THE ERNDIM SPECIAL ASSAYS SCHEME

PROFITS, PITFALLS, DRAWBACKS AND EXPECTATIONS

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ERNDIM special assays

- Because multi-component analysis is not enough
- Keeps track of novel developments
- Gives both analytical and diagnostic confidence
- Serves urine as well as plasma

The Quantitive ERNDIM schemes

	Nr. participants
• Amino acids	193
• Organic acids	66
• Purines / pyrimidines	49
• Special assays serum	151
• Special assays urine	149

Available test for the basic biochemical screening of inborn errors of metabolism

	Test	U	P	CSF		Test	U	P	CSF
1	Amino acids	X	X	X	12	Polyols	X		
2	Organic acids	X			13	Sialotransferrins		X	
3	Acylcarnitines	X	X		14	Sterols		X	
4	Purines pyrimidines (orotic acid)	X			15	Anions (thiosulfate)	X		
5	Oligosaccharides	X			16	Creatine-guanidine-acetate	X	X	X
6	Mucopolysaccharides	X			17	Neurotransmitters	X		X
7	Sialic acid	X			18	Pterins	X		X
8	Homocysteine		X		19	Methyltetrahydrofolate			X
9	Very long-chain fatty acids		X		20	Polyunsaturated fatty acids*		X	
10	Phytanic / pristanic acid		X		21	Sulfatides	X		
11	Bile acids / alcohol				22	Plasmalogens*			

Special assays Serum

2000	No	2006/2007	No
Lactic acid	57	Lactic acid	105
Pyruvic acid	46	Pyruvic acid	91
3-OH-butyric acid	39	3-OH-butyric acid	91
Acetoacetic acid	28		
Carnitine free	53	Carnitine free	91
Carnitine total	46		
Cis-4-decenoic acid	7		
C22:0	32	C22:0	64
C24:0	32	C24:0	64
C26:0	33	C26:0	65
Phytanic acid	32	Phytanic acid	58
Phenylalanine	45	Pristanic acid	30*
		Pipecolic acid	28
Homocysteine	64	Homocysteine	105
7-Dehydrocholesterol	18	7-Dehydrocholesterol	44
Uric acid	34	Creatine	31
		Gyanidinoacetic acid	29
		Galactose	6*
		Methylmalonic acid	34*

Special assays Urine Participants

	2000	2006		
Lactic acid	33	62		
3-OH-butyric acid	26			
Carnitine free	39	60		
Carnitine total	36			
5-HIAA	19	24		
Homovanillic acid	27	37		
Hydroxyproline	25	27		
Guanidinoacetic acid	5	43	Creatine	42
Mucopolysaccharides	46	82		
Sialic acid	11	20		
Pyroglutamic acid	20			
Succinylacetone	17	49		
Orotic acid	45	93		
Orotidine	120			
Uracil	27			
Uric acid	38	56		
			Pipecolic acid	17

DO THE ERNDIM ANALYTE LEVELS MIMIC THE EXPECTED PATIENT LEVELS?

AN EXAMPLE

- Patient K, a girl, was referred to a local hospital at the age of 4 months because of marked hypotonia, a rather slow development, and absence of eye contact.

SELECTIVE SCREENING OF INBORN ERRORS COMPRISED THE FOLLOWING

- URINE
- Amino acids
- Organic acids
- Phenolic acids
- Purines/pyrimidines
- Oligosaccharides
- Mucopolysaccharides

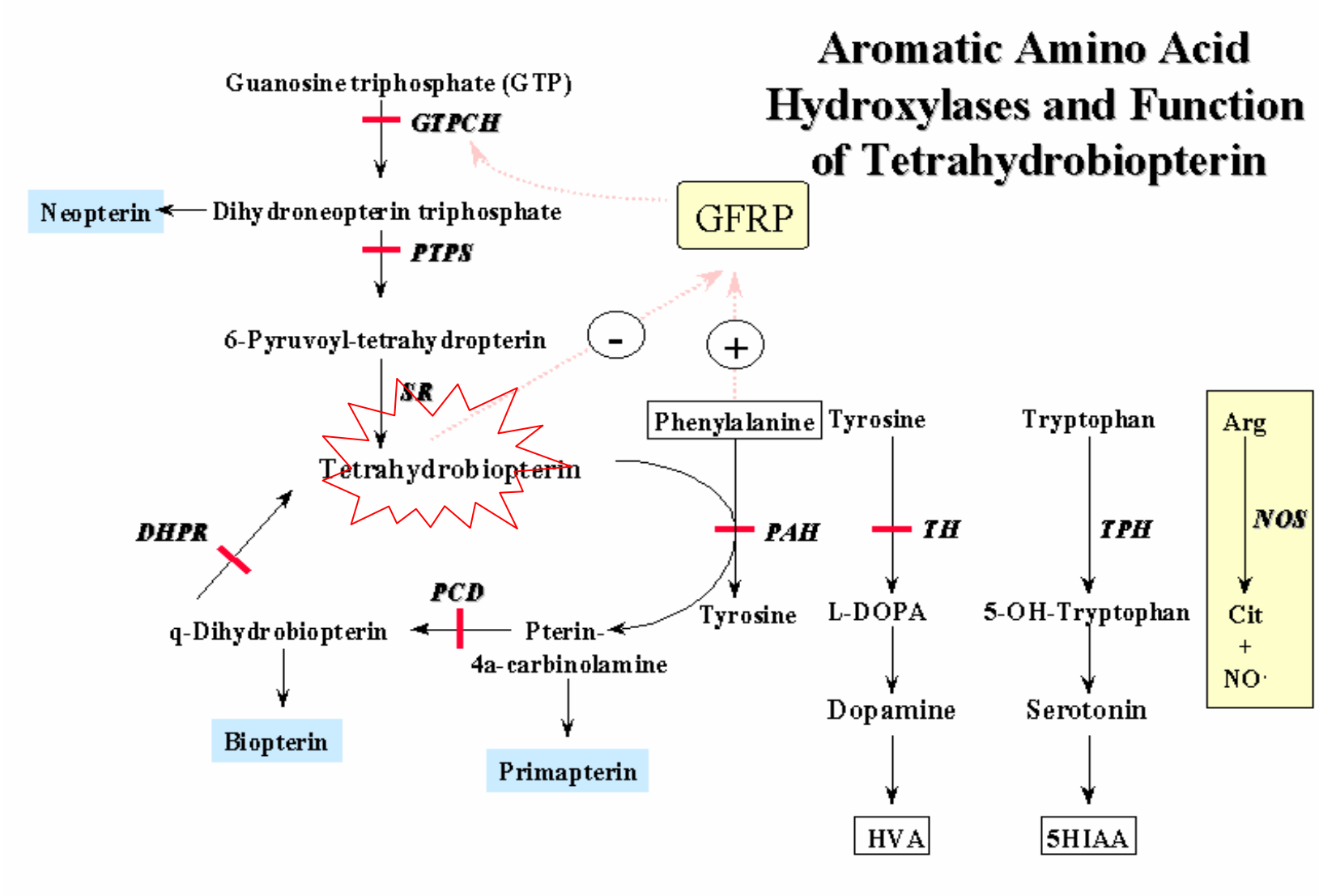
- PLASMA
- Amino acids
- Acylcarnitines
- VLCFA
- Phytanic/Pristanic acid

AN EXAMPLE

Phenolic acids in the urine (mmol/mol creat.)

