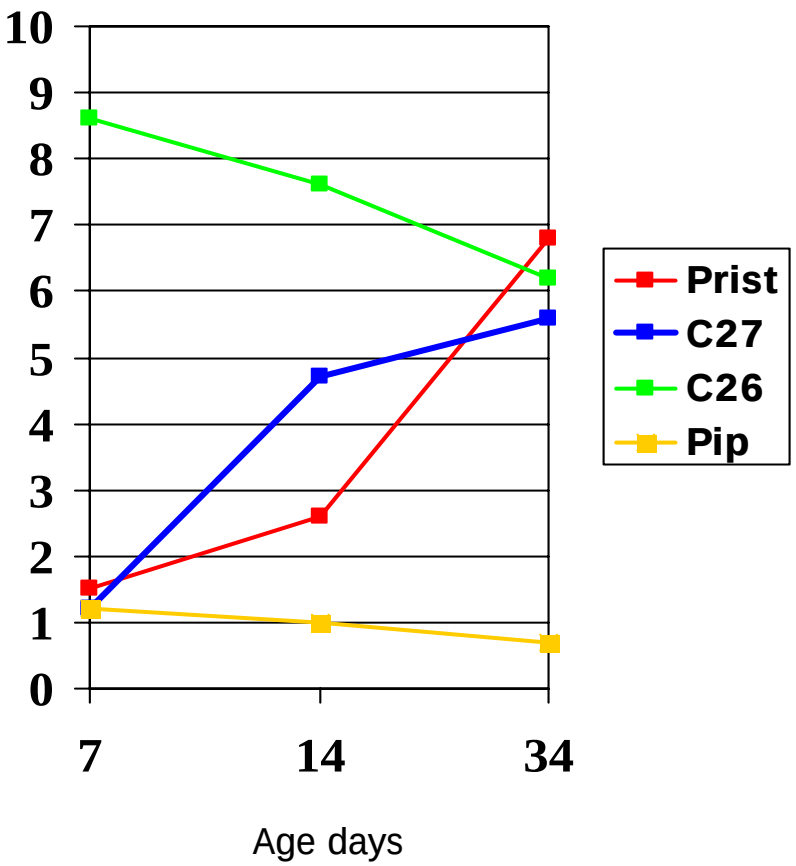
Peroxisomal Fatty Acid ß-oxidation in Human Peroxisomes and its Deficiency



Fatty acid oxidation in peroxisomes



Neonatal evolution of D-bifunctional protein deficiency



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 plasmalogens.

