LYSOSOMAL ENZYMES

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Clinical Genetics

Leading the way in genetic issues

viapath

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Introduction

- Enzyme activity
- Background & history of EQA scheme (ESGLD/ ERNDIM)
- Current ERNDIM scheme.
- Discussion
- Diagnostic issues with fluorimetric substrates

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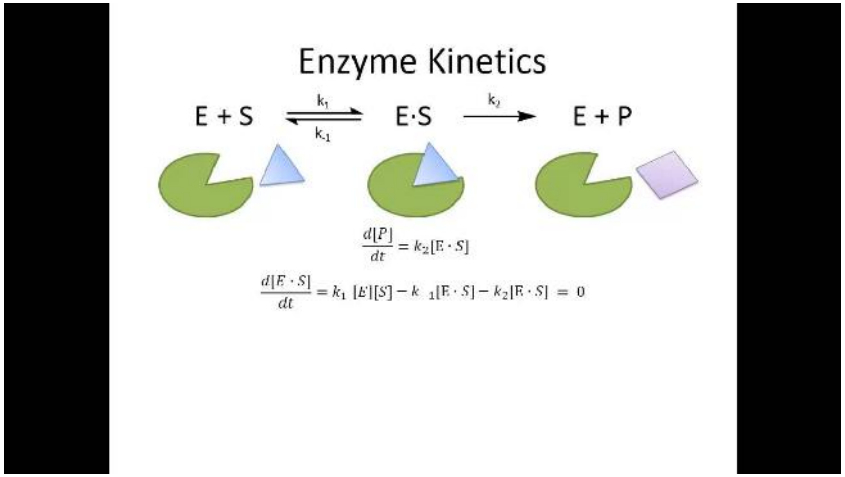
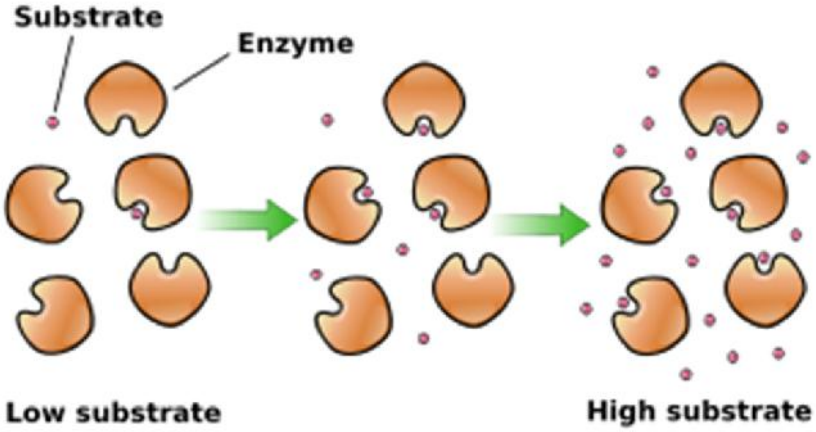
ENZYME KINETICS





Enzyme activity or how much enzyme is present. Two ways: disappearance of substrate or the appearance of product in time. (velocity) Measuring the appearance of product usually more accurate

Fixed-Timed or discontinuous assay With fixed-timed assays, one can measure many assays simultaneously.

Kinetic or continuous assay measure the appearance of product in real-time. Dis-advantage only one reaction can be measured at a time, advantage is the convenience of easily measurable reaction rates.

Initial rates measured. v_0 must be both reproducible and dependent on $[E]_t$. Valid assay conditions exist if the product has consumed less than 5% of the substrate.

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ENZYME ACTIVITY MEASUREMENT: REQUIREMENTS

- **pH is constant**: Enzymes have specific pH ranges of activity due to structural sensitivity to proteins which cause enzymes to be sensitive to pH changes. A prepared buffer solution is used to keep the pH constant so that the pH resist changes. Good buffers do not cross membranes; do not absorb light; are chemically stable; and are biochemically inert.

- **V_0 is proportional to the concentration of the enzyme, $[E]_t$.** An assay is only valid when a plot of V_0 vs $[E]_t$ is linear because the enzyme must be the only limiting factor to the substrate concentration. Additionally, when V0 and [E]t are proportional, the presence of effectors are measurable and one can determine if an inhibitor is competitive, noncompetitive, or uncompetitive.