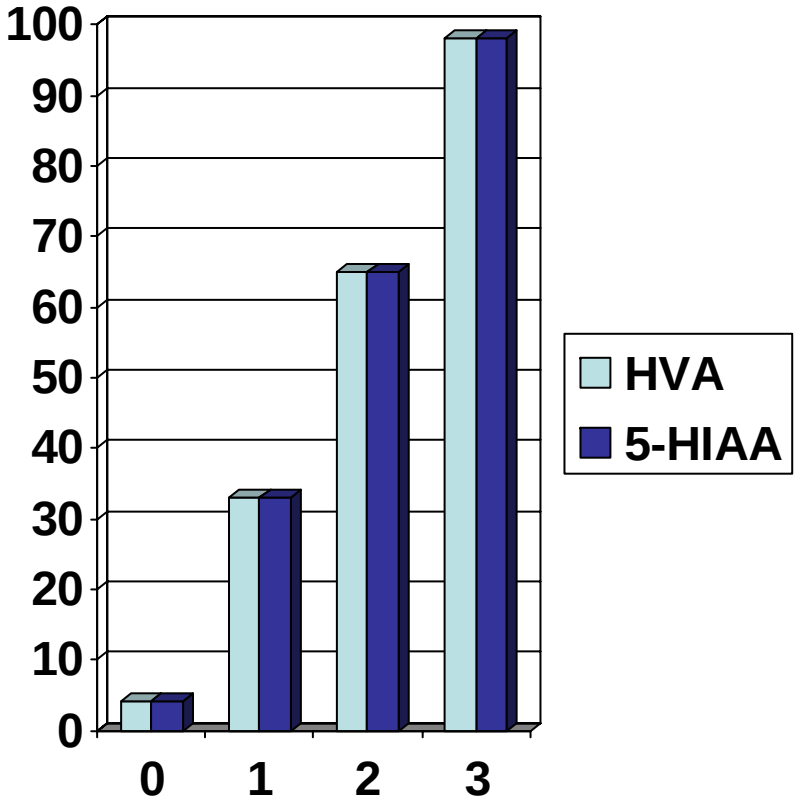
Analyte	Patient K	Controls
Homovanillic acid	<3	2.7-19.9
5-HIAA	4.8	2.2-20.0
Vanillactic acid	n.d.	n.d.

Creatinine in this urine was 1.1 mmol/l



Diagnosis: tyrosine hydroxylase deficiency

PHENOLIC ACIDS IN THE SPECIAL ASSAYS URINE PROGRAM



- The levels of HVA and 5-HIAA are depicted. Decreased neurotransmitter formation in patients usually results in levels less than 10 µmol/l.
- Accordingly, the scheme may be adjusted to accommodate lowered neurotransmitter levels.

WHY DO WE NEED NEW ANALYTES?

Is there a need for pristanic acid in addition to phytanic acid?

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

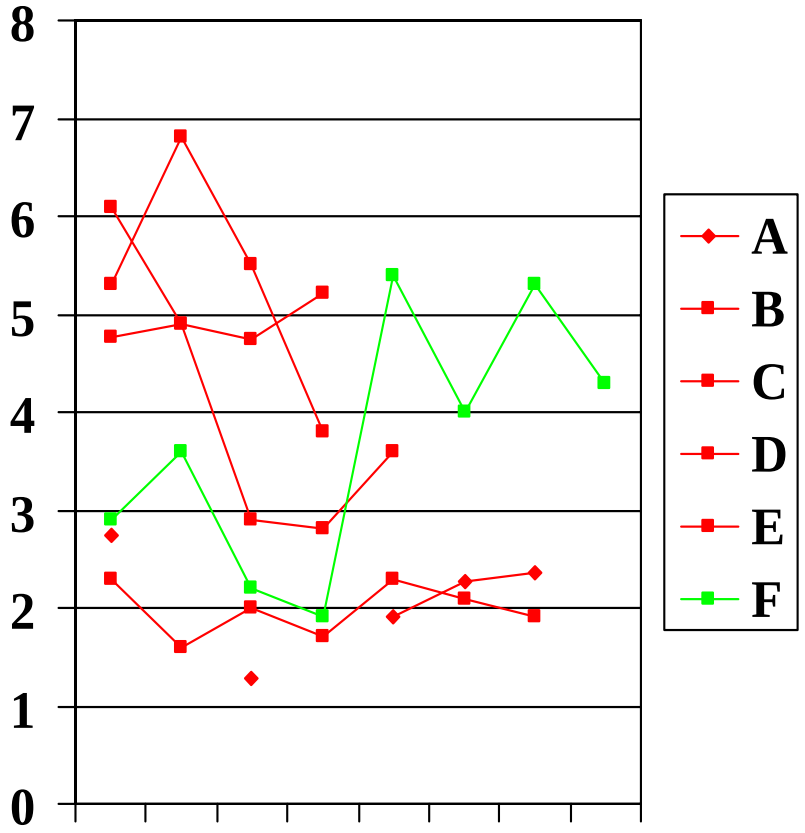
Bile

- C_{27}-bile acids

The VLCFA levels in ERNDIM-SA in relation to the reference range of healthy controls

	C22	C24	C26
Native	60	51	0.62
Add 1	75	73.5	3.92
Add 2	90	106	7.22
Add 3	105	128.5	10.52
Reference	40-119	33-84	0.45-1.32

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.

Characteristic basic biochemical findings in the various disorders of peroxisomal function

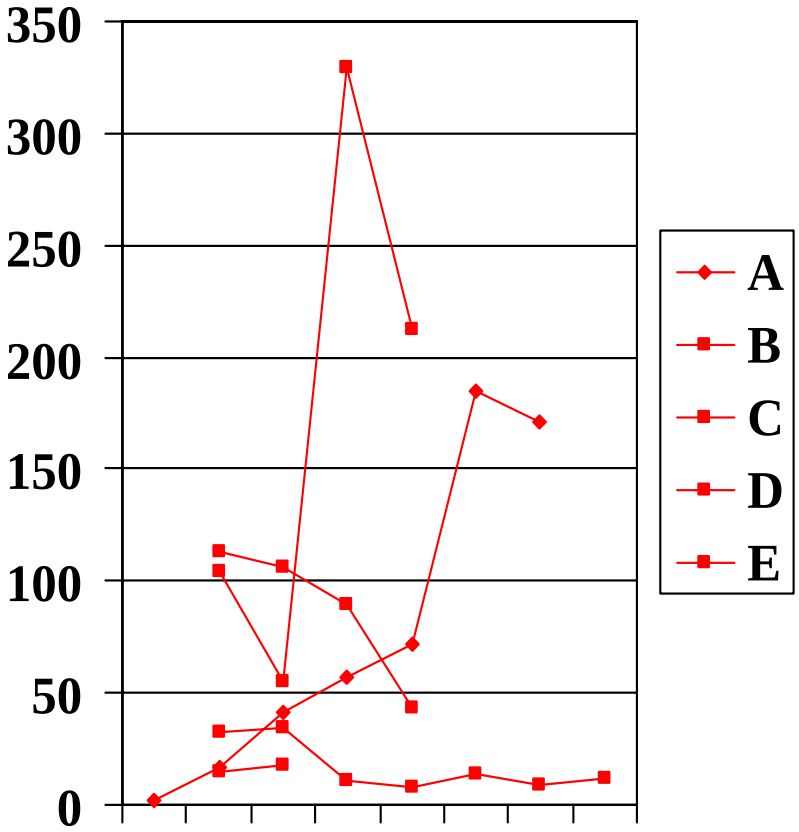
Disorder	VLCFA	C27 bile acids	Phytanic acid	Pristanic acid	Pipecolic acid	Plasmalogens (ery's)
PBD severe	↑	↑	↑	↑	↑	↓
PBD mild	↑	↑	↑	↑	↑	n
RCDP	n	n	↑	↓	n	↓
RCDP mild	n	n	↑	↓	n	↓-n
Bifunctional protein	↑	↑	↑	↑	n	n
X-ALD male	↑	n	n	n	n	n
X-ALD female	n-↑	n	n	n	n	n
Refsum	n	n	↑ ↑	↓	n	n
Racemase	n	↑	n- ↑	↑	n	n
SCPx	n	n-↑	n- ↑	↑	n	n
Acyl-CoA oxidase def.	↑	n	n	n	n	n

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.

