Screening for ALD using MS/MS

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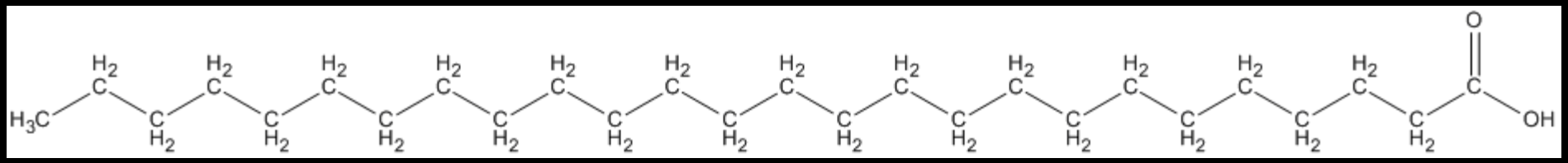
Joseph Orsini, Ph.D.
joseph.orsini@health.ny.gov
518-473-8366



Condition Information

- Causes damage to: the myelin sheath and the adrenal gland
- Accumulation of saturated very long chain fatty acids (VLCFAs: C26:0)
- Lack of a transporter protein that moves VLCFA into peroxisomes for degradation
- Affects predominantly males; females can have mild disease; rarely cerebral disease in females
- Frequency: 1/17,000 – 1/20,000 births
- Expect 12 to 15 cases annually in New York
- X-Linked, mutations in ABCD1 gene

Three Major Types of Adrenoleukodystrophy

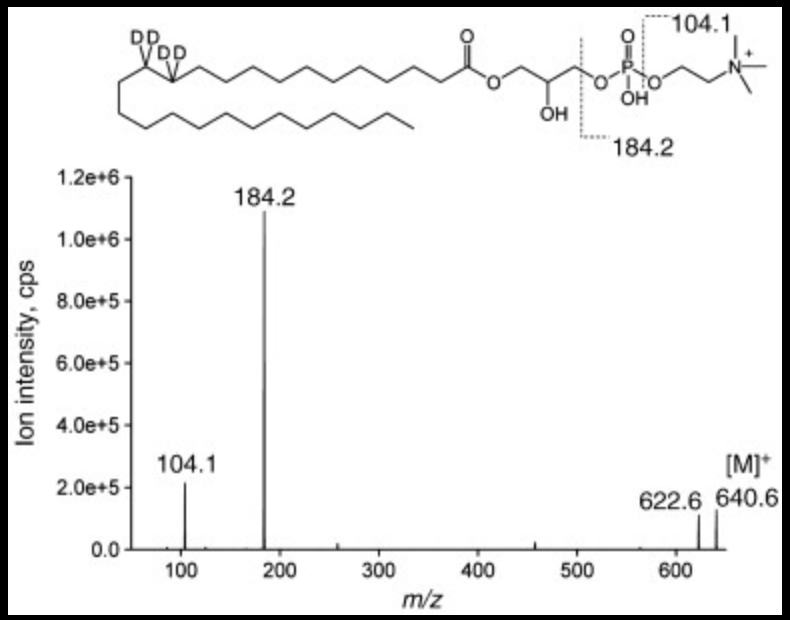
- Childhood cerebral form (4-8 years/45%): hyperactivity, vision problems, loss of verbal understanding, regression in school, handwriting, seizures, aphagia

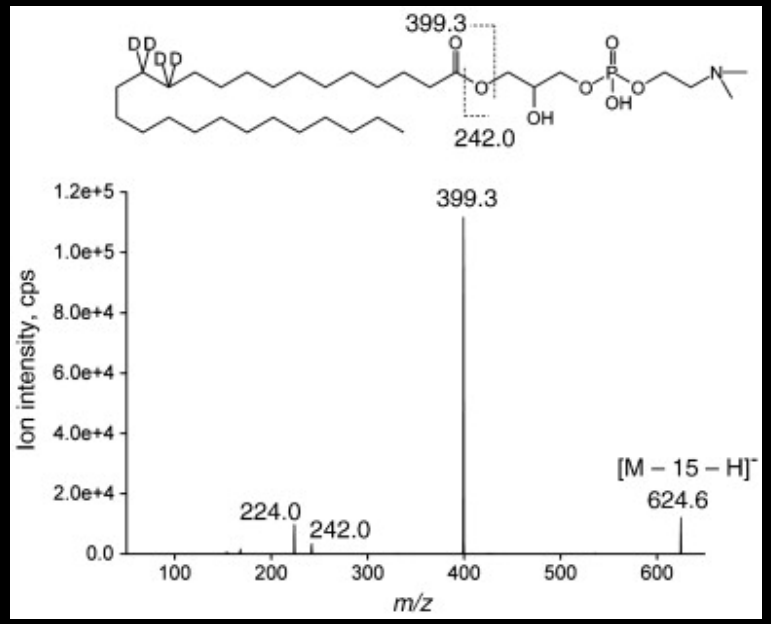
- Adrenomyeloneuropathy (males in their 20's/35%): muscle weakness, difficulty thinking quickly, poor sight memory; uncontrolled urination