Peroxisomal Parameters

CSF

- Pipecolic acid

Plasma

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

Erythrocytes

- Plasmalogens DMA

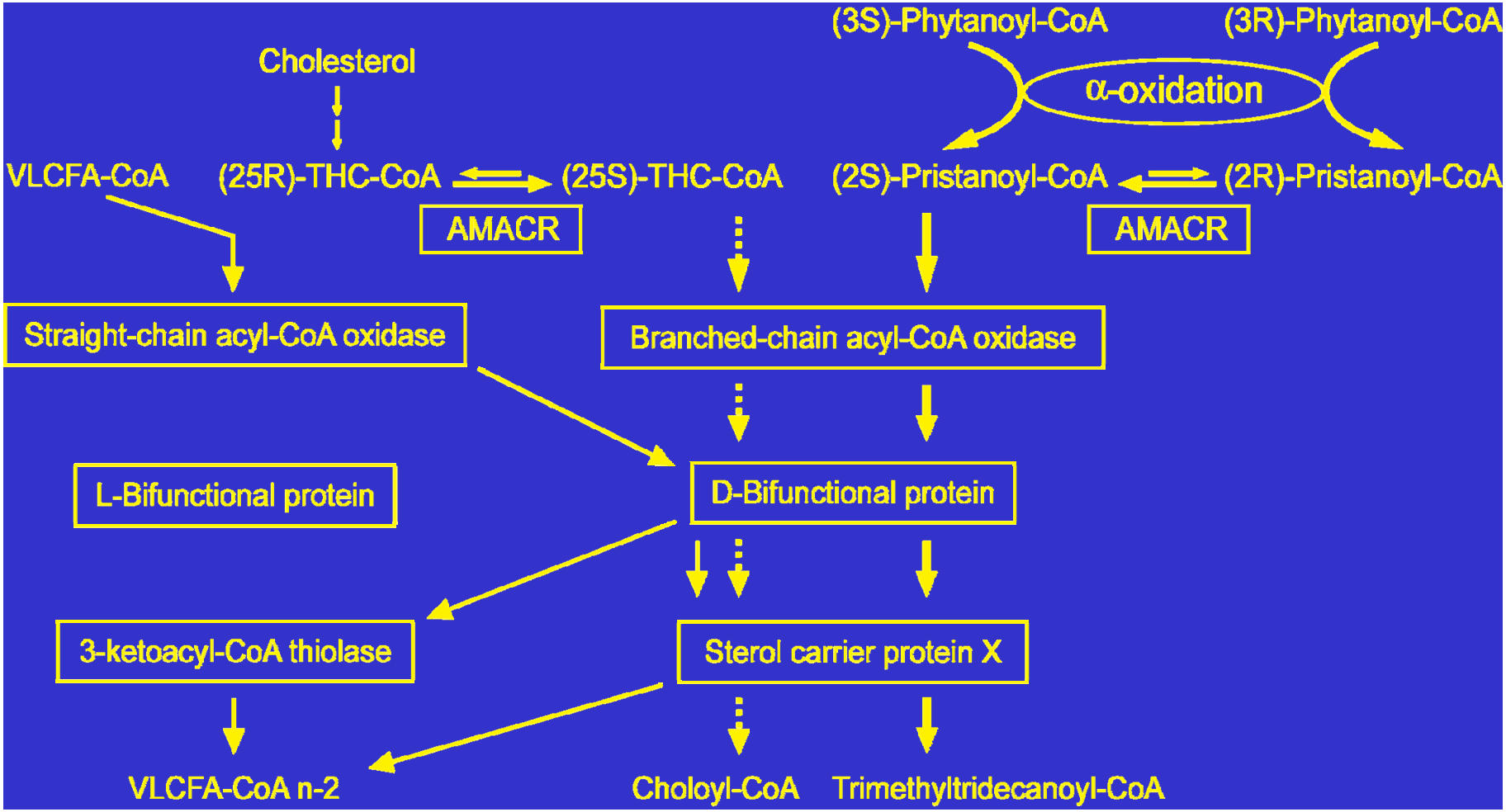
Bile

- C_{27}-bile acids

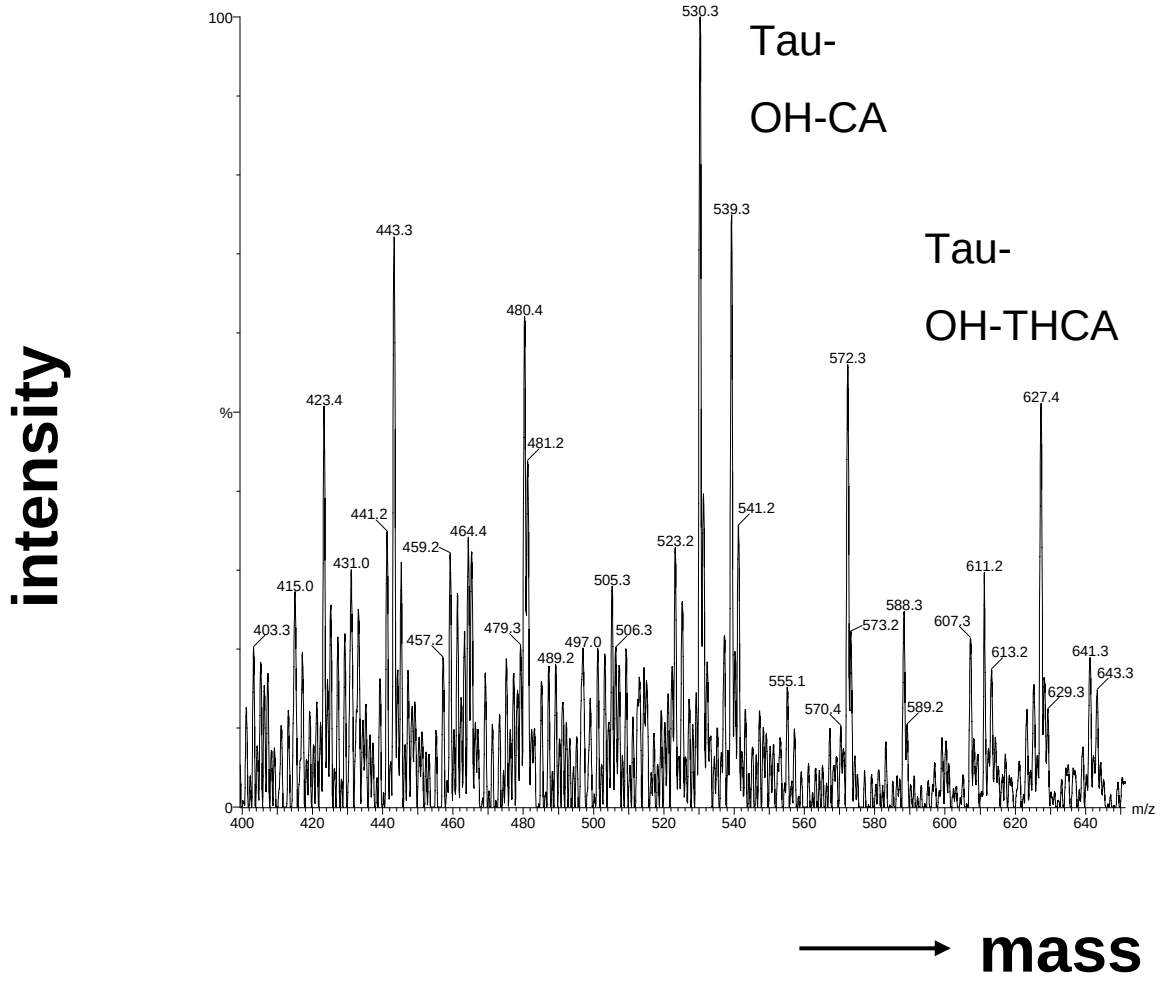
Urine

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