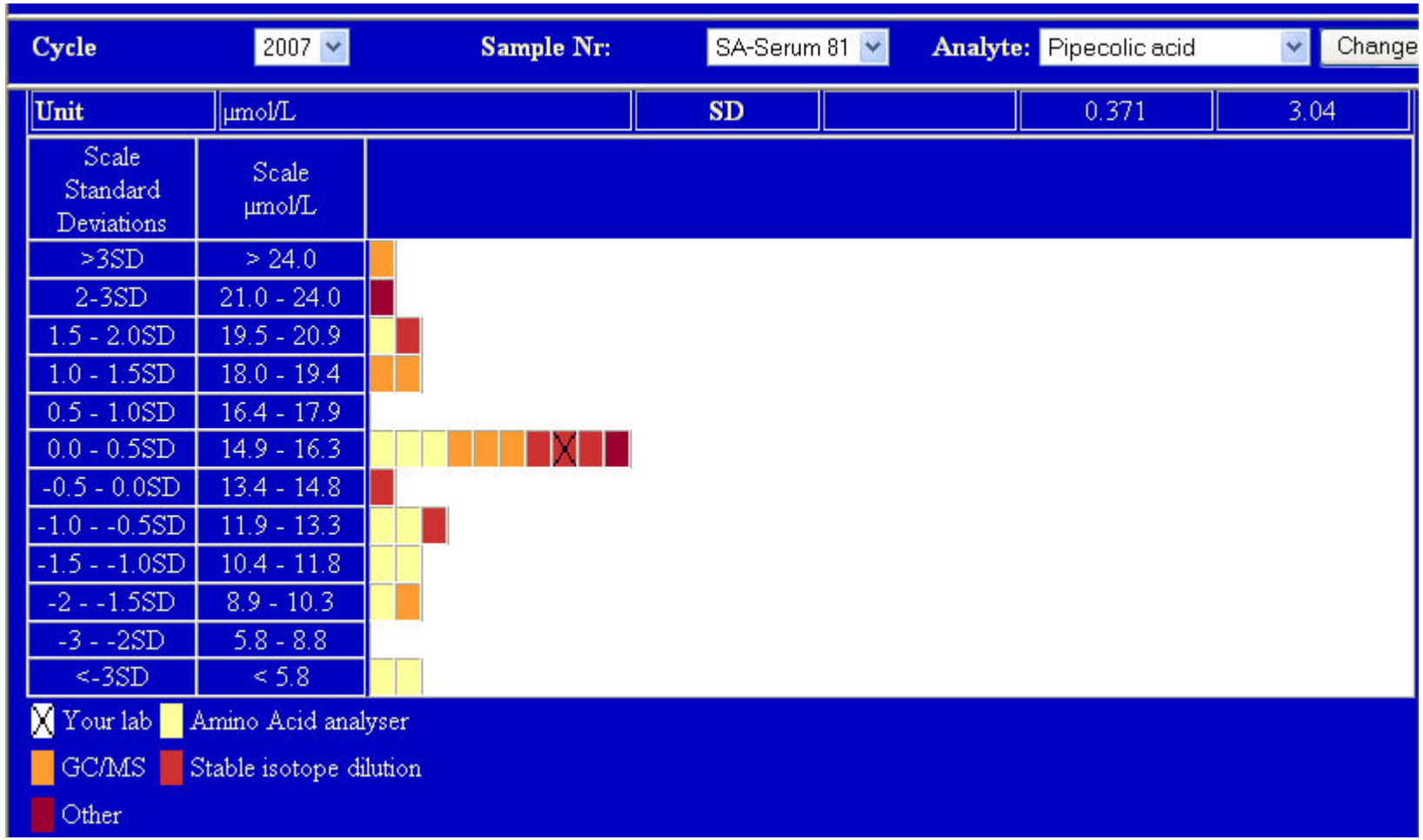
Pipecolic acid my be increased in

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

but NOT in

- Isolated peroxisomal enzyme defects

PIPECOLIC ACID IN PLASMA



A wide dispersion of data was observed, especially in the AAA results. Tandem MS is a promising technique in this area; it allows a rapid diagnosis of the antiquitin defect.

Adult Peroxisomal Disease

In addition to adrenoleukodystrophy and Refsum disease there are now novel disorders:

- Peroxisomal racemase deficiency
- Sterol carrier protein X (SCPx) deficiency ('thiolase')
- Acyl-CoA oxidase deficiency

Patient

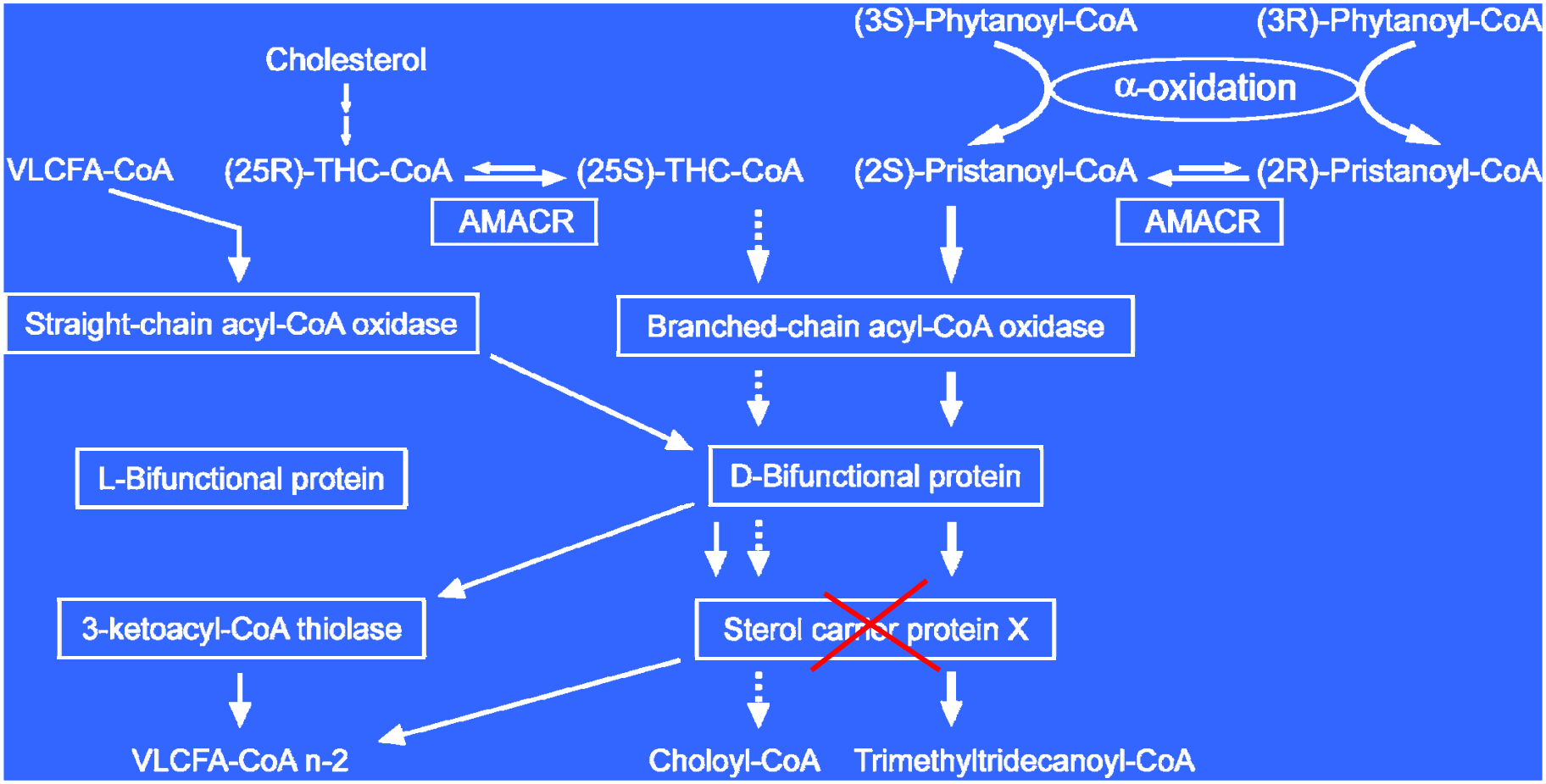
CLINICAL SYMPTOMS

- 45-year-old Caucasian male
- One healthy brother, one brother with similar symptoms
- Age 17: spasmodic torticollis with dystonic head tremor
- Age 29: azoospermia and hypergonadotrophic hypogonadism
- Age 44:
    - Hyposmia, hypoacusis, nystagmus
    - No abnormalities upon ophthalmologic investigations
    - MRI: leukencephalopathy with involvement of thalamus and pons
    - NVC of lower extremities: motor and sensory neuropathy
    - Slight cerebellar ataxia

Biochemical characterization (I)

