White Cell Enzymes – what are they?

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White cells are an easily obtainable source of Lysosomes

- Intracellular organelles
- contain hydrolytic enzymes at acid pH
- contain no DNA
- most enzymes targeted by mannose-6-phosphate recognition signal

Lysosomes

Are the bulky molecule recycling and disposal centre for the cell

Rubbish (macromolecules)
is engulfed whole by the lysosome

Is sorted and broken down according to chemical structure by a series of lysosomal enzymes. The resulting reusable small molecules are transported out of the lysosome by specific carriers

Major Pathways Catalysed

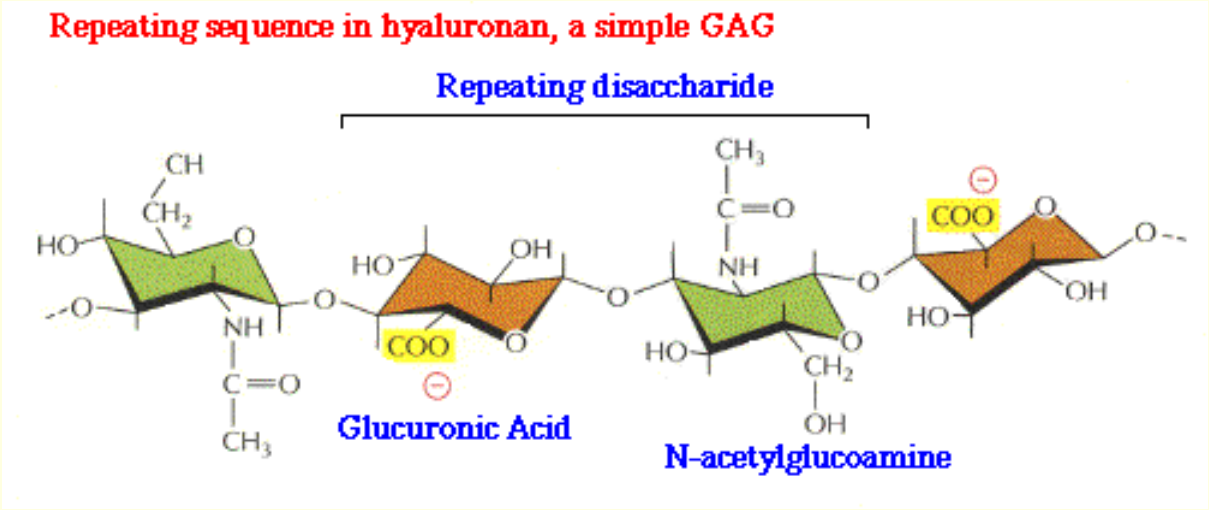
Stepwise degradation of

- Glycosaminoglycans (mucopolysaccharides)
- Glycolipids
- Glycoproteins

Each pathway is catalysed by a series of lysosomal enzymes.

- Deficiency causes a specific disorder
- 50-60 in total. Some diagnosed by lysosomal enzyme profile

Glycosaminoglycans (previously known as mucopolysaccharides)



Chondroitin sulphate	Normal
Dermatan sulphate	Hurler, Hunter, Maroteaux Lamy
Heparan sulphate	Sanfilippo
Keratan sulphate	Morquio

Glycolipids

essential components of cell membranes

GM1 ganglioside	GM1 gangliosidosis
GM2 ganglioside	Tay Sachs, Sandhoff
sphingomyelin	Niemann Pick A or B
galactocerebroside (galactosylsphingosine ceramide)	Krabbe
Glucocerebroside	Gaucher
ceramide	Farber
cholesterol esters	Wolman, CESD
sulphatides	Metachromatic Leucodystrophy