- **V0 is corrected for non-enzymatic conversion**: V_0(corrected) = V_0(+enzyme) – V_0(-enzyme). A control factor must be measured while conducting enzyme assays in order to ensure accurate calculations. For non-enzyme controls, buffers are used in place of enzymes.

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ENZYME ACTIVITY

- **Salt Concentration**: of importance for proper enzyme folding and substrate binding. This can effect the enzyme's ability to catalyze a reaction.

- **Enzyme and Substrate Concentration**: Precise enzyme and substrate measurements must be calculated to ensure optimum activity.

- **pH Dependence**: Most enzymes have an optimal pH at which their reaction is catalyzed the fastest. Often enzymes operate at a maximum activity when the pH is close to the pk_a of the enzyme's active site.

- **Inhibition**: Inhibitors can decrease reaction rates via binding to the enzyme in the active site, products of the reaction can often inhibit the enzyme by binding the active site.

- **Activators**: Activators increase the activity of an enzyme. Additional chemicals may be needed to achieve necessary concentrations of activators, reactants, and co-factors.

- **Temperature dependence**: Many enzymes have an optimal temperature that can be found by measuring reaction rates with varying temperatures. Reaction rates usually increase with temperature, however high temperatures usually denature and give no activity of the enzyme.

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ENZYMOLOGY FOR DIAGNOSTIC TESTING

- **Specificity** Measurement of only the enzyme responsible for product formation
- **Sensitivity** Measure enzyme activity in sample (without prior purification)
- **Stability** Reproducibility
- **Convenience** High throughput

Requirement for enzyme diagnostics

- To distinguish enzyme activity in an affected patient from unaffected controls.

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LYSOSOMAL ENZYME SCHEME HISTORY

Pilots “large scale” OvD ESGLD
2006 (36) cultured fibroblasts
2007 (46)
2008 (55)Protein +MU standard
2009 (59)EBV +leucocytes+ DBS

Complicated to send cultured flasks
Many fibroblasts were not alive on arrival

Difficult to obtain enough blood from patient
EBV easy to culture in bulk

HISTORY

2010(58)EBV ERNDIM
2011(61)EBV KS
2012(64)Fibroblasts pellet+DBS
2013(71)Fibroblasts
2014(76)Fibroblasts+DBS
2015(74)Fibroblasts
2016(74)Fibroblasts
2017(74)Fibroblasts

Low enzyme activity EBV
EBV not used in diagnostics and labs not familiar with the enzyme levels in this material.

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ACTIVITY ENZYMES CTRL EBV CELL LINE

ALL PARTICIPANTS 2010 SCHEME

–	Act.EBV (% ctrl leuco)	Range participants
• α-L-iduronidase	12 ± 15	1.6-69%(0.08-12 nmol/h)
• Galactocerebrosidase	16 ± 16	2-70%(0.4-15 nmol/17h)
• Sphingomyelinase	206 ± 137	1.3-795%(2-127 nmol/17h)
• α-N-acetylglucosaminidase	79 ± 32	26-284%(0.5-44 nmol/h)
• β-Hexosaminidase A + B	41 ± 29	14-204%(49-2212 nmol/h)
• β-Hexosaminidase A	42 ± 25	12-152%(5-1737 nmol/h)
• Galactose-6S sulfatase	6 ± 5	0.6-19%(0.12-19 nmol/17h)
• β-Galactosidase	25 ± 17	9-126%(0.06-136 nmol/h)
• β-mannosidase	26 ± 12	3-56%(3-118 nmol/h)
• β-Glucuronidase	36 ± 26	9.8-131%(6.6-369 nmol/h)