	Patient	Control subjects	
		Median + IQR	Range
Plasma			
Phytanic acid	10.1	2.4 ± 3.0	0-9
Pristanic acid	**39.8**	**0.2 ± 0.3**	**0-3.1**
DHCA	0.1	0 ± 0	0-0.13
THCA	0.1	0 ± 0	0-0.09
C26:0	1.34	0.74 ± 0.26	0.46-1.31
Fibroblasts			
C26:0	0.31	0.29 ± 0.12	0.18-0.38
C26:0 β-oxidation	1,048	1,438 ± 484	1,025-2,994
Pristanic acid β-oxidation	131	1,402 ± 533	691-2,178
Phytanic acid α-oxidation	46	58 ± 22	32-173

β-oxidation in peroxisomes



Sterol carrier protein X - deficient patient

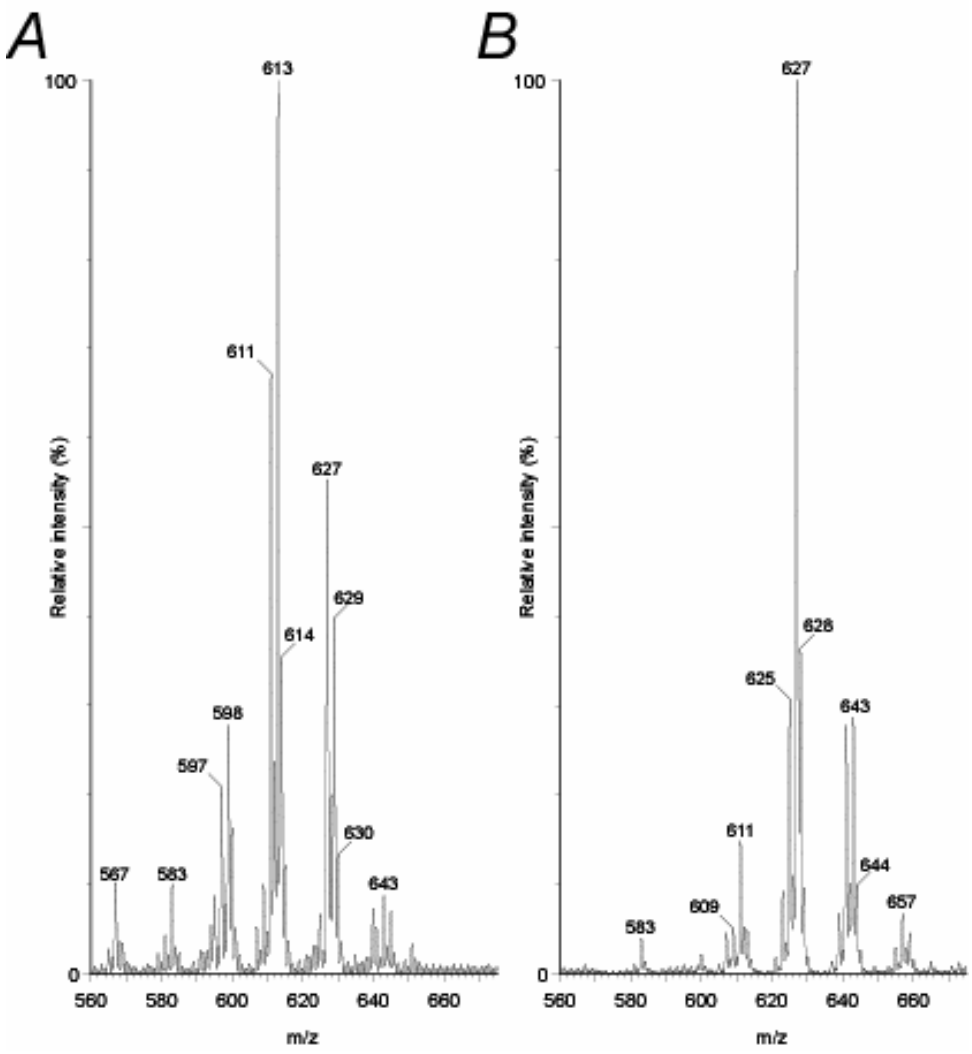
- Nine months after start of a low-phytanic acid diet, the pristanic acid levels had decreased to 6.2 µM.

- Since the beginning of the diet no progression of symptoms have been observed and a follow up MRI showed no increase in leukencephalopathy.

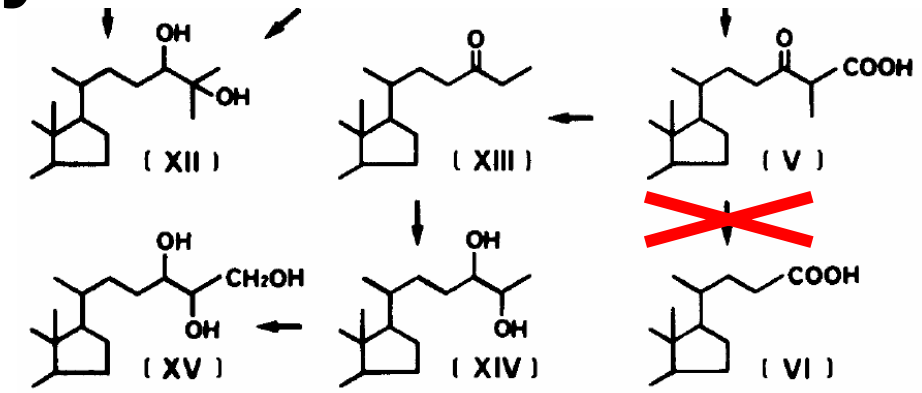
- Excretion of abnormal bile alcohol glucuronides.

- S.Ferdinandusse et al. Am.J.Hum. Genet.78(2006)1046-1052

Bile acid analysis in urine



SCPx CTX



Glucuronide conjugates:

- m/z 611 cholestanetetrol, or pentahydroxy-27nor-cholestan-24one
- m/z 613 27-nor-cholestanepentol
- m/z 627 cholestanepentol, or hexahydroxy-27nor-cholestan-24one
- m/z 629 27-nor-cholestanehexol

Aaron

Aaron H. was a 9-year-old boy who had always been healthy; his psychomotor development was normal.

Following circumcision he did not feel very well, his behaviour became aggressive, and he had hallucinations.

Subsequently he vomited and gradually became lethargic.

An EEG was abnormal (slow activity in the frontal area).

A cranial CT showed no abnormalities.

An MRI revealed white matter changes (not specified).

Aaron

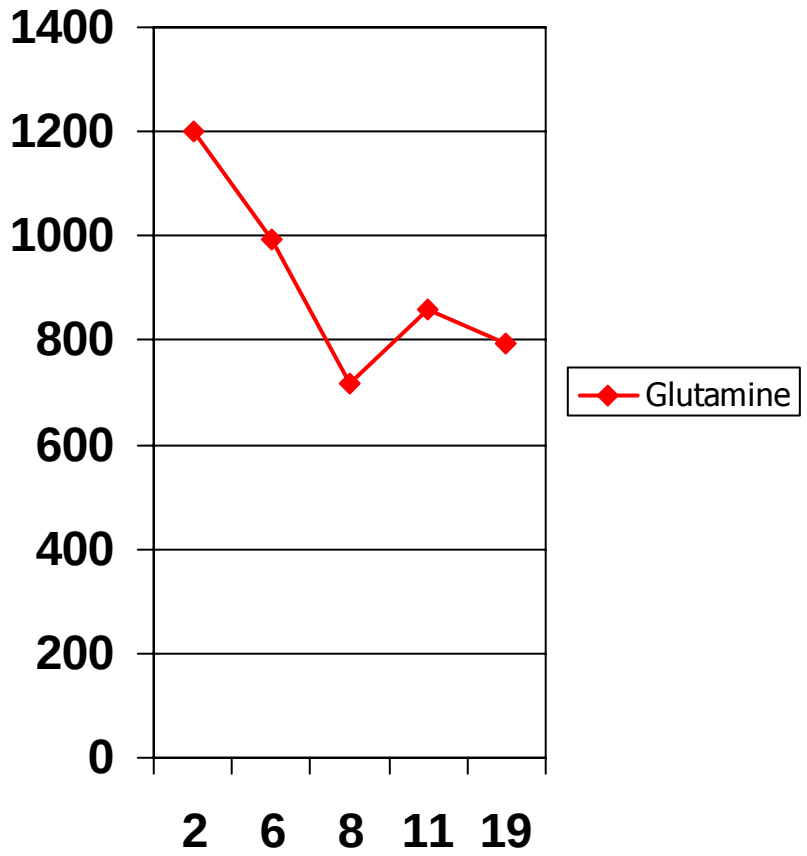
Blood glucose	:	Normal
Electrolytes	:	Normal
Blood lactate	:	1.0 mmol/L
Blood ammonia	:	9 μmol/L
CSF	:	No abnormalities (lactate 1.5 mmol/L)

Aaron

The patient became comatose and was transferred to the ICU of a University Hospital.