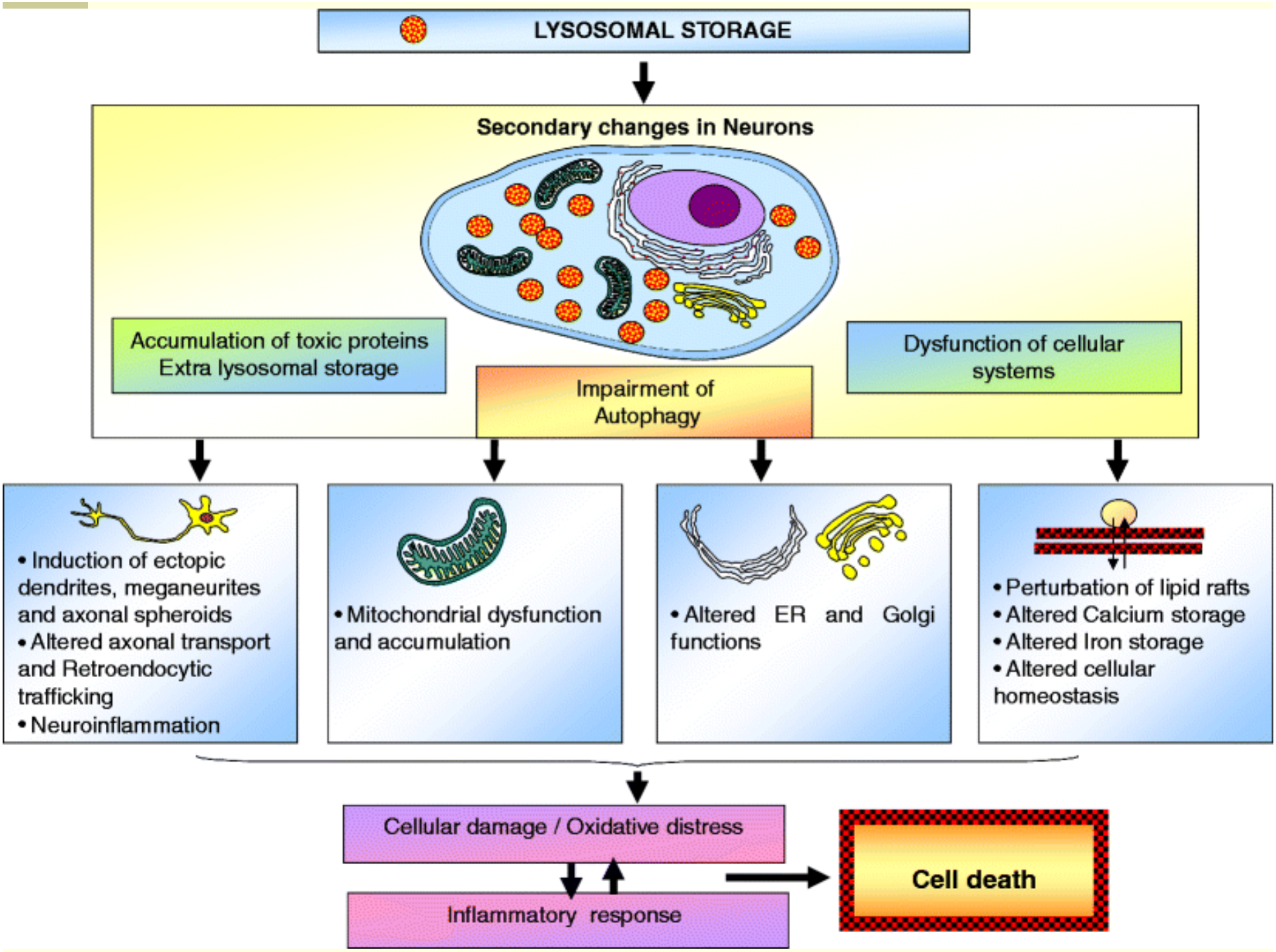
Glycoproteins

important constituents of connective tissue
tend to present with coarse features

- oligosaccharide side chains
  - a and b mannosidosis
  - fucosidosis
  - sialidosis
  - aspartylglycosaminuria

Lysosomal storage disorders

The substrate of the specific enzyme accumulates


LYSOSOMAL STORAGE
Secondary changes in Neurons
Accumulation of toxic proteins
Extra lysosomal storage
Impairment of
Autophagy
Dysfunction of cellular
systems
• Induction of ectopic dendrites, meganeurites and axonal spheroids
• Altered axonal transport and Retroendocytic trafficking
• Neuroinflammation
• Mitochondrial dysfunction and accumulation
• Altered ER and Golgi functions
• Perturbation of lipid rafts
• Altered Calcium storage
• Altered Iron storage
• Altered cellular homeostasis
Cellular damage / Oxidative distress
Inflammatory response
Cell death

Clinical Presentation

Lysosomal enzyme deficiencies result in

- lysosomal engorgement with unmetabolised substrate
- excretion of unmetabolised substrates

Presentation depends on

- which tissue is most active in metabolising that specific substrate (liver, spleen, brain)
- or on specific toxicity of the stored material

Specific Features

- startle response
- cherry red spot
- vacuolated lynphocytes or storage cells

characteristic dysmorphic features

- organomegaly
- developmental regression
- dysmorphic features

May lead straight to diagnostic enzyme test

But findings may not be specific of a single disorder (eg trivial names for disorders Hurler, pseudo Hurler)

Non specific Presentation – Investigation Strategy

Same clinical syndrome may result from different enzyme deficiencies (eg Tay Sachs & Sandhoff Disease, MPSIII San Filippo Types A, B C and D)

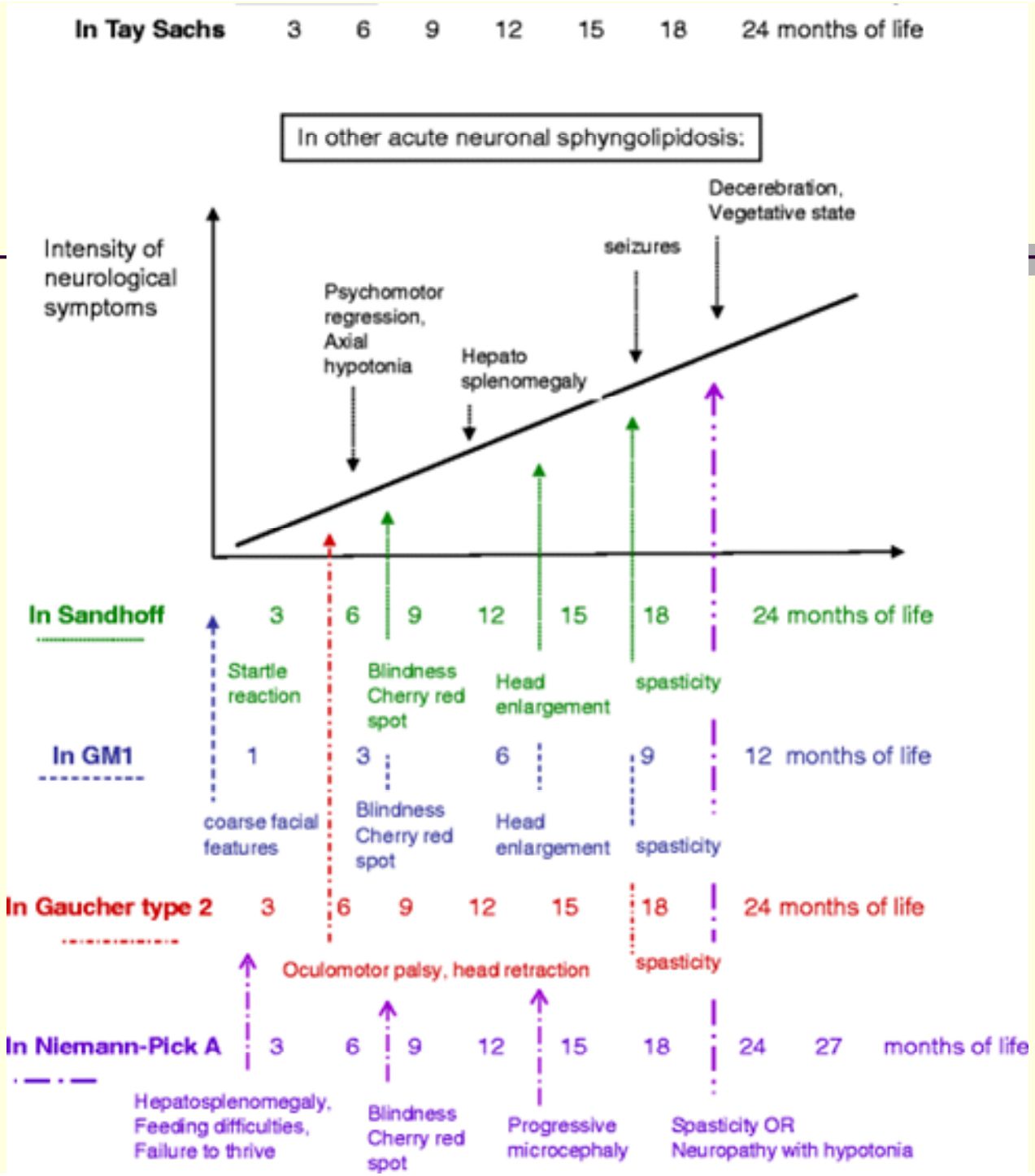
Same enzyme deficiency may present with a spectrum of clinical severity and age of onset (see Tables on next 2 slides)