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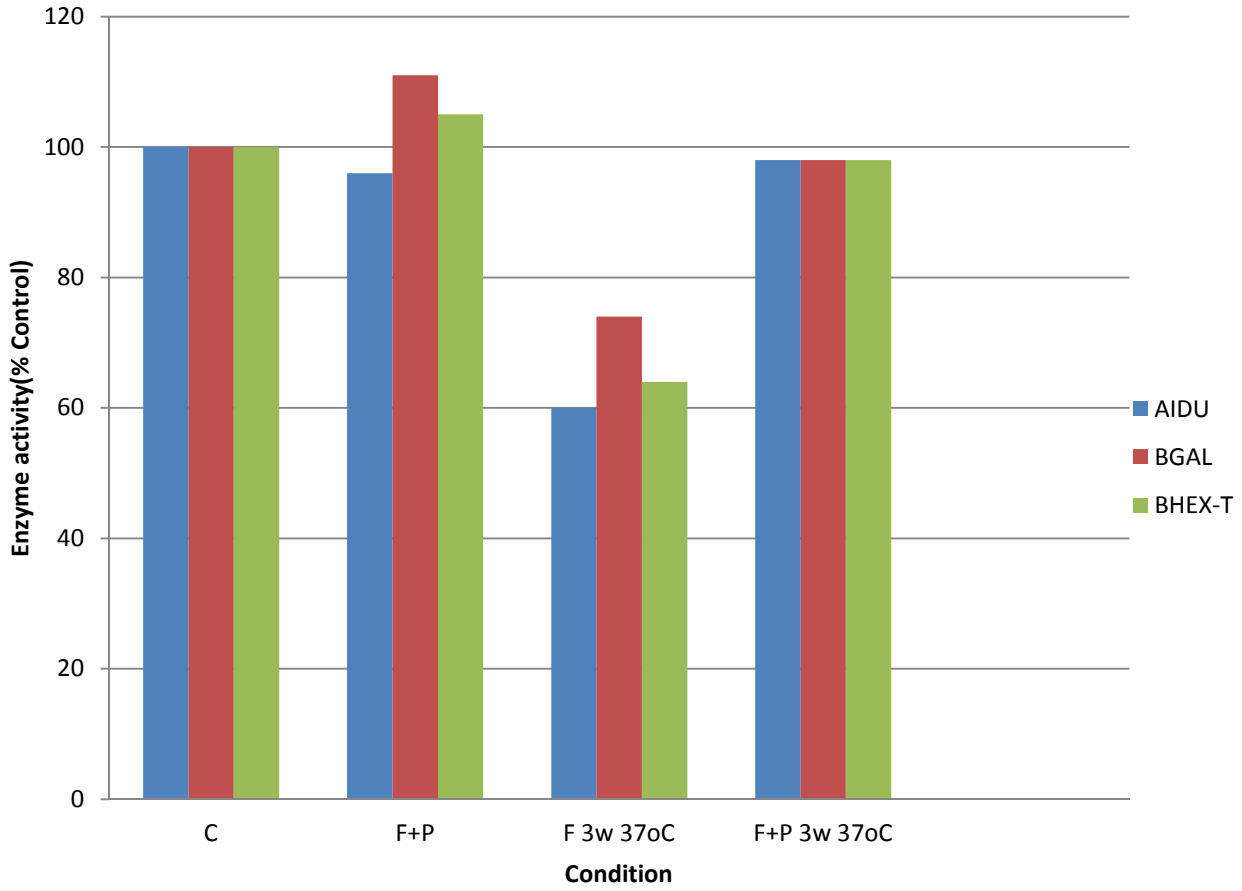
FIBROBLAST HOMOGENATES

STABILITY OF LYOPHILISED LYSOSOMAL ENZYMES



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ORGANISATION

- SELECTION OF ENZYMES IN SCHEME
- SELECTION OF SAMPLES IN SCHEME
- CULTURING ENOUGH FIBROBLASTS FOR ALL THE PARTICIPANTS (ABOUT 3 – 4 months)
- HOMOGENISATION OF FIBROBLASTS
- SUBDIVISION AND LYOPHILISATION
- SHIPMENT TO PARTICIPANTS

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LYSOSOMAL DISORDERS

Sphingolipidosis	**Oligosaccharidosis**	**Mucopolysaccharidosis**
Gm1-gangliosidosis	Mucolipidosis	MPS-I
Gm2-gangliosidosis	Fucosidosis	MPS-II
Fabry	α-Mannosidosis	MPS-III A-D
Gaucher	ß-Mannosidosis	MPS-IV A-B
Niemann-Pick A/B/C	Aspartyl-glucosaminuria	MPS-VI
MLD	Pompe	MPS-VII
Krabbe	Schindler	
CLN		
Wolman		

- What enzymes in the scheme?