Based on the severe, but unexplained clinical presentation, a selective screening for inborn errors of metabolism was started.

Aaron
Amino acids in plasma

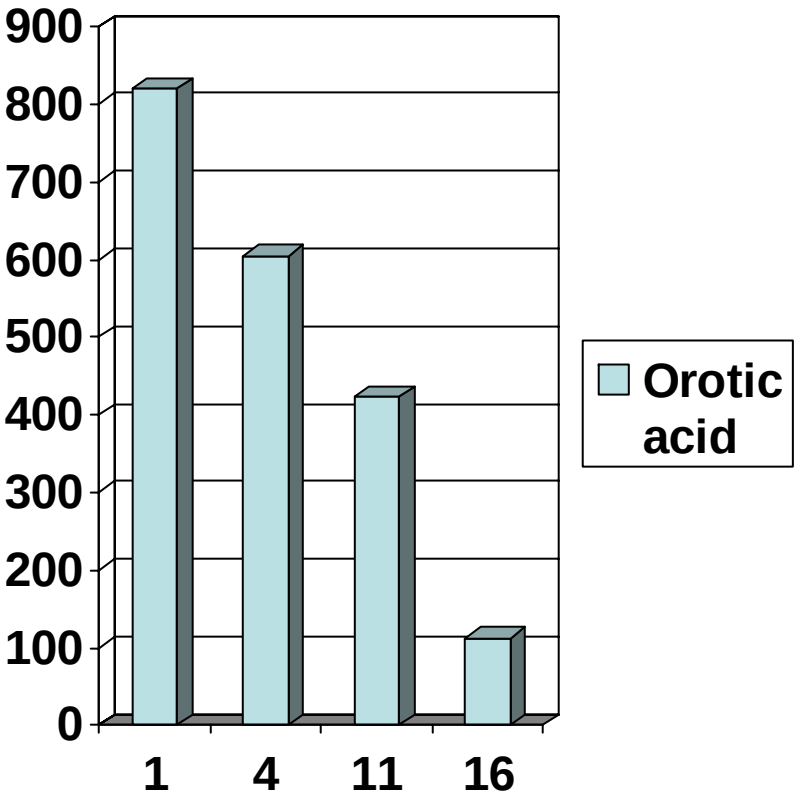


Glutamine (controls < 700 μmol/L) is formed from excess ammonia and glutamate.

Plasma citrulline was repeatedly in the range of 10-12 μmol/L, which is low.

This suggests that the citrulline-forming reaction from ornithine and carbamyl phosphate (OCT) did not function.

Aaron
Orotic acid in urine

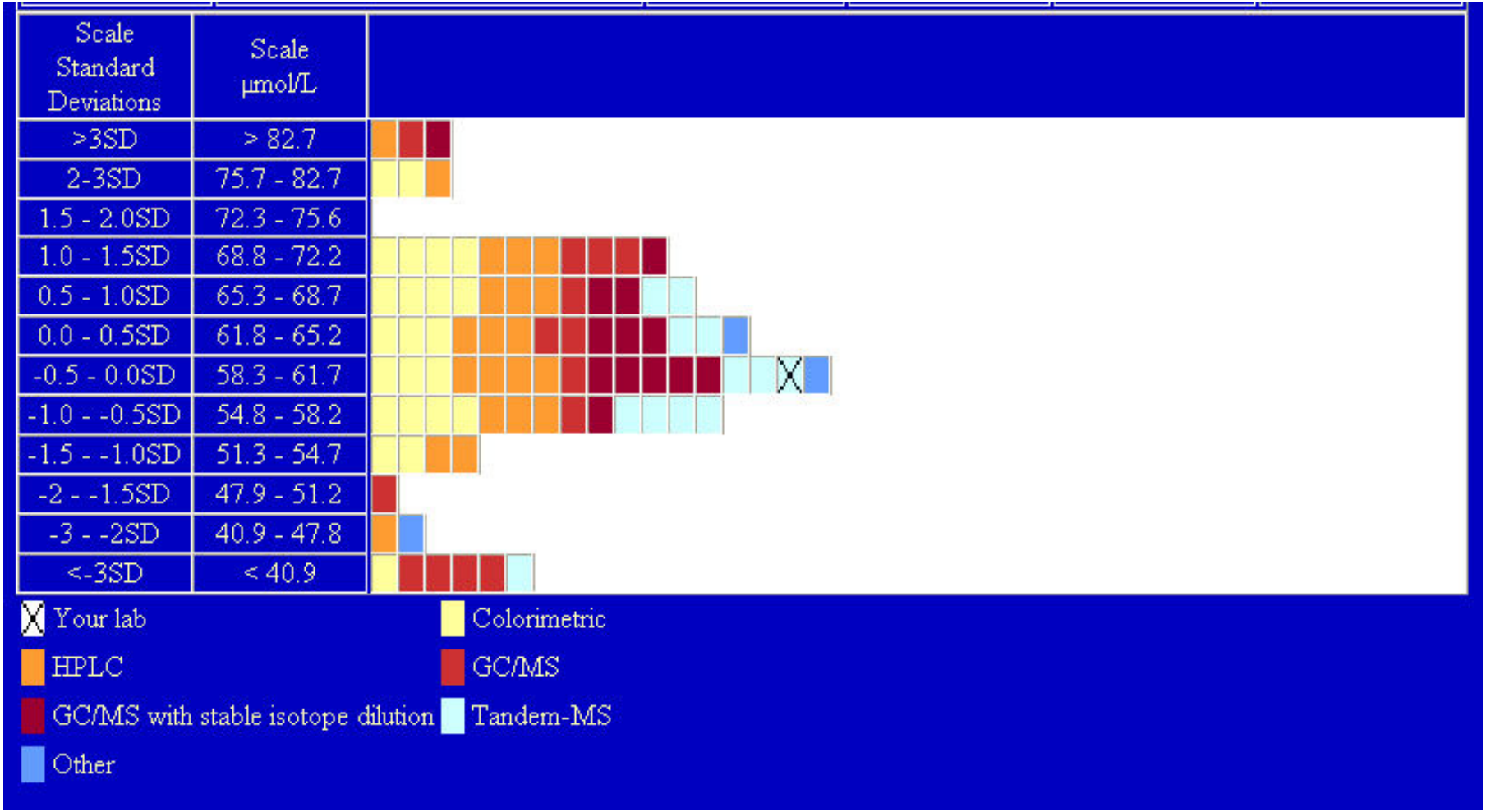


Organic acid analysis in the urine showed an excessive orotic acid excretion.