Lysosomal storage disorders are individually rare

Few clinicians will have extensive experience of them

Patients usually first present to community or non specialist services

	Early infantile deterioration	Psychosis, behavioral	Cherry-red spot	Eye-movement disorder	Optic atrophy or retinopathy	Dysphagia, stridor, reflux	Myoclonus	Seizures	Cerebellar ataxia	Extra pyramidal	Axial hypotonia	Upper motor neuron	Lower motor neuron	Neuro pathy	Systemic involvement
GM2 type B, early onset	+		++		+			+			+	+			
GM1, infantile	+		+					+			+	+			++
Gaucher type 2	+			Oculomotor palsies		++						+			+
Niemann-Pick type A	+		+		+						+			+	+
ML-II (I-cell disease)	+														+++
Galactosialidosis, severe type	+		++					+			++	++			+++
Fucosidosis type 1	+							+				+++			+++
Krabbe, infantile	++				+	++		+				+++		++	
GM2 type B, late onset				abnormal saccades	+							+	+		
Gaucher type 3				abnormal saccades			++	+	+	+		+			+
Niemann-Pick type C				Supranuclear gaze palsies					+	+		+		+	+
GM1, late-infantile								+			+	+			+
GM1,adult										++					
Niemann-Pick type B			+						+	+		+		+	
Farber	+		+					+				++	+	+	
MPS I H	+	+			+							++	+	+	+++
MPS II		++			+			+				+	+	+	+++
MPS III		+++			+			+				++	+		+
MSD							+	+				++		++	++
Galactosialidosis, juvenile type			++		+		++	+	++					+	+
Alpha-mannosidosis		+							++						++
Beta-mannosidosis		+						+	+			+		++	++
Fucosidosis type 2							+	+		+		+++			+
Sialidosis type II			++				+	++	++			++			+++
Sialidosis type I			+++		+		+++	+++	+						
Aspartylglucosaminuria		++					++	++							
Schindler disease	+				++		++	++				+++			
INCL	+				+++		+++	+++	+			++			
LINCL					+++		+++	+++	+	+		+			
JNCL		+			+++		++	++	+	+		+			
ANCL		++					+	+		+		+			


In Tay Sachs 3 6 9 12 15 18 24 months of life
In other acute neuronal sphyngolipidosis:
Intensity of neurological symptoms
Psychomotor regression, Axial hypotonia
Hepato splenomegaly
seizures
Decerebration, Vegetative state
In Sandhoff 3 6 9 12 15 18 24 months of life
Startle reaction
Blindness Cherry red spot
Head enlargement
spasticity
In GM1 1 3 6 9 12 months of life
coarse facial features
Blindness Cherry red spot
Head enlargement
spasticity
In Gaucher type 2 3 6 9 12 15 18 24 months of life
Oculomotor palsy, head retraction
spasticity
In Niemann-Pick A 3 6 9 12 15 18 24 27 months of life
Hepatosplenomegaly, Feeding difficulties, Failure to thrive
Blindness Cherry red spot
Progressive microcephaly
Spasticity OR Neuropathy with hypotonia

Strategy for Investigation

Specific features may suggest a specific disorder

– request specific enzyme eg

- Pompe (cardiomyopathy),
- Fabry (male with painful extremities, unexplained renal failure, angiokeratomas)

Otherwise

Urine Mucopolysaccharide screening test

Blood lysosomal enzyme profile

Evidence of storage???

– but ask any haematologist – vacuolated wbcs on peripheral films seen in much commoner disorders than lysosomal storage diseases.

Other enzymes selectively (Battens, NPC etc)

Mucopolysaccharides(Glycosaminoglycans)