- Addison's disease: lack of steroid hormones (cortisol and aldosterone); decreased appetite, low blood pressure, increased pigmentation, muscle wasting, vomiting, coma

Assay approach

- C26:0 is the marker, used in diagnostic testing for ALD
- C26:0 is elevated in dried blood spots, so use C26:0 LPC (lysophosphatidylcholine) (Raymond, Jones, Moser, 2007)
- Electrospray MS/MS, positive mode or negative mode
- Unknown interferent(s) using positive mode (need to follow first tier with second tier HPLC-MS/MS)





Some assay options

1. HPLC-MS/MS for C26:0 LPC. May work for smaller labs, improved accuracy, pick up more carriers (Hubbard, et. al., 2009)

2. First tier: FIA-MS/MS: Screen for C26:0 LPC (and 20,22,24); second tier HPLC-MS/MS. Requires additional capacity (Sandler et. al.; 2012)

3. Combine with amino acid/acylcarnitine assay. Similar to aa/ac approach, requires using an un-derivatized AA/AC method (under development, AC/C26:0-LPC, Sandler, et. al; 2012)

4. Negative ion mode analysis of C26:0-LPC (De Jesus, Haynes; 2012)

5. Combine with LSD assay (Matern, unpublished), followed by second tier HPLC-MS/MS: for NY, no extra capacity required for first tier, “simplest for us to implement”

New York State Assay
(LSD and ALD)

Punch 3-mm specimen, add 200 µL methanol with d4-C26:0 LPC

↓ **1 hour extraction**

Remove 25 µL of extract and combine with LSD extract (optional)*

↓

Analyze samples, 1.5(1.0) minutes per sample/Marker is C26:LPC (C20,22,24,26)

↓

Follow screening algorithm

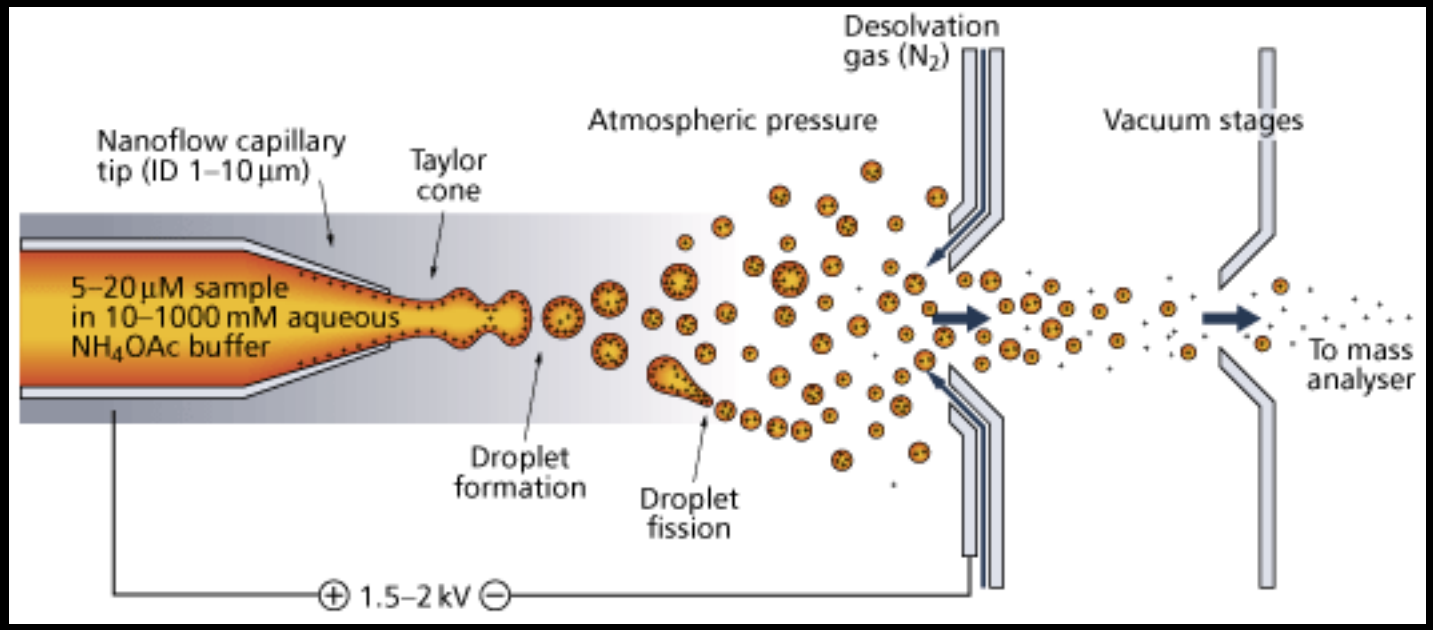
* Important to combine quickly with LSD extract.