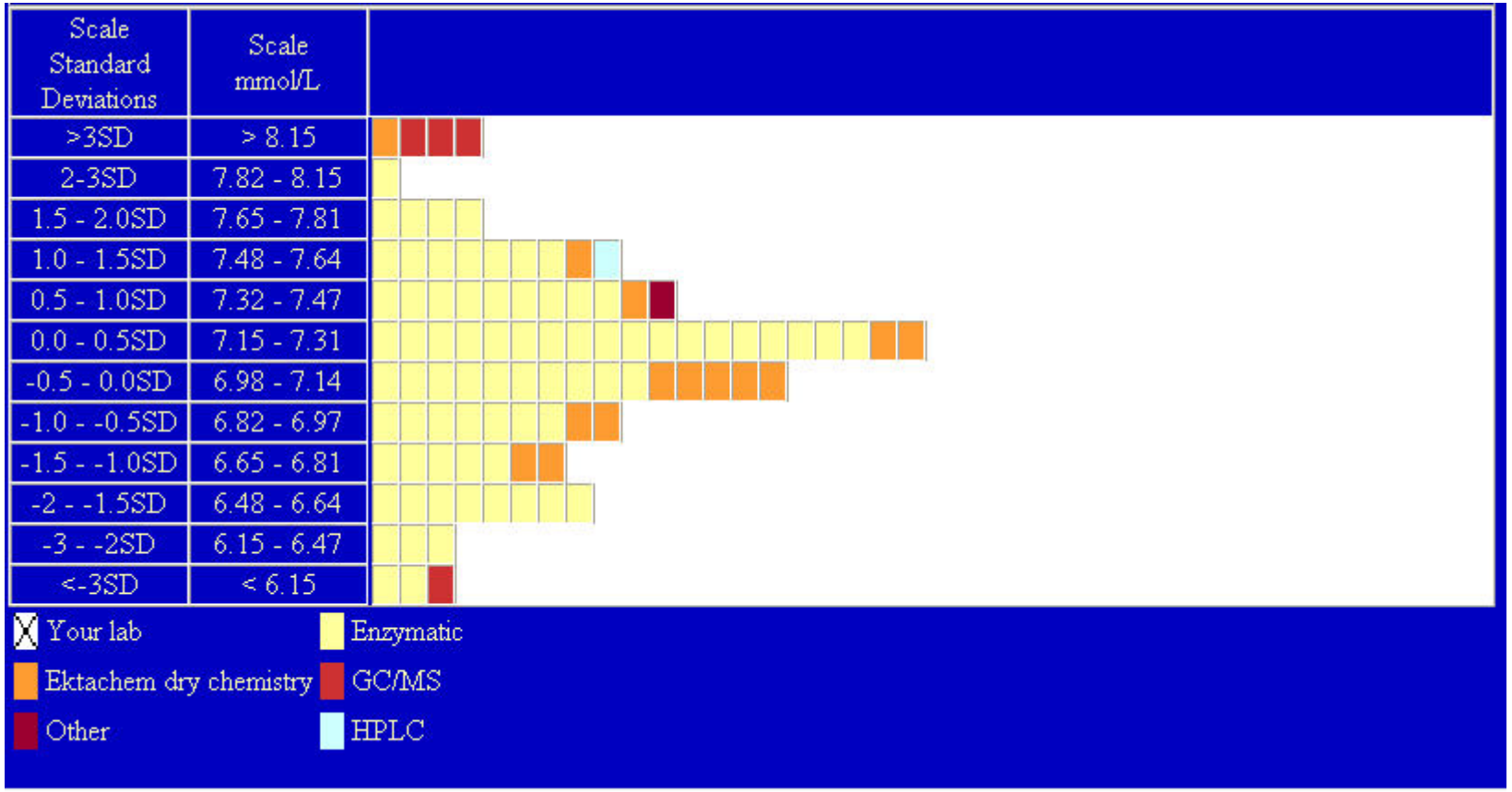
Subsequent pyrimidine analysis demonstrated increased uracil and uridine.

Ammonia was shunted into the pyrimidine biosynthesis and breakdown pathway, which has a limited capacity.

OROTIC ACID IN URINE



The interlab CV of this assay was 97% in 2006. One would like to pinpoint the ‘best’ method, but neither technique performs truly outstanding, perhaps with exception of tandem-MS.



The performance of the serum lactate scheme is satisfactory, one may conclude that chromatographic methods in this assay may give misleading results

Which carnitine levels would you like as a check of your performance?

Patient and methods

A few hours after birth the girl developed seizures and apnoea, which required mechanical ventilation and anti-epileptic medication.

On physical examination no other abnormalities were found.

Major laboratory abnormalities:

hypoglycaemia (2.0 mmol/L),

lactic acidaemia (7.9 mmol/l),

hyperammonaemia (200 μmol/L),

mildly elevated transaminases (ASAT 136 U/L, ALAT 187 U/L),

mild elevation of creatine kinase (448 U/L)

Neonatal Period (contin.)

- Development of progressive hepatomegaly
- I.V. glucose was given, followed by a high-energy diet
- Episodic vomiting occurred
- At 3 weeks the patient became somnolent, hypotonic, hypothermic and subsequently presented with convulsions, apnoea, and bradyarrhythmia
- Referral to University Hospital

Routine Clinical Chemistry

- Glucose 2.8 mmol/L
- Lactate 3.2 mmol/L
- Ammonia 286 $\rightarrow$ 1368 μmol/L
- CK 1760 U/L

Metabolic Investigations

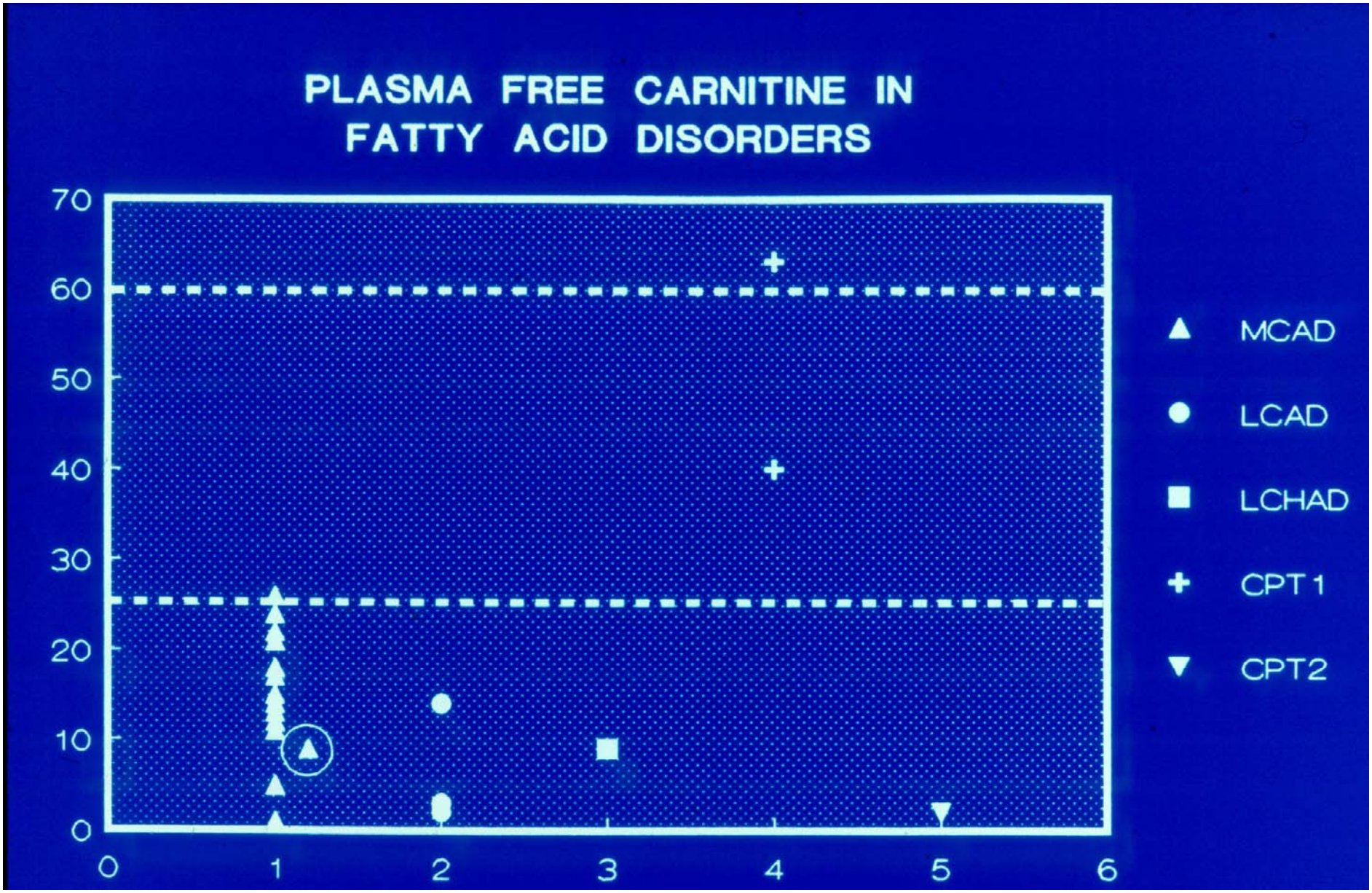
CAA – normal plasma amino acids, including glutamine

COA – intermittent dicarboxylic aciduria (C_6-C_{12})