14 deficient enzymes

1st line screen urine quantitative dye binding test for excess MPS

Good for

MPS1 (Hurler/Scheie),

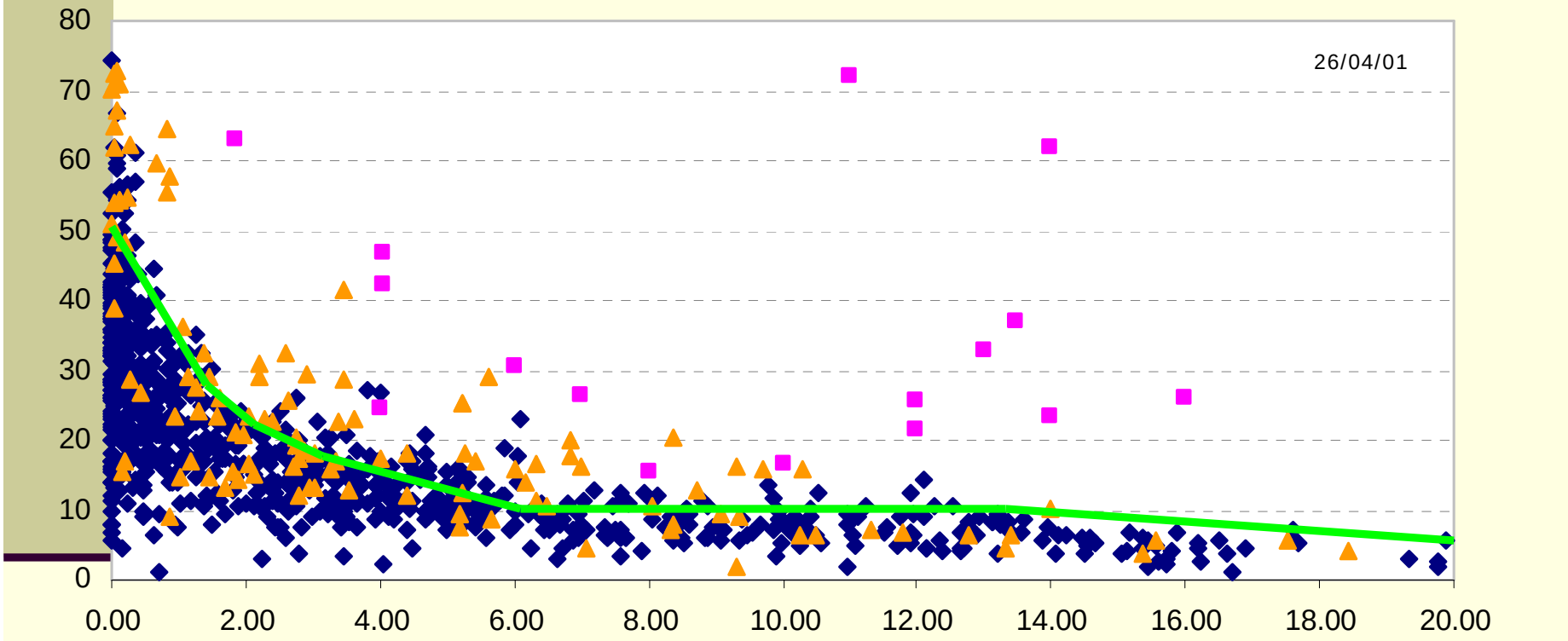
MPS II (Hunter),

MPS III (San Filippo)

Won't detect Mucolipidoses, and other lysosomal storage disorders, that may present with similar clinical presentation

Not reliable in babies <3months (high excretion due to rapid growth & tissue turnover) or dilute urine samples (creatinine <1.0mmol/L – additive errors in calculating ratios. How reliable are non enzymatic methods in urine at this concentration?)

DMB MPS all data
860 blue diamonds = reported as within ref range,
121 orange triangles =eph done and NAD, 23 pink squares= abnormal eph & MPS diagnosis confirmed



Age (Years)

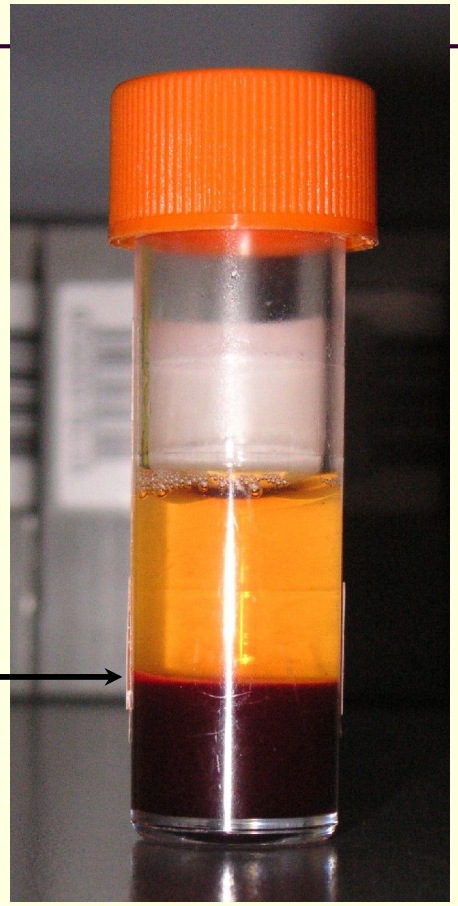
Lysosomal enzyme profile (11 enzymes)

Prepare Leucocyte pellet

- 5mL EDTA blood is the MINIMUM
- Differential lysis of red cells
- Requires several incubations & centrifugations – 45 min minimum
- Yield of mixed leucocytes depends on age of sample, patient's white cell count

ENORMOUS improvement in pellet quality by preparing leucocytes locally and transporting pellets on dry ice, compared to overnight (at best) transport of whole blood to specialist lab before leucocyte isolation.

Pretend this is
a PINK top



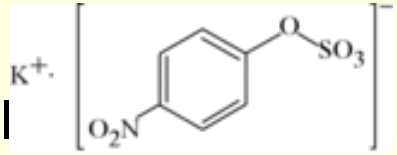
Leucocyte layer

Prepared leucocyte pellet
Stored –70’C pending analysis

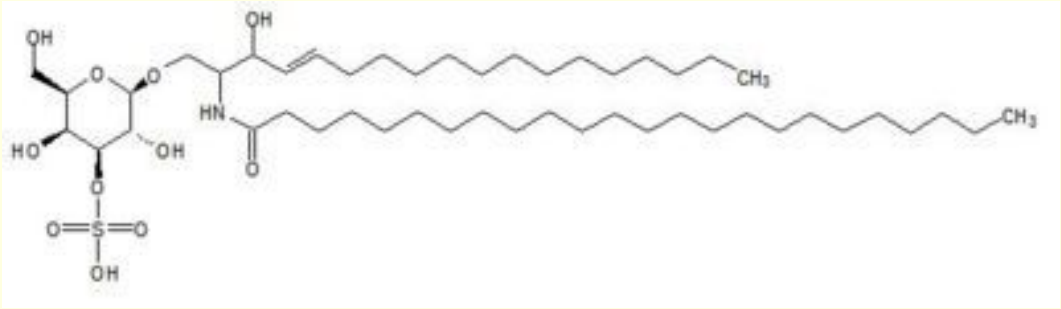
Cells and lysosomes are disrupted by carefully controlled sonication

Substrates for measuring lysosomal enzymes

- Fluorimetric tag & artificial substrate – 4MU
  4-Methylumbelliferyl β-D-glucopyranoside
  4-Methylumbelliferyl β-D-galactopyranoside
  4-Methylumbelliferyl α-D-galactopyranoside
- Colorimetric tag & artificial substrate – p-nitrophenol