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ERNDIM WEBSITE

USING LYOPHILISED FIBROBLASTS

6 samples:

- 75 laboratories
- Measure activity of 10 enzymes + protein
- Enter specific activity and % mean control.
- Tickbox for diagnosis

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RESULTS

	Lab	ALL	n
L01. Protein (mg/vial)	0.230	0.330	61
L041. Arylsulphatase-B (nmol/h/mg)	523	262	41
L042. Arylsulphatase-B (% mean control)	75.0	108	37
L06. alpha-Galactosidase (nmol/h/mg)	41.0	35.3	50
L061. alpha-Galactosidase (% mean control)	80.0	76.5	43
L08. beta-Galactosidase (nmol/h/mg)	828	536	59
L081. beta-Galactosidase (% mean control)	75.0	91.0	49
L10. alpha-Glucosidase (nmol/h/mg)	86.0	77.0	38
L101. alpha-Glucosidase (% mean control)	76.0	76.6	34
L11. beta-Glucosidase (nmol/h/mg)	367	238	52
L111. beta-Glucosidase (% mean control)	84.0	104	45
L14. beta-Hexosaminidase A+B (nmol/h/mg)	8390	4699	55
L141. beta-Hexosaminidase A+B (% mean control)	60.0	88.0	46
L18. alpha-Iduronidase (nmol/h/mg)	263	162	48
L181. alpha-Iduronidase (% mean control)	122	113	44

→

Lab specific activity (nmol/hr/mg ptn)

All labs will have developed own reference ranges or run assays with simultaneous in-assay controls. Inter-lab variability and not directly comparable.

% mean control

Many labs do not use fibroblasts. In 2017 scheme: 10 – 19% labs have not entered a value.

However, this probably more useful for inter lab comparison.

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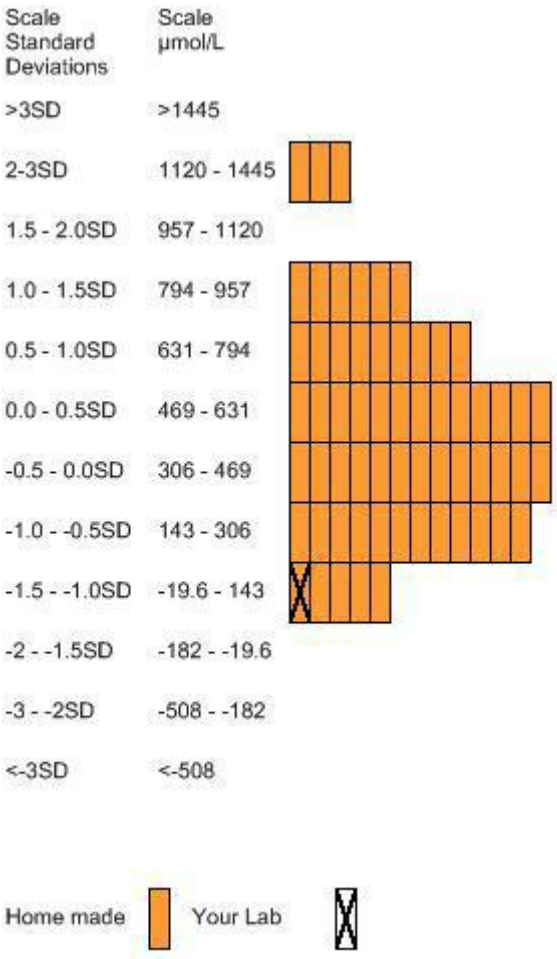
ENZYME ACTIVITIES (nmol/h/mg)

BGAL 2 nmol/h/mg

– LAB C



Judgement red flags analogous to metabolite scheme?

N=60
Mean±sd
522±263

QUANTITATIVE WITHIN THE LIMITS
QUALITATIVE ?????