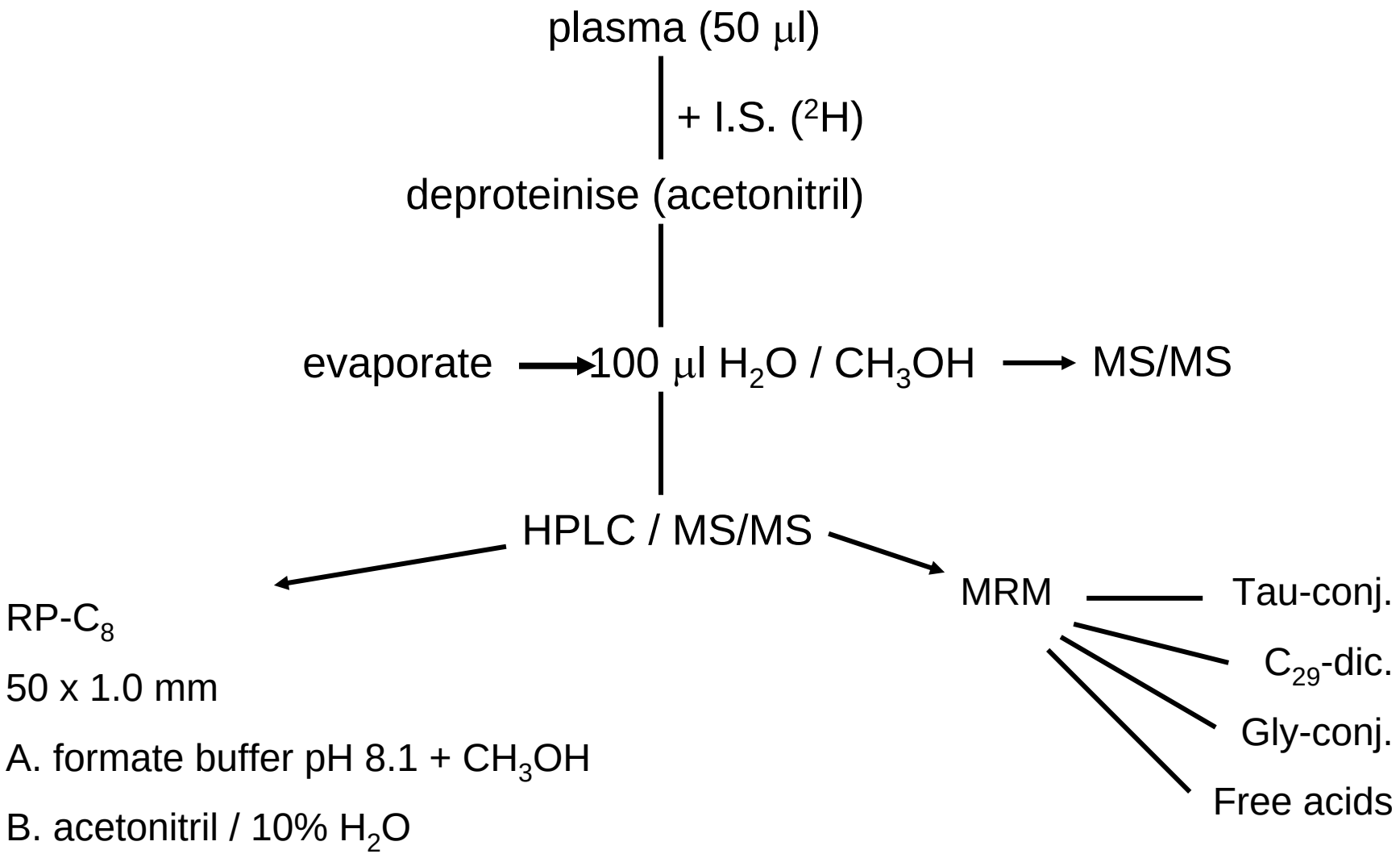
Fatty acid oxidation in peroxisomes



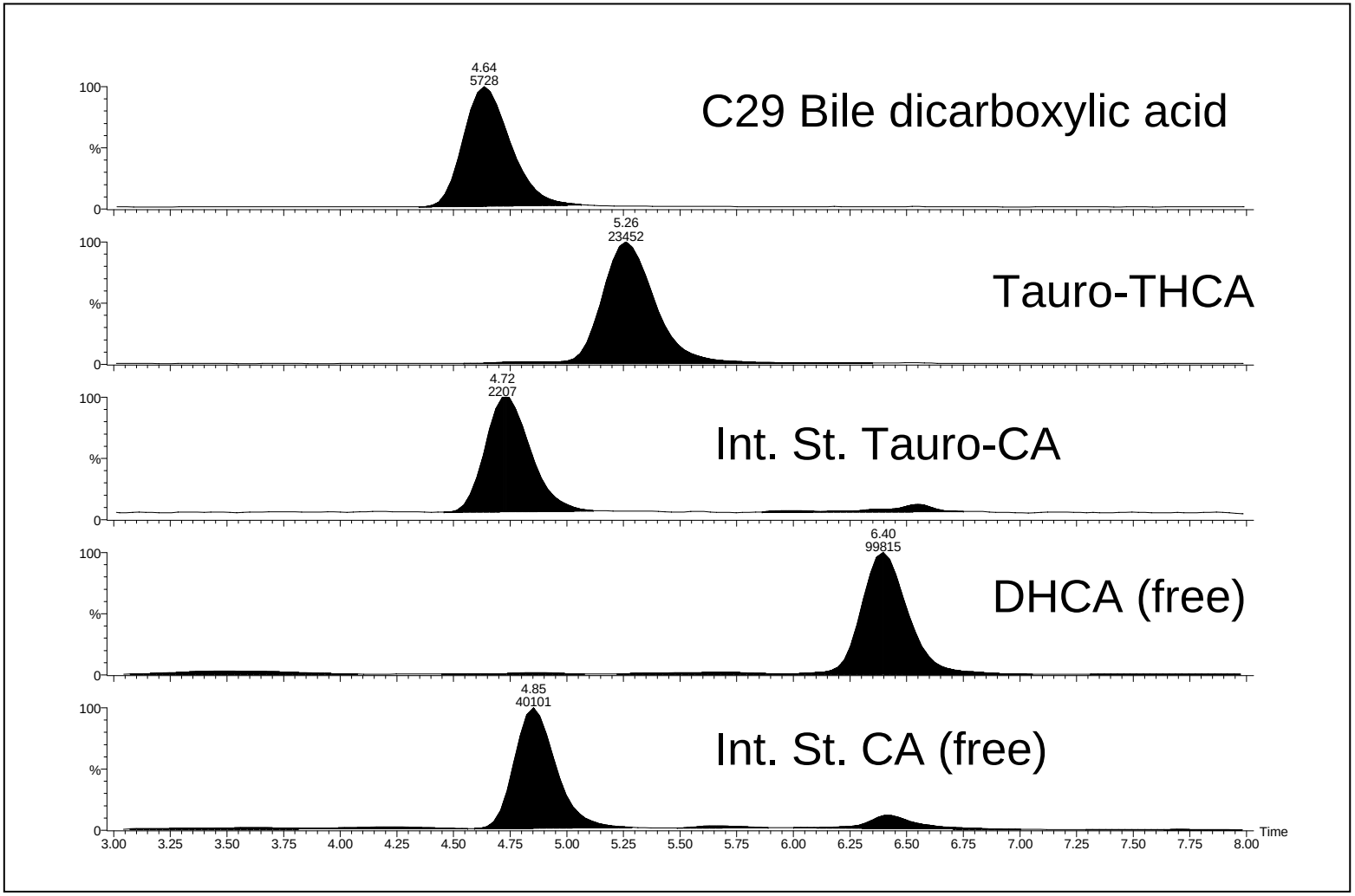
Peroxisome Biogenesis Defect
Bile Acids in urine (tandem-MS)



Analysis of Plasma C_{27}-bile acids



LC-MS/MS of plasma bile acids