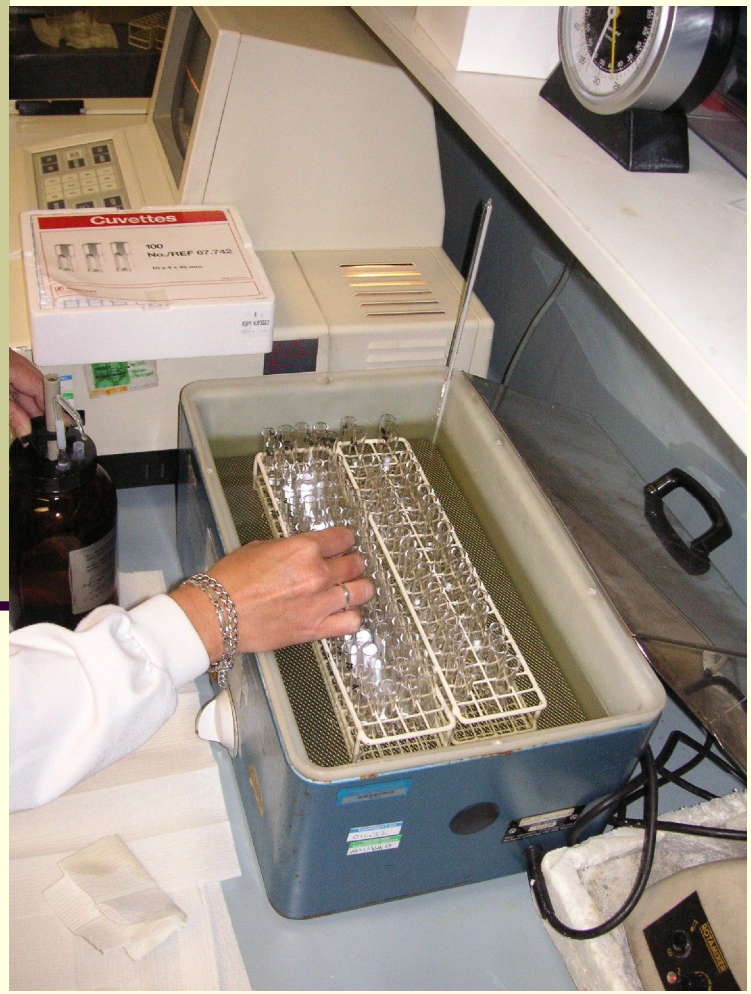


Despite looking nothing like the natural substrate below, the "aryl"sulphatase enzyme is fooled

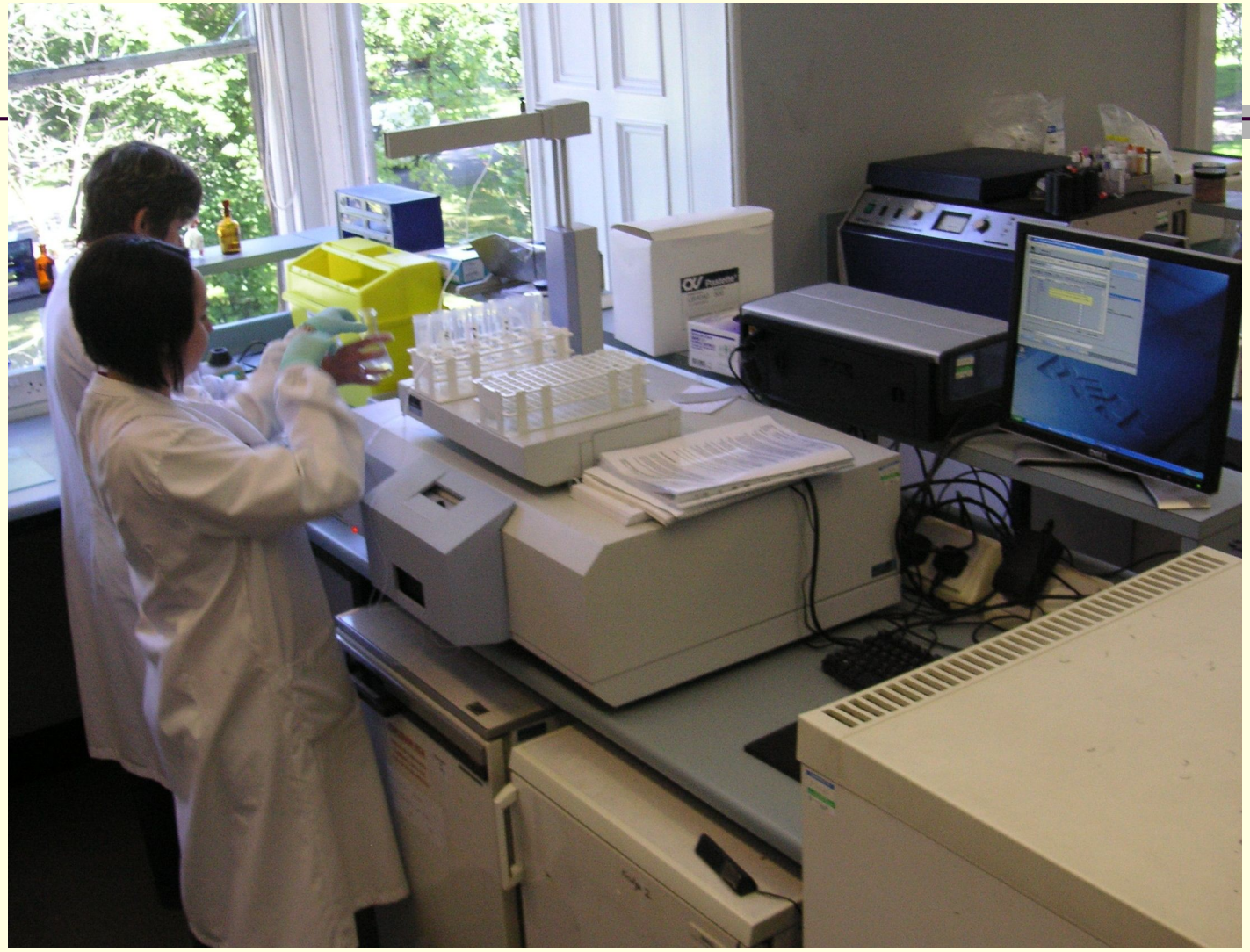


- Radioactive tag – natural substrate with ^{3}H or ^{14}C

Pipette sonicate dilutions into tubes.
7 patients = >200 individual tubes.
Start enzyme reaction by timed addition of appropriate substrate.
Incubation times depend on enzyme.
Stop reactions at same timed intervals by addition of an inhibitor