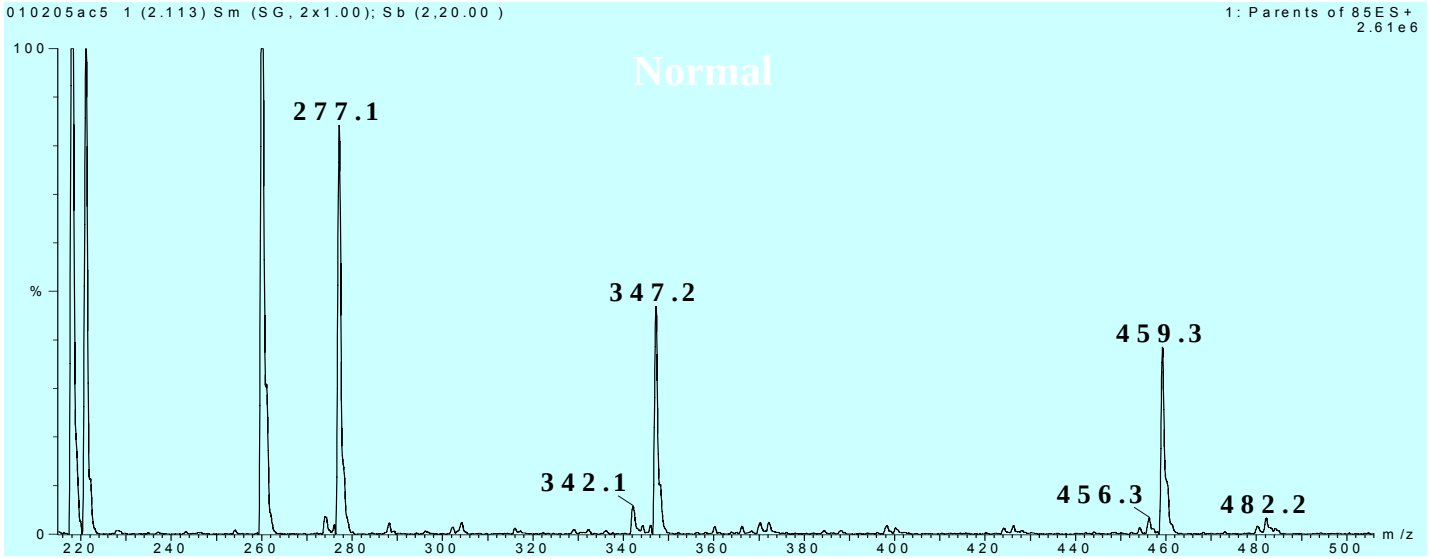
Orotate – normal

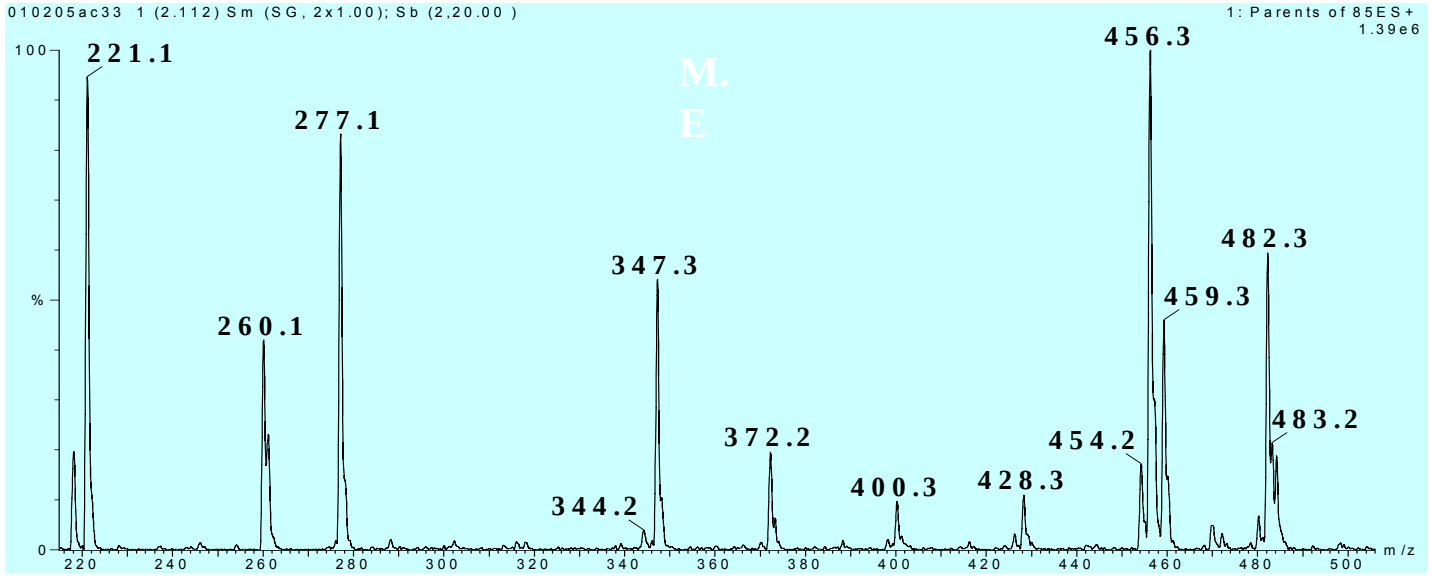
Free carnitine – 4.9 μmol/L

Acylcarnitines – Strong elevation of 16:0, 18:0, 18:1, 18:2

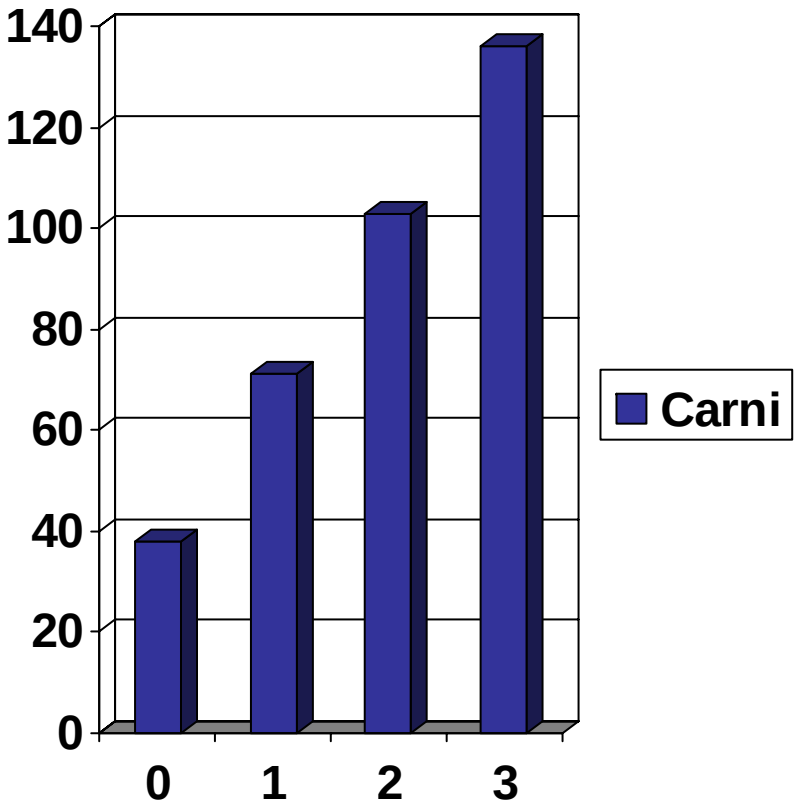

PLASMA FREE CARNITINE IN
FATTY ACID DISORDERS
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
MCAD
LCAD
LCHAD
CPT1
CPT2