Peroxisomal Parameters

CSF

- Pipecolic acid

Plasma

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

Erythrocytes

- Plasmalogens DMA

Bile

- C_{27}-bile acids

Urine

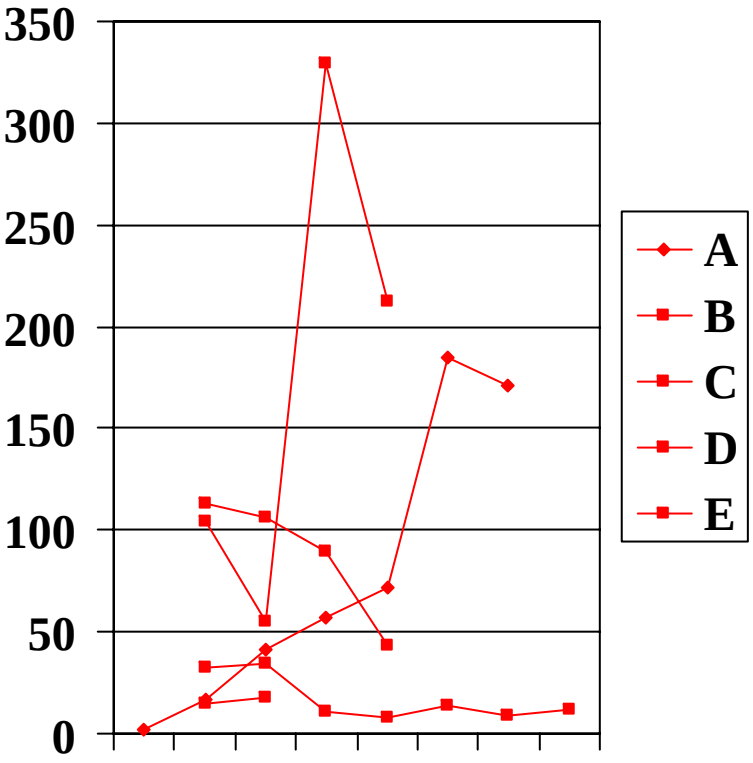
- C_{27}-bile acids
- Pipecolic acid
- Dicarboxylic acids
- Oxalic acid