Technical challenges in ALD Screening

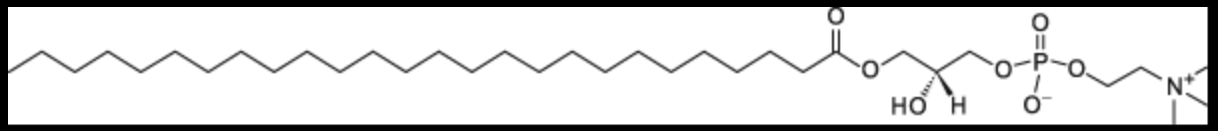
- Reproducibility issues: need to minimize evaporation (initial versus retest)
- Edge Effects on plates (evaporation, increased ionization suppression, corrected)
- Adding C26:0-LPC MRM channel to LSD test: – lost GALC-IS/P signal (memory effect - corrected)
- Adding ALD extract to GALC: Linearity of GALC affected - slope 1.5 (normally 1.1, corrected)
- ALD and LSD extracts need to be combined quickly (avoid evaporative loss, which leads to ionization suppression)

- C26:0 LPC sticks to glass
- solubility issues with C26:0 LPC (minimal water)
- C26:0 requires a lot of energy to ionize
- Ionization suppression

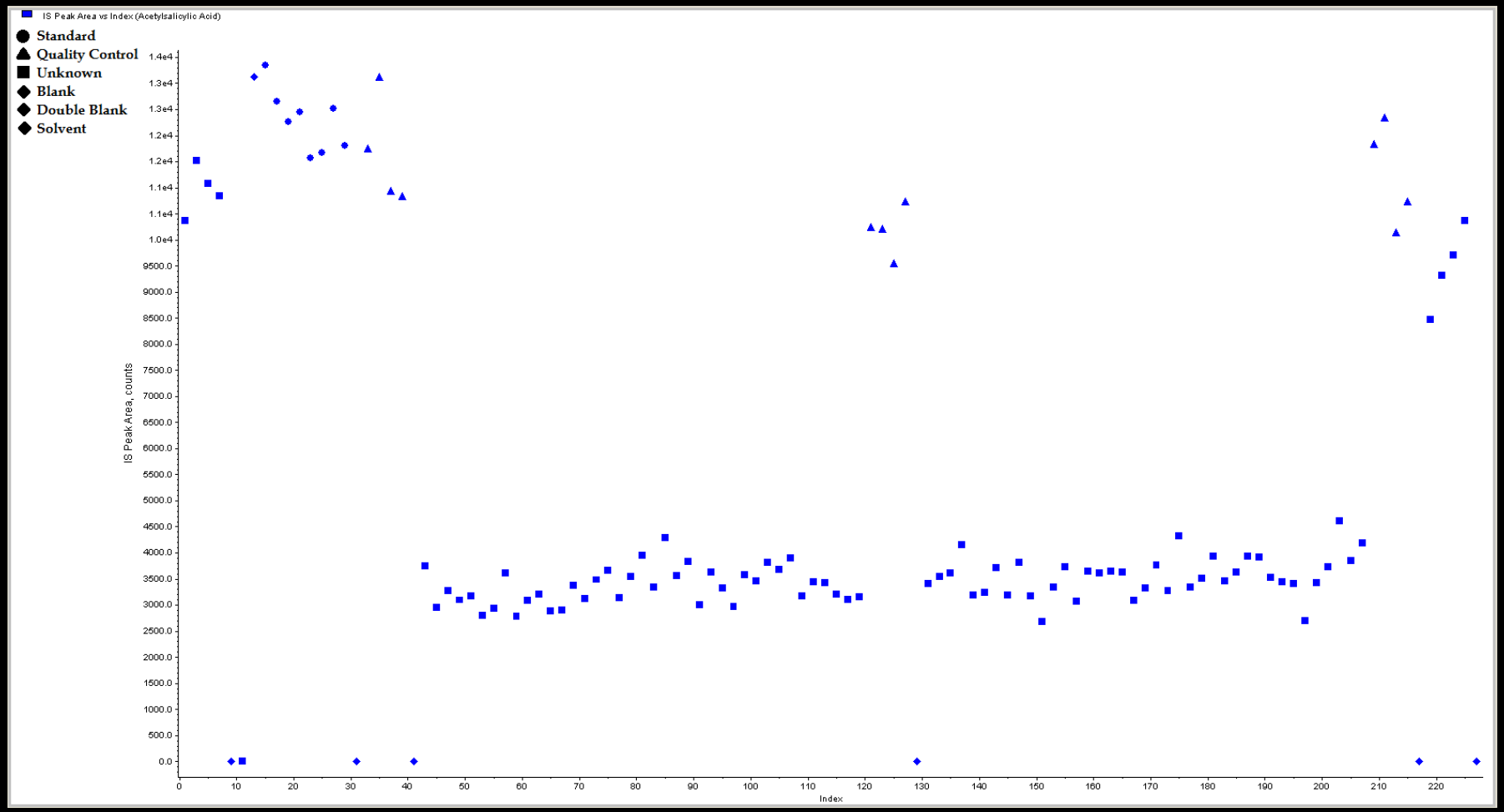
MS/MS Electrospray ionization



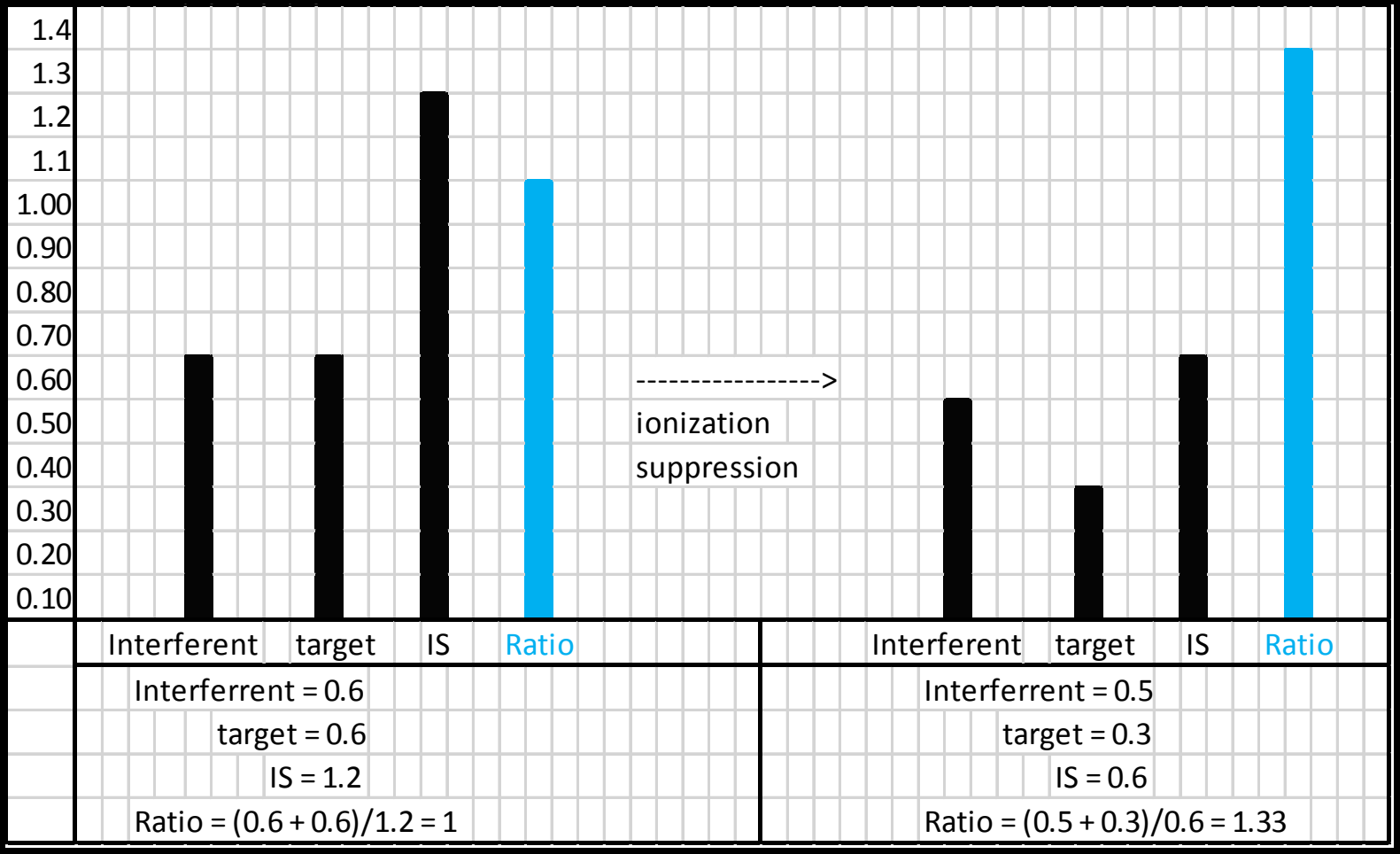
Ionization suppression is an issue: solution is to use deuterated internal standards: ionization suppression effect is the same on IS and target compound



Ionization Suppression



Ionization Suppression: example



	Interferent	target	IS	Ratio
	Interferrent = 0.6	target = 0.6	IS = 1.2	Ratio = (0.6 + 0.6)/1.2 = 1

	Interferent	target	IS	Ratio
	Interferrent = 0.5	target = 0.3	IS = 0.6	Ratio = (0.5 + 0.3)/0.6 = 1.33