Acylcarnitines in Plasma





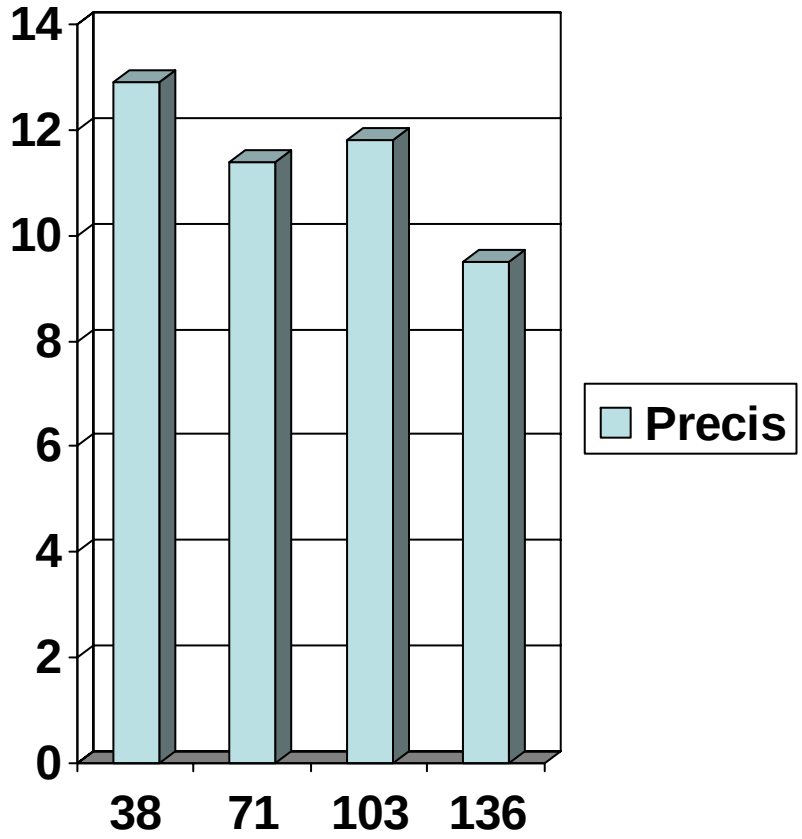
THE CARNITINE LEVELS OF SERUM SPECIAL ASSAYS 2006



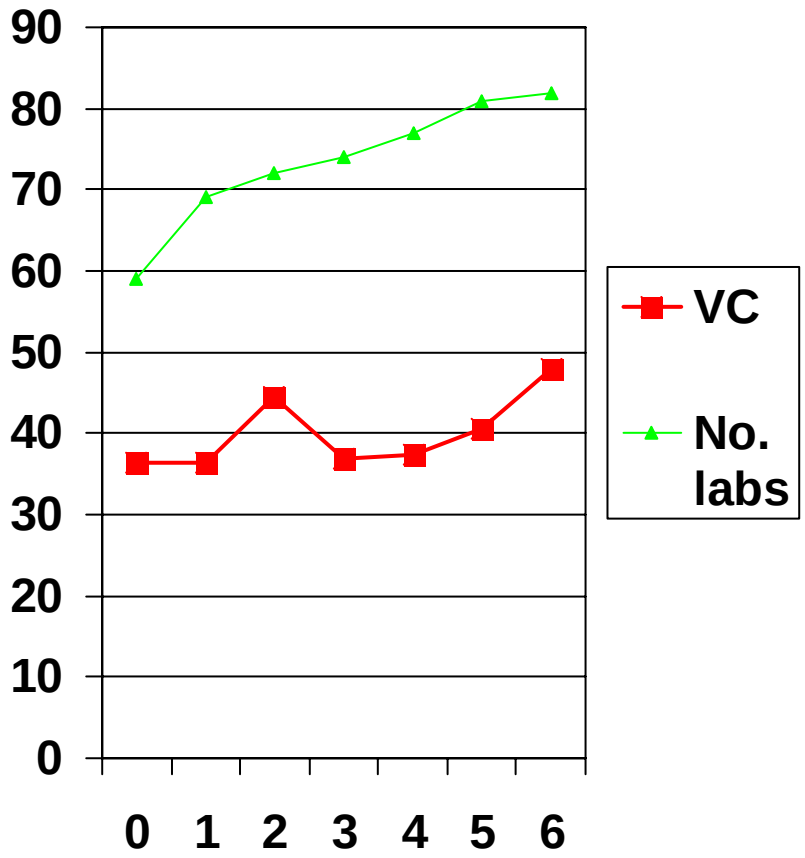
- Ideally, carnitine deficient values, i.e. those below 20 µmol/l, are the target of our labs.
- The current scheme does not give the possibility of checking our performance at the desired level.

THE PRECISION OF THE SERUM CARNITINE ASSAY IN THE SA-SCHEME

- The overall precision of all labs had a slightly downward trend with the increase of the added carnitine level.
- One may predict that the precision may be even worse when the scheme goes for ‘carnitine deficiency’ concentrations



THE INTERLAB VC: MUCOPOLYSACCHARIDES



- The DMBU-test gives quite variable results, possibly as a result of the lack of standardization.
- Not all labs use the same calibration standard and ERNDIM has changed the product which is added to the SA-urine.
- More labs does not mean better performance.

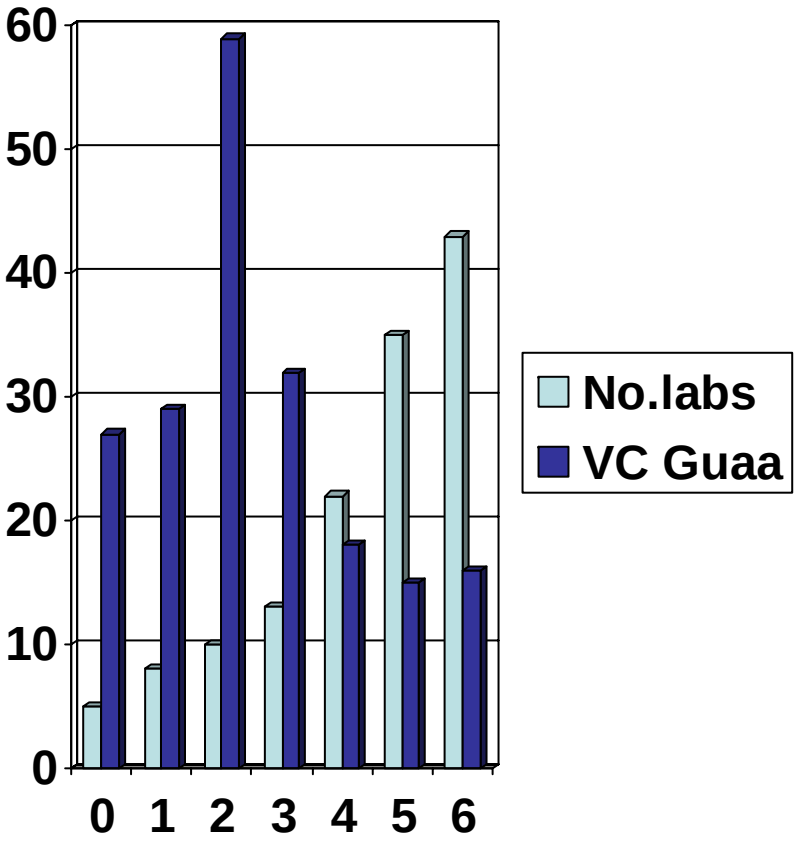
AN IDEAL SYSTEM: HOMOCYSTEINE

Year	Precision (%)	Linearity	Recov. (%)	Interlab VC (%)
2000	27.7	.914	76	14.1
2001	6.7	.996	99	10.1
2002	5.8	.996	101	8.9
2003	5.7	.996	101	9.3
2004	5.2	.996	103	8.8
2005	7.1	.994	103	11.0
2006	5.7	.995	105	9.9

Sulfur amino acids in plasma

	Methionine μmol/L	SAM nmol/L	SAH nmol/L	Hcy (tot.) μmol/L
CBS def.				
Pat 1	224	1607	1185	260
Pat 2	96	184	142	304
MAT def.	200-1300	48-120	10-15	30-45
GNMT def.	400-700	2500	40-70	13
SAH-hydr.def	400-550	1900	530-770	15
Controls male	16-36	80-214	15-31	8-18
female		66-128	10-23	6-15

A SCHEME SHOULD IMPROVE WHEN THE NUMBER OF PARTICIPATING LABS INCREASES



- Guanidinoacetate started with five labs only.
- When the number of participants exceeded 15, a definitive improvement of the interlab VC became apparent.
- Similar findings were obeserved for creatine which was introduced four years later.

Special assays: future expectations

- Not only high, but also lowered levels
  - **carnitine**
  - **neurotransmitters**
- Keep up with novel disorders
  - **sugar alcohols**
  - **bile acids**
- Take care of unstable substances
  - **acylcarnitines**
  - **biopterin**
  - **SAM / SAH**
  - **pyruvate**
- Expand the range of existing disorders
  - **mevalonic acid**
  - **cholestanol**
- Other fluids/cells/tissues
  - **erythrocytes**

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