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

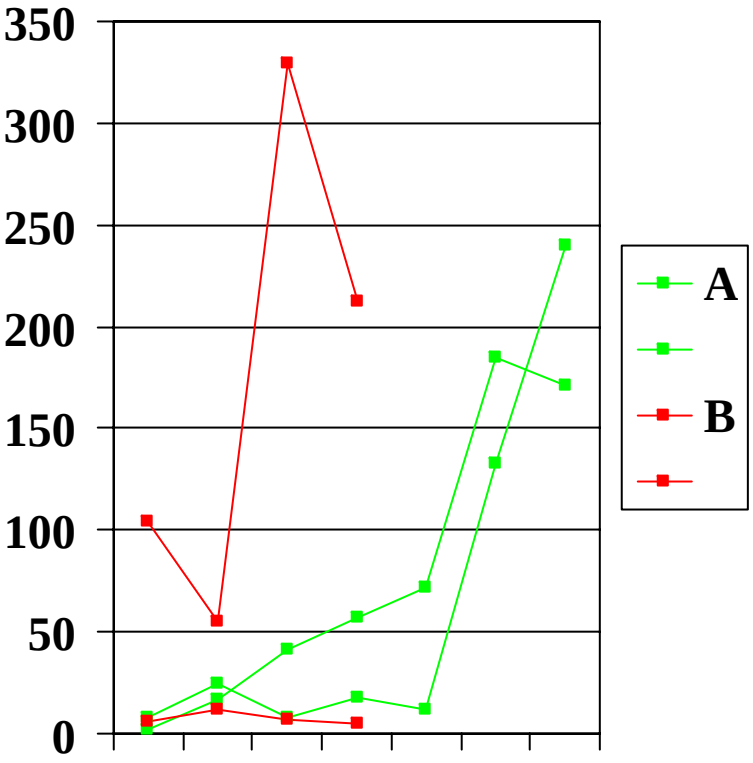
There is a wide variation of plasma Pipecolic acid in Zellweger



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 my be increased in

- Peroxisome biogenesis defects
- Vitamin B_6-responsive convulsions (antiquitin defects)
- Hyperlysinemias
- Hyperprolinemia type 2
- Unexplained conditions