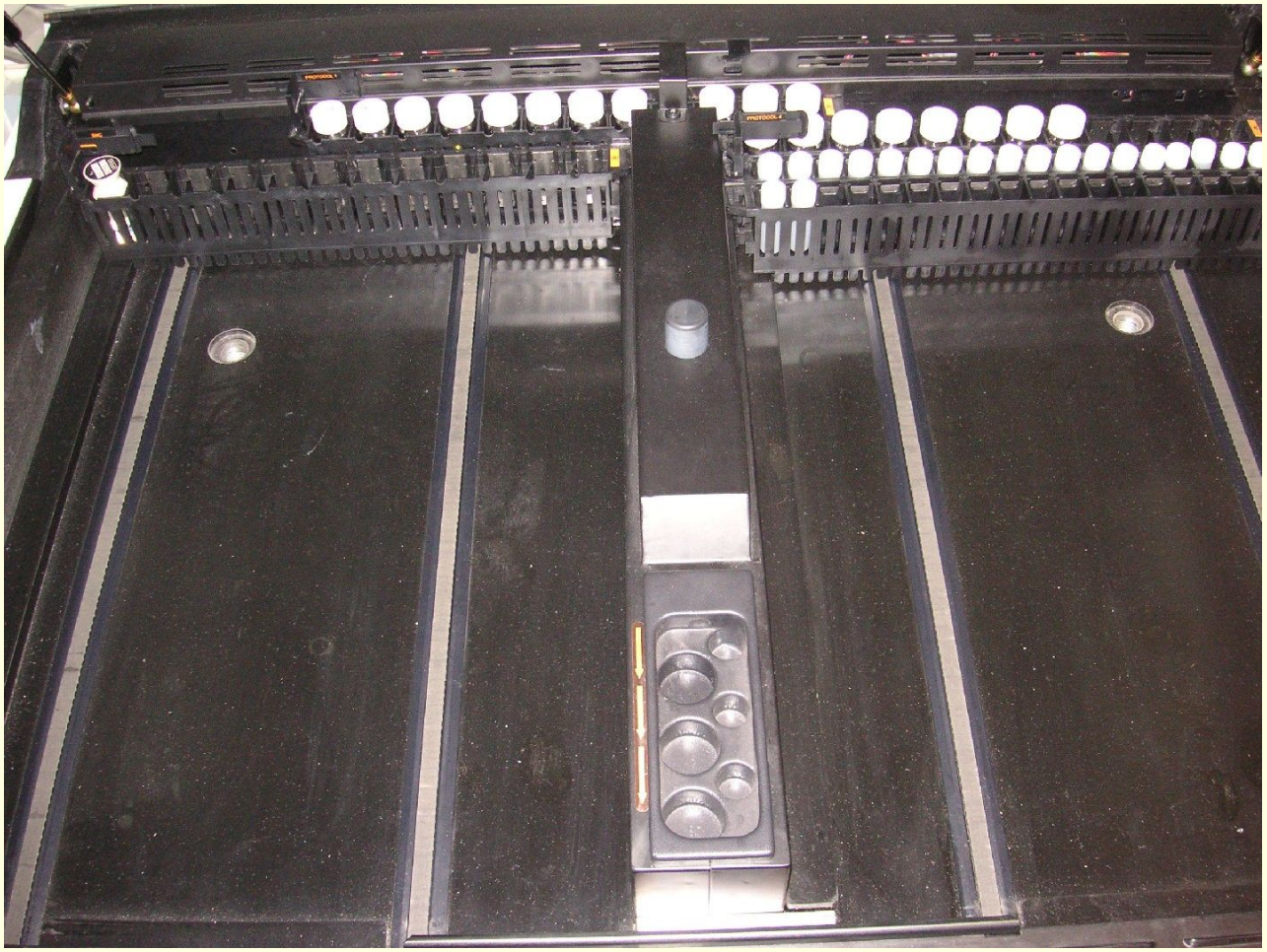


Fluorimetric and colorimetric enzyme reactions are read directly



Radioactive assays require further manual steps (phase separation or precipitation) to separate product from unreacted substrate.

Separated radioactive product is then measured overnight on a scintillation counter