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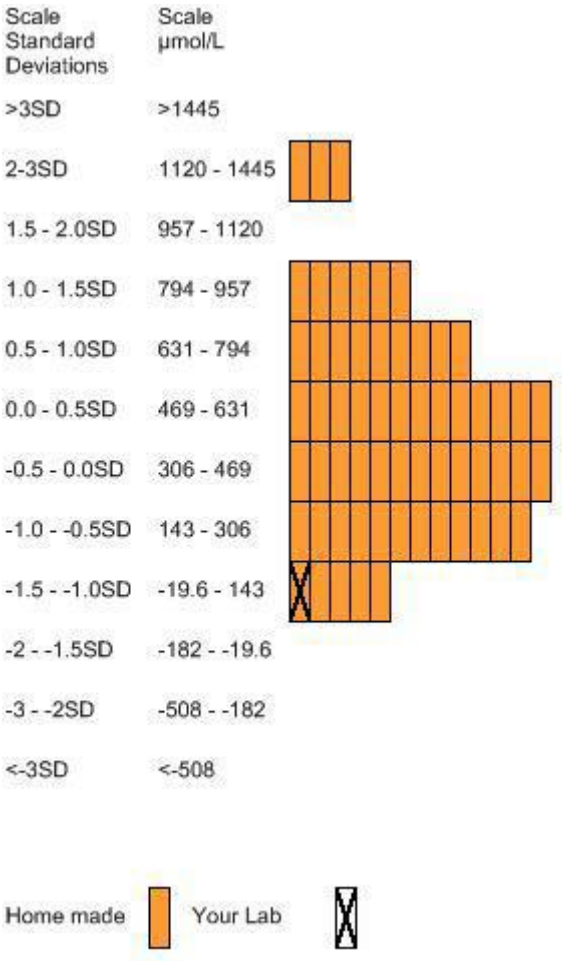
RELATIVE ENZYME ACTIVITIES(%)

BGAL 0.6 %

– LAB C



N=60
Mean±sd
98±67

Diagnosis incorrect

- Enzyme activity control sample <15% mean control range
- Enzyme activity patient sample >30% mean control range

QUALITATIVE FALSE POSITIVE

QUANTITATIVE NO RED FLAG

Red flags cannot be used in enzyme scheme

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INDICATION OF DIAGNOSIS

Interpretation of the results	
Your Score :	
Mean Score all Labs :	0.10
Frequency :	

Code	Description
1	No obvious enzyme deficiency
2	beta-Hexosaminidase A+B deficiency
3	alpha-Galactosidase deficiency
4	beta-Galactosidase deficiency
5	alpha-Glucosidase deficiency
6	beta-Glucosidase deficiency
7	beta-Hexosaminidase A deficiency
8	alpha-Iduronidase deficiency
9	Galactose-6-sulphate sulphatase deficiency
10	Galactosylceramidase deficiency
11	Sphingomyelinase deficiency

- Useful for labs to see correct results.

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Discussion

- Are fibroblasts an appropriate material for the LSD scheme?
- Are the number of samples (6) and submissions (3) appropriate?
- Is the website appropriate for recording the data?

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SCORING SYSTEM

Nr.Enzymes	ASB	AGAL	BGAL	BGLU	AGLU	BHEX-T	AIDU	IDU2S	GALC	SM	PROT	Nr. satisfactory enzymes	Score
5(22)	2	4	4	4	0	0	4	0	0	0	2	4	20
7(30)	4	4	4	4	4	4	0	4	0	0	2	7	30
6(26)	2	0	4	4	0	0	4	4	0	4	2	5	24
9(38)	4	4	4	4	4	4	4	4	0	4	2	9	38
10(42)	4	4	4	4	4	4	4	4	4	4	4	10	42
9(38)	4	4	4	2	4	4	2	0	4	4	4	7	34

Quantitative Red flags+Qualitative (Reproducibility + Diagnosis)
Reproducibility 2 pt for every enzyme and protein
CV>60% 0 pt; 35<CV<60 1 pt
Diagnosis 2pt for every enzyme
Sample not measured, 6 samples for every sample -16% of total points
Diagnosis not correct 0 pt,
Diagnosis correct but incorrect level measured enzyme activity 1 pt
Diagnosis not correct but correct level measured enzyme activity 1 pt

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Scoring scheme:

Diagnosis (2) and CV (2)

Participants score >60% (average all enzymes) to be satisfactory performer.

Most important is to obtain correct diagnosis

Affected patient missed or unaffected patient given incorrect diagnosis.