but NOT in

- Isolated peroxisomal enzyme defects

Peroxisomal Parameters

CSF

- Pipecolic acid

Plasma

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

Erythrocytes

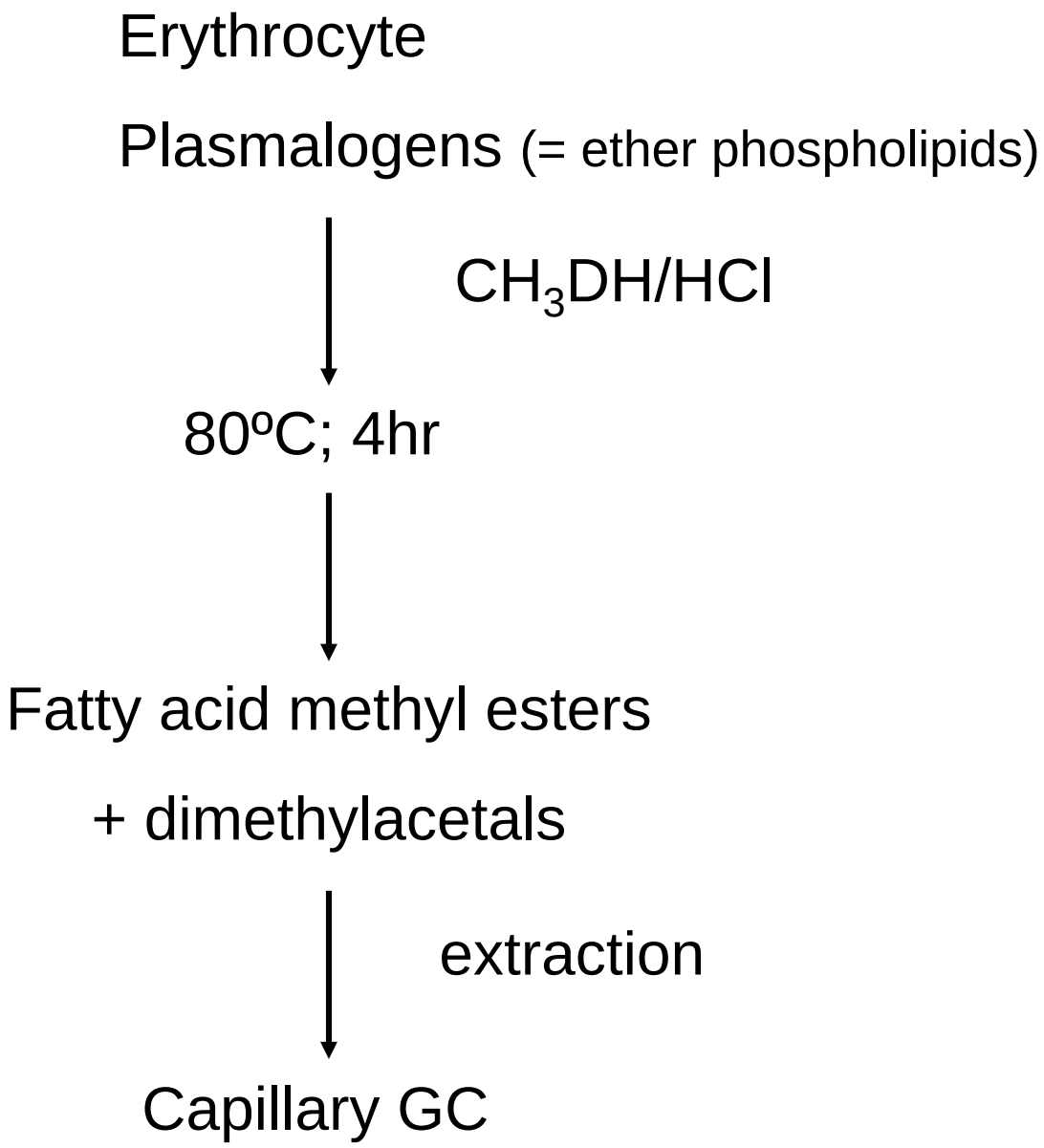
- Plasmalogens DMA

Bile

- C_{27}-bile acids

Urine

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


Erythrocyte
Plasmalogens (= ether phospholipids)
CH_3DH/HCl
80ºC; 4hr
Fatty acid methyl esters
+ dimethylacetals
extraction
Capillary GC

The PEX 7 spectrum

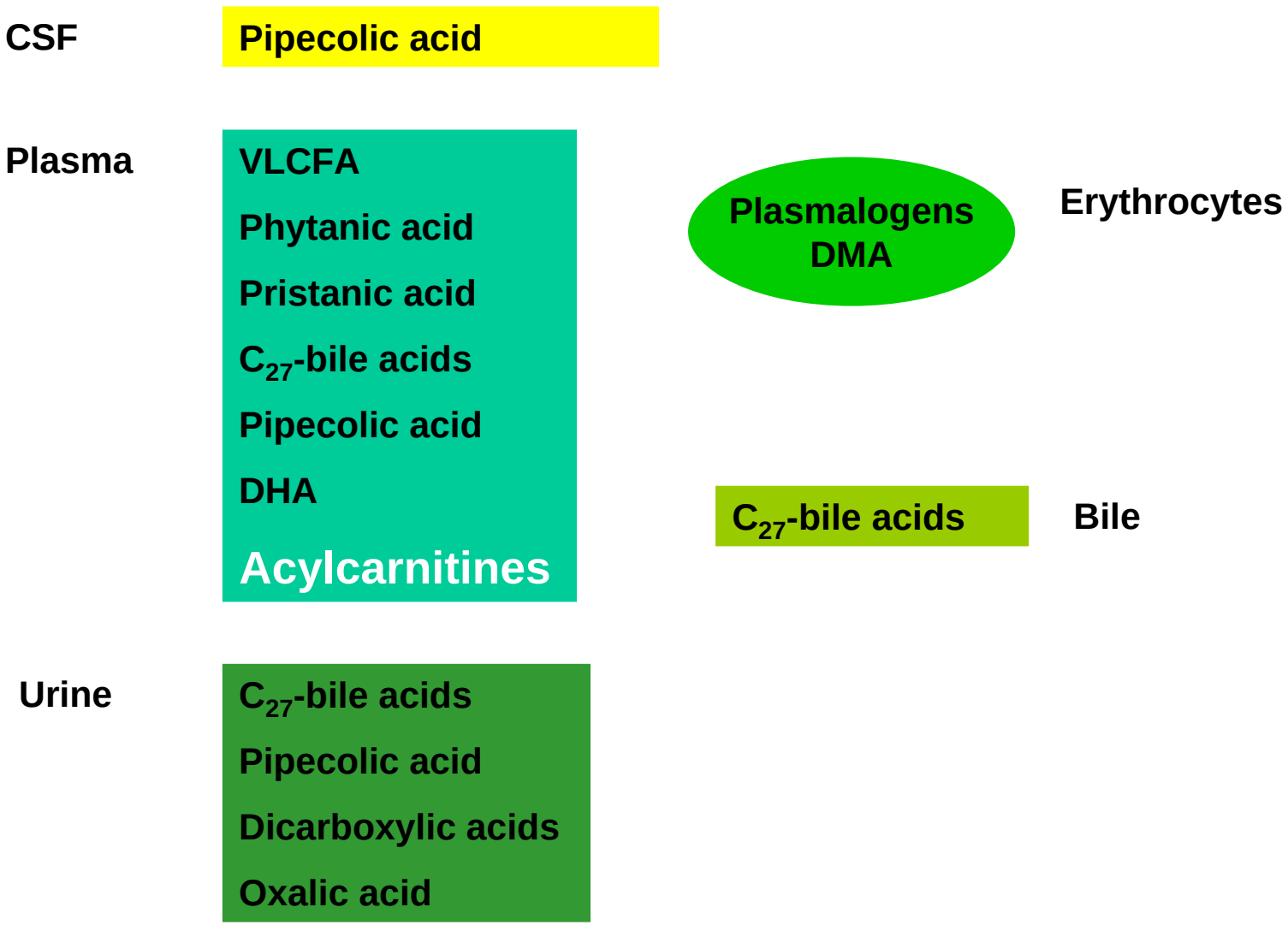
Clinical presentation	Biochemical presentation	
	Phytanic	Plasmalogens
Refsum	↑	N
MR + cataract	↑	↓-n
Severe RCDP	↑	↓