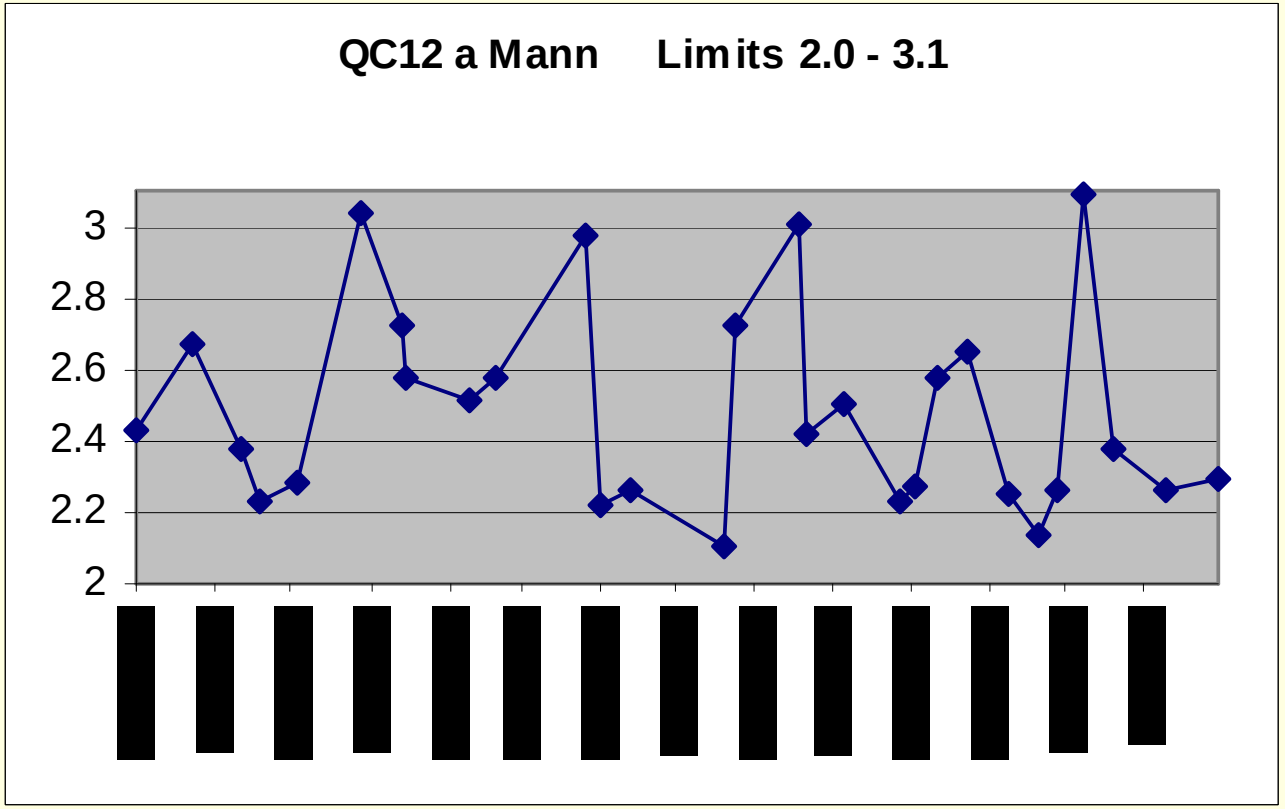


Reporting

- Protein is measured in all samples, and the enzyme activity reported /mg protein

IQC

Obtain white cell concentrate from Blood Transfusion Service
Bulk prepare white cell pellets
Same method as for patient samples
Store -70'C
Analyse 1 in each batch



n	mean	SD		2SD limits		CV	
6	**2.51**	**0.3**		**1.90**	**3.11**	**12.1**	26/01/10 es
13	**2.53**		0.3	**1.99**	**3.07**	**10.6**	27/05/10 JK
29	**2.48**		0.3	**1.92**	**3.05**	**11.3**	05/01/11JK

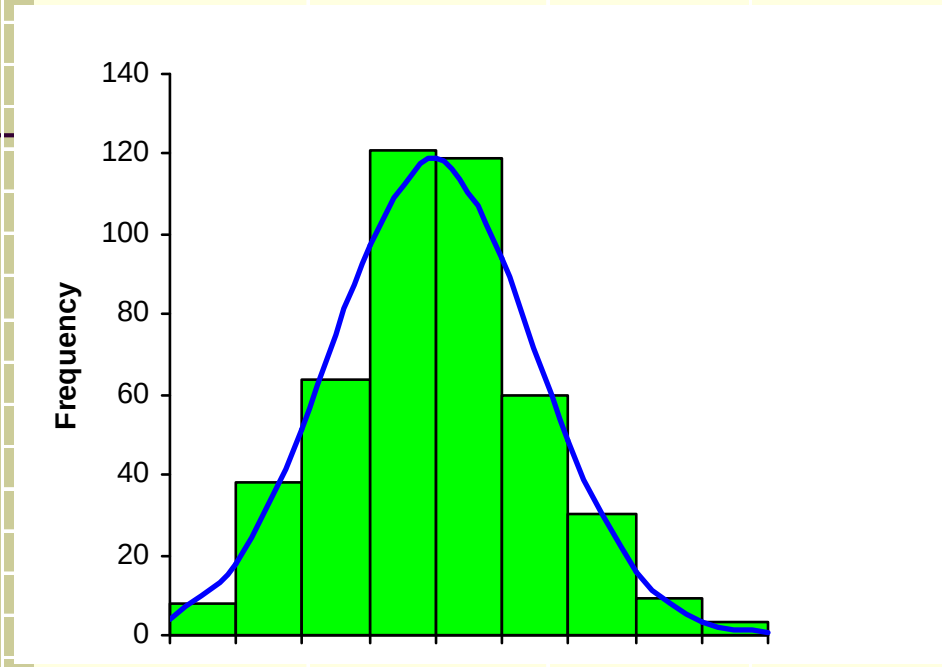
EQA

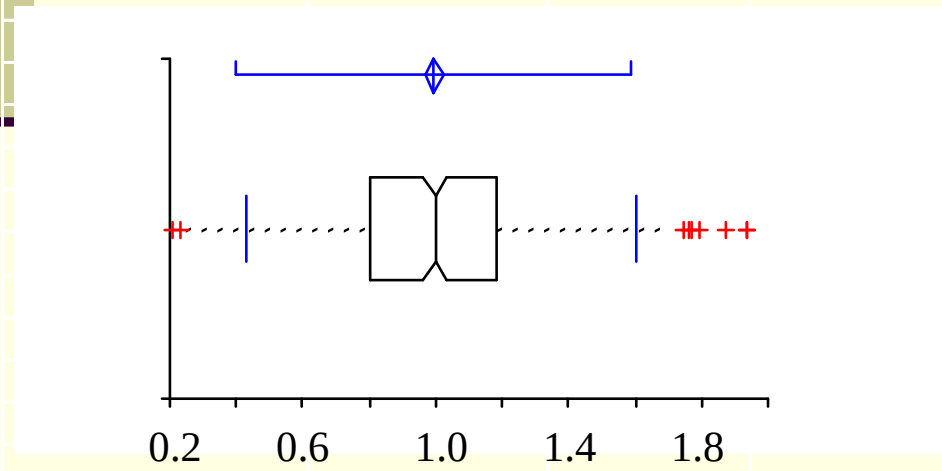
- ERNDIM lysosomal enzyme scheme
- ~ 80 participants worldwide
- Few teething problems
- To obtain enough material to ship to all participants 2011 scheme used transformed lymphocytes from diagnosed patients and controls.
- Enzyme activity in lymphocytes may be very different from mixed leucocytes eg a-iduronidase ~10%.
- Report results as % mean normals

Reference ranges

- Lysosomal enzyme activities are not usually changed by intercurrent illnesses
- Possible to use patient data from those not diagnosed to construct reference range
- In-house methods – many different suppliers of substrates
- Recheck stability of reference range at regular intervals or following known changes of supplier or instrumentation.