- Interferent and target have same MRM – add together
- Ionization suppression affects IS and target compounds the same. Affect on the interferent is less
- The measured ratio is higher as a result

Ionization Suppression (100%, 50%,25% ALD Extract added to LSD extract)

100% ALD ext	galc_P	galc_IS_cnt	galc_P_cnt	c26	d4c26	c20	gaa_P	gaa_IS_cnt	gaa_P_cnt
LSD000_1	0	81430	154	0.16	6886	0.2	0	463100	58
LSD005_1	0.06	77070	4938	0.15	6947	0.15	0.05	442100	22970
LSD010_1	0.12	70260	8770	0.22	5837	0.23	0.11	425100	45640
LSD050_1	0.7	63320	44180	0.2	5664	0.27	0.54	391700	213100
LSD100_1	1.35	71630	96690	0.17	5969	0.17	1.09	424700	463900
LSD200_1	2.7	68670	185100	0.13	6255	0.19	2.19	440500	964700
LSD500_1	6.53	51860	338400	0.17	4791	0.21	5.33	335700	1788000
Average		**69177**	**96890**	**0.17**	**6050**	**0.20**		**417557**	**499767**

50% ALD ext	galc_P	galc_IS_cnt	galc_P_cnt	c26	d4c26	c20	gaa_P	gaa_IS_cnt	gaa_P_cnt
LSD000_1	0	114100	188	0.14	5848	0.16	0	623100	160
LSD005_1	0.06	113300	6615	0.13	5756	0.15	0.05	610200	32600
LSD010_1	0.12	135300	16410	0.16	5314	0.18	0.11	684800	75020
LSD050_1	0.66	118600	77750	0.12	5762	0.17	0.54	645500	350000
LSD100_1	1.34	112800	151000	0.14	5692	0.13	1.1	619300	680700
LSD200_1	2.6	103600	269500	0.15	4754	0.16	2.17	599500	1299000
LSD500_1	6.56	82950	544500	0.18	4764	0.19	5.35	502700	2688000
Average		**111521**	**152280**	**0.15**	**5413**	**0.16**		**612157**	**732211**

25% ALD ext	galc_P	galc_IS_cnt	galc_P_cnt	c26	d4c26	c20	gaa_P	gaa_IS_cnt	gaa_P_cnt
LSD000_1	0	257800	399	0.11	3070	0.19	0	1033000	202
LSD005_1	0.05	250600	13610	0.16	2995	0.16	0.05	1027000	53590
LSD010_1	0.11	262500	27660	0.13	2805	0.17	0.11	1035000	111900
LSD050_1	0.58	248200	143800	0.09	2876	0.16	0.54	987600	535300
LSD100_1	1.23	220300	271700	0.11	3007	0.18	1.09	928000	1010000
LSD200_1	2.47	192300	475100	0.1	2423	0.16	2.14	855700	1834000
LSD500_1	5.96	173300	1032000	0.13	2298	0.16	5.28	751200	3965000
Average		**229286**	**280610**	**0.12**	**2782**	**0.17**		**945357**	**1072856**

- LSD IS counts go up with decreasing amount of ALD extract
- GALC ratios more affected than GAA ratios (GAA had deuterated IS)
- ALD counts do not scale with dilution
- ALD ratios tend to get lower as decrease amount of ALD extract used
- Lower volumes – magnify evaporation issues during plate processing

Population Statistics (12/30/13 – 1/29/15)

C26:0-LPC (uM)	
mean	0.23
StDev	0.066
max	2.78

ALD N = 281647 samples		
C26:0	**Count**	**Yr-Count**
>0.35	12794	11356
>0.4	5026	4461
>0.5	782	694
>0.6	230	204
Birthrate for NY = ~240,000		
First tier cutoff = 0.4 μM		