Score each enzyme individually?

Discussion

- Is the scoring system appropriate?
- Is it more appropriate to mark enzymes individually?
- Are the enzymes offered satisfactory – should other enzymes be included?
- Is it more appropriate for the LSD enzyme scheme to be a qualitative (diagnostic proficiency) scheme?

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Qualitative scheme

- Enzyme results
- Enzyme deficiency consistent with diagnosis of X
- Normal result which would make diagnosis of Y highly unlikely.
- Any further comments to confirm diagnosis etc.

Other issues

- Labs have own reference ranges (or may quote simultaneous controls).
- What are labs using to report % mean values (seem to be discrepancies!). ? Useful to report using sample 1 as normal reference sample.
- Labs may be using different substrates (radiolabelled natural substrates/ fluorescent substrates).

This scheme needs to be simplified!

Qualitative scheme

- Enzyme results
- Enzyme deficiency consistent with diagnosis of X
- Normal result which would make diagnosis of Y highly unlikely.
- Any further comments to confirm diagnosis etc.

Discrepancies with artificial substrates.

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NIEMANN PICK A/B
Q292K MUTATION

- Affected patient with Q292K mutation included in scheme.
- Normal activity observed with artificial substrate.
- 50% of participants missed this diagnosis
- Overcome by addition of lyso-sphingosine (van Diggelen et al)

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Discrepancies between natural/artificial substrates

- Traditionally diagnosis of Krabbe disease relied on measurement of enzyme deficiency using a radiolabelled natural substrate (>30yrs).
- More convenient fluorimetric substrate implemented in some labs; (6-hexadecanoylamino-4-methylumbelliferyl- beta-D-galactoside available from Moscerdam & Glycosynth).
- Potential for false positives – pseudodeficiencies.
- Potential for false negatives – normal GALC activity against artificial substrate.
- Similar situation with Niemann Pick A/B.

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In 2017 ERNDIM Scheme:

- 3 participants measured a **normal level** of galactocerebrosidase activity in an **affected patient sample.**

- 6 participants measured a **deficiency** of galactocerebrosidase activity in an **unaffected sample.**

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CASE 1a: Diagnosis confirmed by molecular genetics

Patient

Index case is a child with biochemically diagnosed Krabbe disease (measured In leucocytes using the natural substrate for the enzyme[)]:

	Patient	Normal range
Galactocerebrosidase (nmol/h/mg)	0.01	0.4 – 4.0

Screening of the *GALC* gene showed the patient was homozygous for the c.621+2T>C pathogenic variant confirming the diagnosis.

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CASE 1b: Pseudodeficiency

Patient's unaffected mother

Enzyme testing was also carried out for the patient's unaffected mother: results were very low / deficient.

	Patient's mother	Normal range
Galactocerebrosidase (nmol/h/mg)	0.04	0.4 – 4.0

Screening of the *GALC* gene showed: in addition to being heterozygous for the **c.621+2T>C** variant detected in her affected son, this individual was also heterozygous for the **c.550C>T p.(Arg184Cys)** variant.

This variant is present in up to 6% of alleles in the European population, is not associated with enzyme deficiency when expressed in COS-1 cells and is generally regarded to be non-pathogenic.

As this individual is unaffected the observed enzyme deficiency is termed 'pseudodeficiency' and is due to reduced activity associated with c.550C>T.

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CASE 2: Carrier identified by enzyme analysis

Patient with developmental delay who was referred for lysosomal enzyme analysis. (Manchester Willink)

Galactocerebrosidase (nmol/h/mg)	Patient	Normal range
Fluorescent	0.34	0.8 – 4.0
Natural	0.13	0.4 – 4.0

i.e. patient has reduced but clearly not deficient activity. Chitotriosidase normal

GALC gene showed the patient to be heterozygous for the common 30kb deletion and also for the c.550C>T variant. NOT AFFECTED.

Note: The incidence of Krabbe disease in the general population is very low, equating to a carrier frequency of approx. 1/160. However as several hundred patients undergo lysosomal enzyme screening in the larger UK biochemical genetics labs per year we would occasionally expect to pick up carriers.

Fluorescent substrate picking up these carriers and pseudodeficiencies.

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Thank you – any questions?