ASA data 2002-6 used to check RefR (0.5 – 1.7)
1 exclusion:0.06 – diagnosis of Metachromatic Leucodystrophy





n	452
Mean	0.993
95%CI	0.965 to 1.021
Variance	0.0920
SD	0.3034
SE	0.0143
CV	31%
Median	1.000
95.7%CI	0.960 to 1.030
Range	1.73
IQR	0.38

Percentile	
2.5th	0.427
25th	0.800
50th	1.000
75th	1.180
97.5th	1.600

Carrier Detection

Mean enzyme activity of carriers 50% mean of non carrier population – but can't reliably identify carriers by enzyme analysis.

successful screening programmes in selected communities

DNA mutation studies used within families

- eg for prenatal diagnosis, alone or in combination with enzyme
- Phenotype/genotype correlations for some disorders

In this family prenatal diagnosis was carried out by mutation studies.

Pseudodeficiency

- First described for arylsulphatase A
- healthy relatives of MLD patients with deficient enzyme activity
- many have common mutation in MLD gene
- population estimates 1:7-14 carriers
- 1:50-200 homozygous for pseudodeficiency
- MLD incidence 1:40,000

Interpretation of Subnormal Arylsuphatase A (ASA) Results

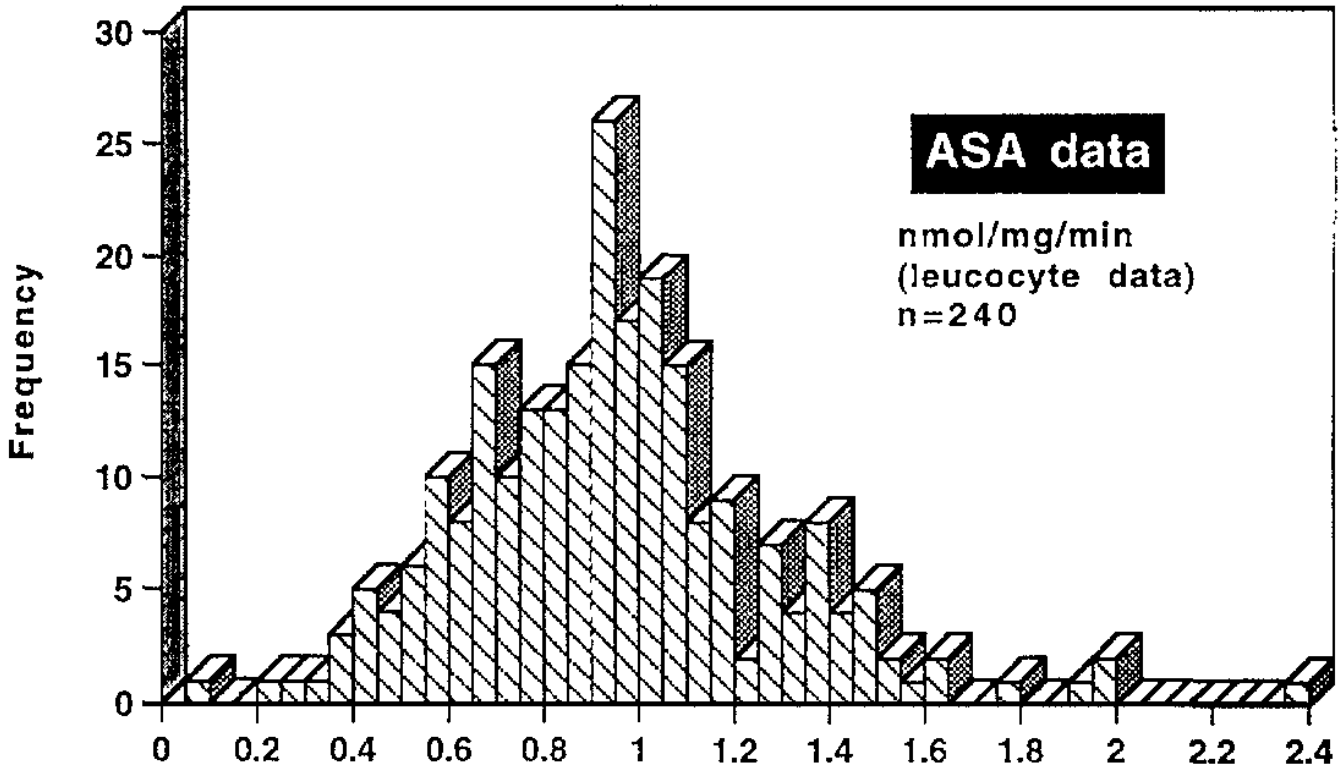


Chart shows results on 240 children assayed by the same method, and not diagnosed Metachromatic Leucodystrophy.

Two patients with results <0.2 were shown to be homozygous for the pseudodeficiency allele.

Not included above are:

Diagnosed MLD patients : 0.12, 0.07, 0.14, 0.1, 0.22, <0.01

Presumed heterozygotes (parents): 0.36, 0.32, 0.60, 0.41, 0.53, 0.64, 0.75, 0.55

Other disorders - not single enzyme

- targeting of enzyme to lysosomes
  - absent mannose 6 phosphate recognition signal
  - I-cell (mucolipidosis II)

  Measure very high levels of lysosomal enzymes in plasma
- transport of small molecules out of lysosome
  - Salla disease, infantile sialic acid storage disease
  - Cystinosis

  Measure storage material in relevant tissue
- multiple sulphatase deficiency
  - features of MLD, Hunter, Morquio

  Measure more than one sulphatase enzyme

Summary

- Lysosomal enzyme profile is a useful panel of tests to investigate a number of storage disorders that may clinically present similarly.
- Usually in early childhood with developmental regression and/or hepatosplenomegaly.
- It does not rule out all storage disorders, let alone all inherited metabolic diseases.
- Deficient enzyme activity is not diagnostic unless backed up by other evidence