Positive Controls

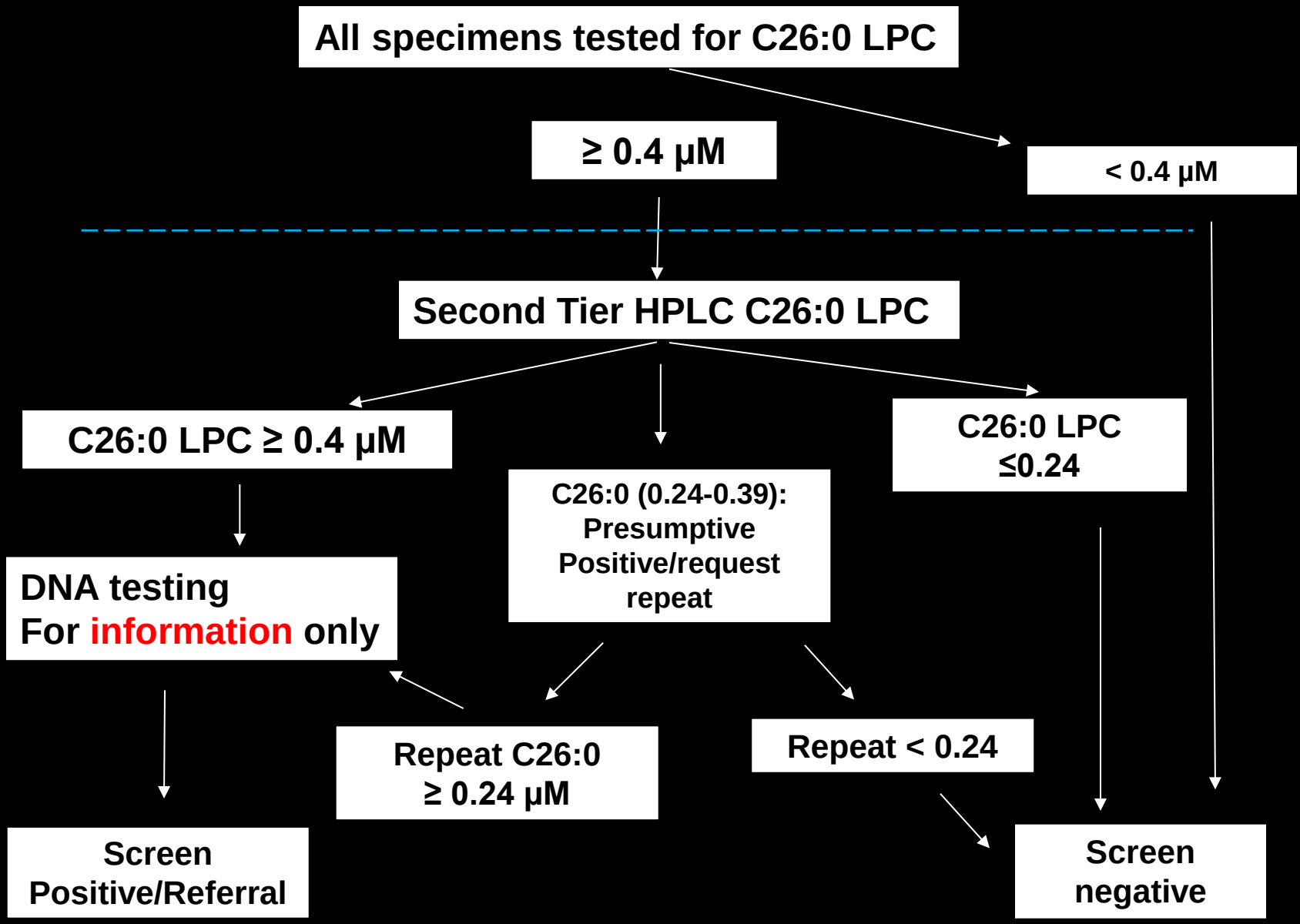
Positive controls, Tier 1 results

Sample ID	Accession #	Condition	C26:0 (µM)
ALD_1	20042872073	ALD	1.2
ALD_2	20131571625	Zellweger	1.75
ALD_3	20042091488	ALD	1.3
ALD_4	20070191816	Zellweger	1.53
ALD_5	20001511848	ALD	0.78
ALD_6	19991892305	ALD	1.08
ALD_7	20021191634	ALD	1.09
ALD_8	20100251314	Carrier	0.78
ALD_9	20041381090	ALD	1.19
ALD_10	20023531007	ALD	1.28

Mayo positive controls

Sample ID	Patient information	C26:0 (µM)
PLSD 041614-04	XALD #67655 7.7 year old male	1.03
PLSD 041614-05	XALD #61933 7.8 year old male	0.48
PLSD 041614-06	XALD #67651 8.8 year old male	0.69