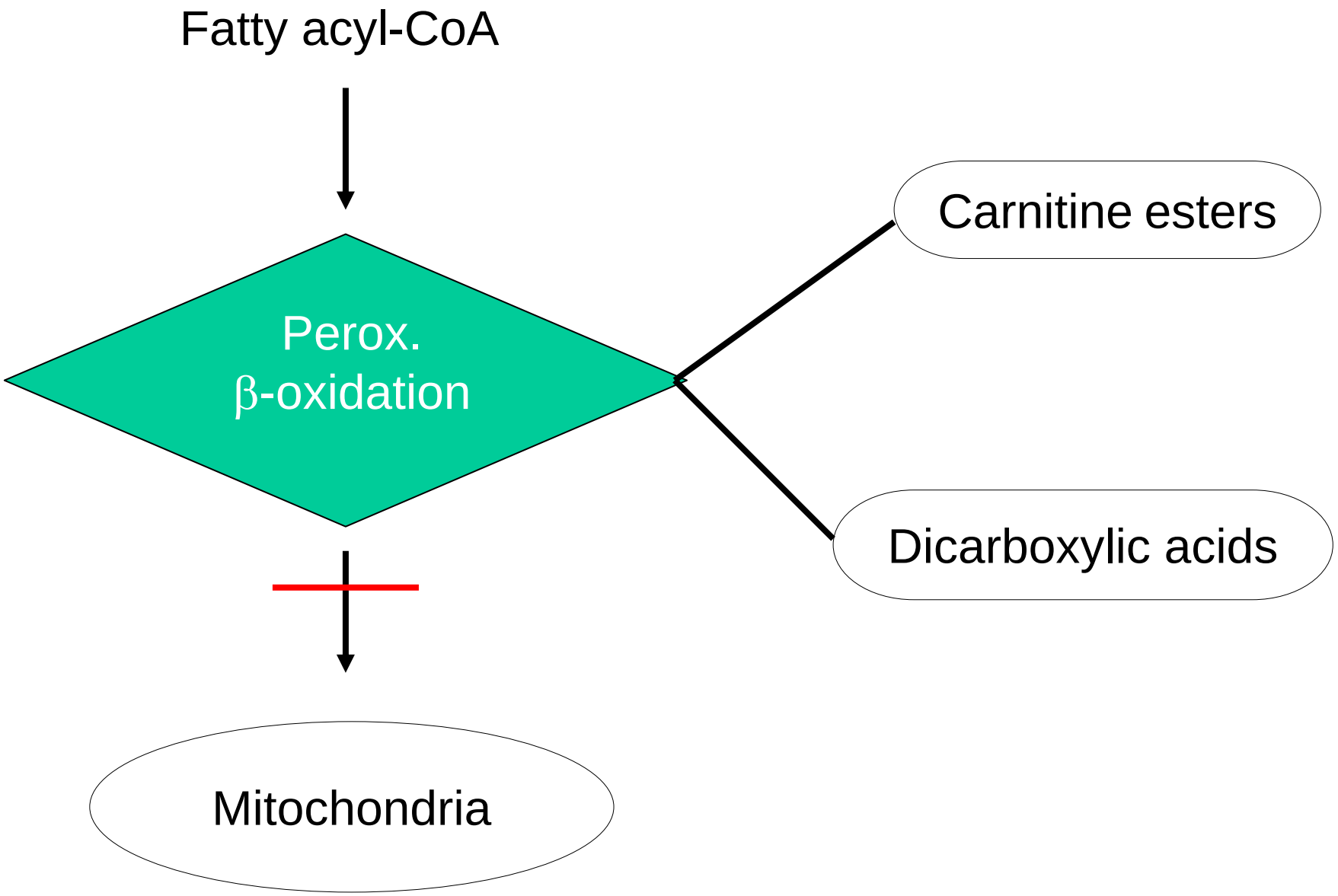
Early increase of Plasma Phytanic acid in RCDP-patients