ALD Screening Algorithm

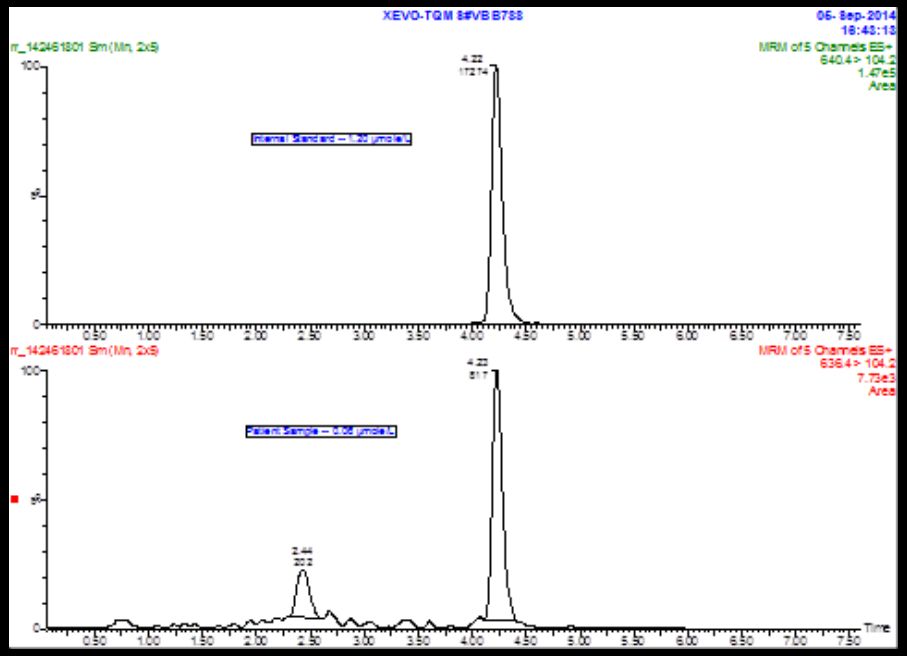


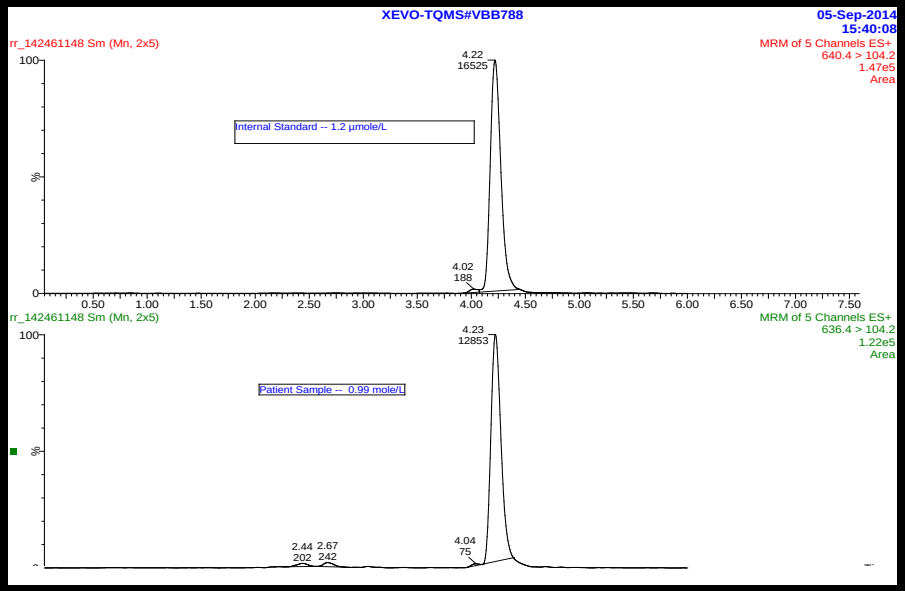
Second Tier: HPLC-MS/MS

Methanol Extract

Column:
Waters Xterra C8
2.5 uM particle size

Linear Gradient
Mobile A: 20:80 (MeOH/H20)
Mobile B: 100 MeOH





Sample ID	Sample Result (µMole/L) 1st Tier	2nd Tier
NB_3223	0.40	0.12
NB_3231	0.41	0.08
NB_3251	0.48	0.11
NB_3286	0.40	0.09
NB_3288	0.42	0.08
NB_3337	0.40	0.07
NB_3364	0.42	0.06
NB_3387	0.40	0.08
NB_3542	0.41	0.08
NB_3544	0.92	0.08
NB_3556	1.09	0.10
NB_3669	0.43	0.07
NB_3948	0.45	0.11
NB_5395	0.41	0.09
NB_5997	0.55	0.10
NB_6009	0.42	0.08
NB_6251	0.42	0.09
NB_6253	0.40	0.07
NB_6771	0.43	0.09
NB_6772	0.53	0.08
NB_6919	0.41	0.05
NB_6981	0.53	0.36
NB_8321	2.44	0.14
NB_8322	1.62	0.07
NB_8326	0.42	0.08
NB_8327	0.64	0.11
NB_8424	1.81	0.08
NB_8439	0.58	0.09
NB_8451	0.51	0.08
NB_8511	0.40	0.09
NB_8599	0.97	0.08
NB_8600	0.50	0.09
NB_8601	0.43	0.09
NB_8846	0.61	0.36
NB_8872	0.41	0.08
NB_8895	0.40	0.14
NB_9001	1.02	0.87
NB_9148	1.06	0.08
NB_9160	0.95	0.10
NB_9172	0.41	0.07
NB_9220	0.43	0.08
NB_9480	0.41	0.08
NB_9589	0.42	0.10
NB_9663	0.40	0.14
NB_9669	0.45	0.09