Patient	Age	Phytanic acid (μmol/L)
1	1 day	0.7
2	3 days	5.8
3	1 week	4.4
4	2 weeks	9.9
5	2 weeks	13.2
6	3 weeks	9.3
Controls *	0-4 months	0.04-5.3

H.J. ten Brink et al – J Lipid Res, 1992

Peroxisomal Parameters




Fatty acyl-CoA
Perox.
β-oxidation
Carnitine esters
Dicarboxylic acids
Mitochondria

Analysis of Acylcarnitines

plasma (50μl)

↓

add internal standards (^{2}H-labelled)

↓

deproteinise / extract with acetonitril

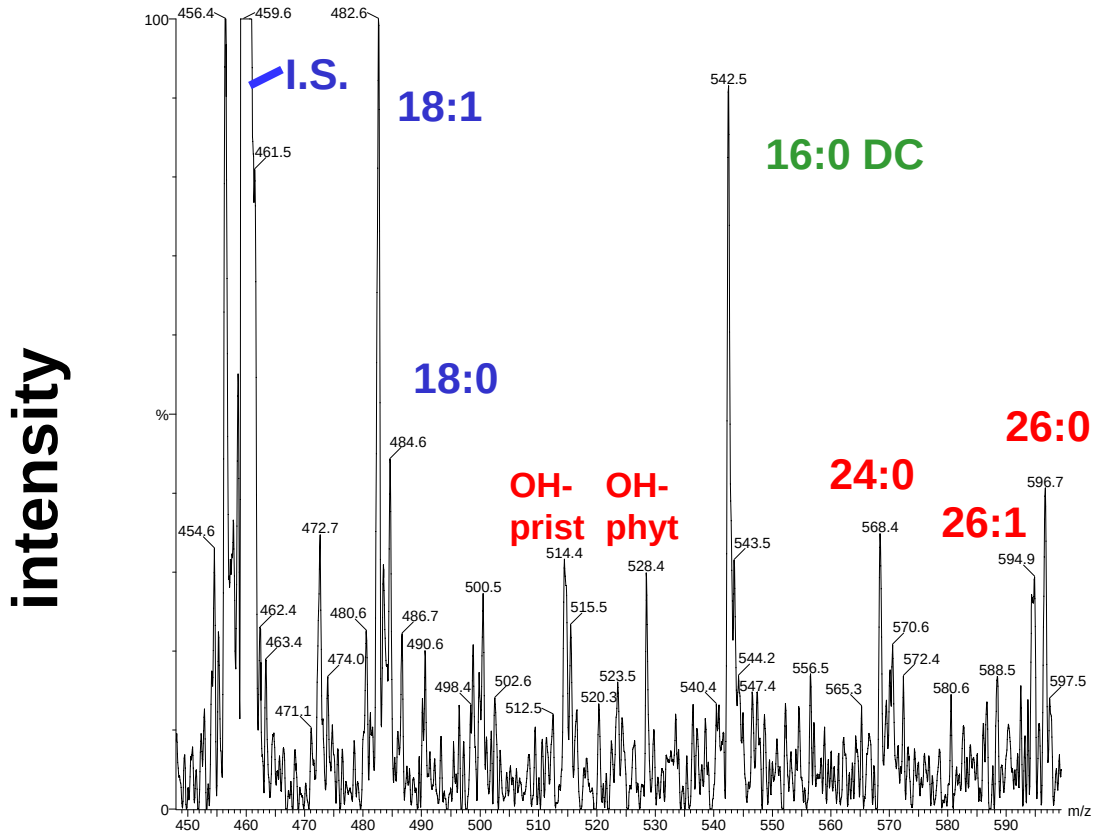
↓

make butyl esters (butanol /acetyl chloride)

↓

electrospray MS/MS (parents of 85)

Peroxisome Biogenesis Defect
Acylcarnitines in plasma (tandem-MS)



mass

Peroxisomal Parameters

CSF

- Pipecolic acid

Plasma

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

Erythrocytes

- Plasmalogens
- DMA

Bile

- C_{27}-bile acids

Urine

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

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. ω-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

S.H. Korman et al. – J. Inherit. Metab. Dis. 2000; 23:425-428

Characteristic Biochemical Genetic Findings in the Various Disorders of Peroxisomal Function

Disorder	C_{24} + C_{26} fatty acid	C_{27} bile acid	Phytanic acid	Pristanic acid	Pipecolic acid	Plasmalogens (ery's)
PBD severe	↑	↑	↑	↑	↑	↓
PBD mild	↑	↑	↑	↑	↑	n
RCDP	n	n	↑	↓	n	↓
Bifunctional protein	↑	↑	↑	↑	n	n
X-ALD male	↑	n	n	n	n	n
X-ALD female	n- ↑	n	n	n	n	n
Refsum	n	n	↑↑	↓	n	n
Racemase	n	↑	n- ↑	↑	n	n

Follow-up studies for the confirmation of peroxisomal disease

1. Fibroblast studies

   - β-oxidation of VLCFA / pristanic acid
   - α-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. <u>One</u> patient had normal plasma parameters, but fibro’s were diagnostic.