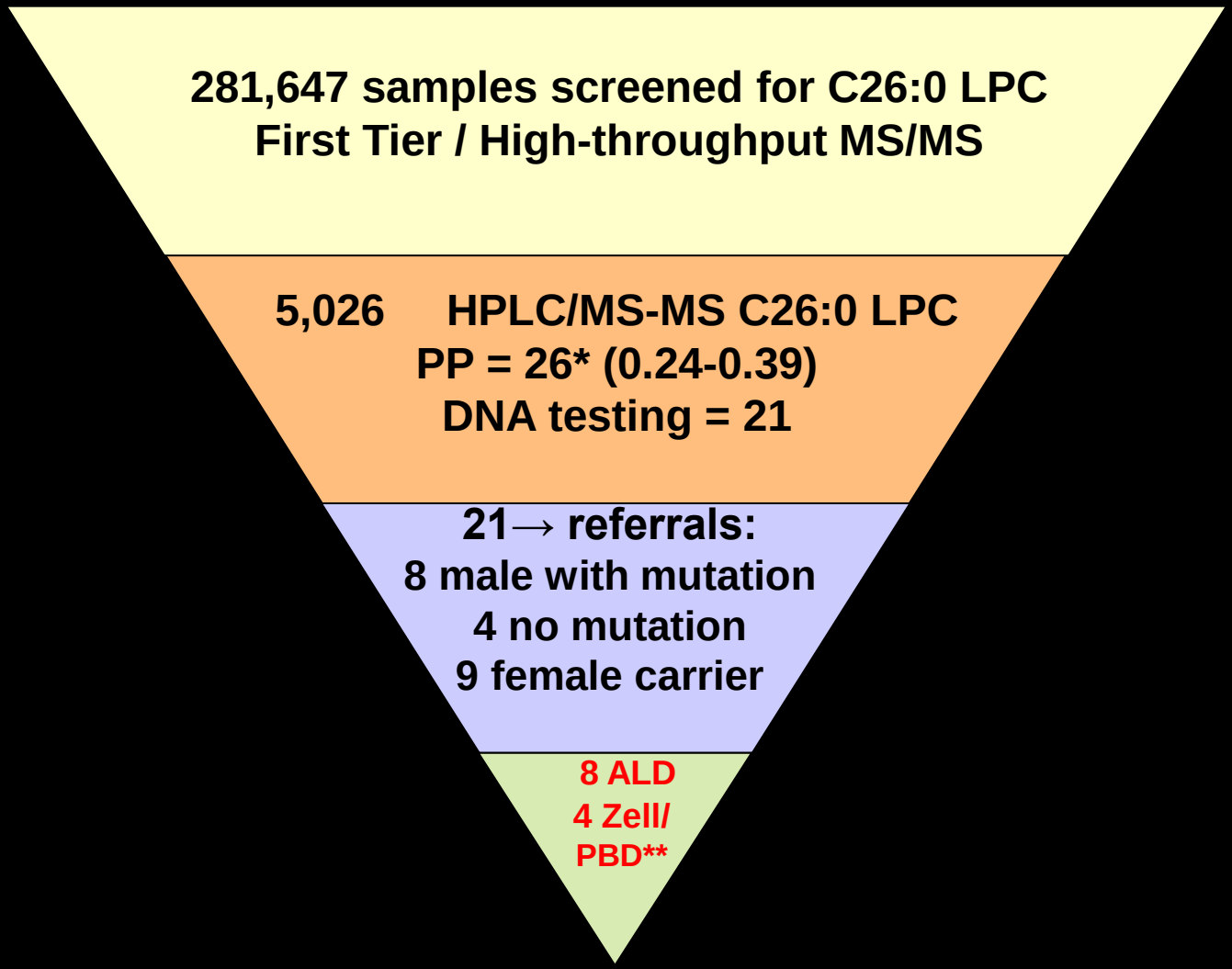
Third Tier: DNA Sequencing

1. Full sequencing of ABCD1 gene
2. Not intended to reduce referrals
3. Helps to Determine
    a. if females are ALD carriers
    b. if males have mutation
    c. if no mutation, consider other PGD

4. Genotype does not correlate with phenotype

New York State Newborn Screening for X-ALD

December 30, 2013 to January 29, 2015



ALD by the Numbers (~250,000 births)

- **Referral rate: 1 in 11,900 or 0.008% of infants screened**
- **Incidence of ALD*: 1 in 31,250 births**
- **Incidence of ALD*: ~1 in 15,625 males**
- **Incidence of PGDs: 1 in 62,500 births**

Too early for stable incidence rates – prediction is 1 in 17,000 to 1 in 20,000 births

* ***Assumption that all with Muts will become symptomatic, not counting female carriers, who may become symptomatic.***

Other challenges in ALD Screening

- **Genetic diversity –** ***(novel variants?; VOUS)***
- **Incomplete genotyping –** ***(undetected variants?)***
- **Later onset condition –** ***(boys and AMN)***
- **Potential for carriers to be symptomatic**
- **Assay doesn’t identify all carriers**
- **Potential for Dad to have AMN**
- **Lack of genotype:phenotype correlation**
- **Lack of correlation of C26 concentration to severity of disease**

Acknowledgements

- Monica Martin: first tier method development
- Mark Morrissey/Cathy Lubowski: second tier method development
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