Endogenous Non-Reducing End Glycosaminoglycans for Newborn Screening and Diagnosis of Mucopolysaccharidoses

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Mucopolysaccharidosis Types I, II, IIIA-D, IVA, IVB, VI, VII

Lysosomal storage diseases due to the deficiency in enzymes that breakdown glycosaminoglycans (GAGs).

Treatments are available for MPS-I, -II, -IVA, -VI, and –VII

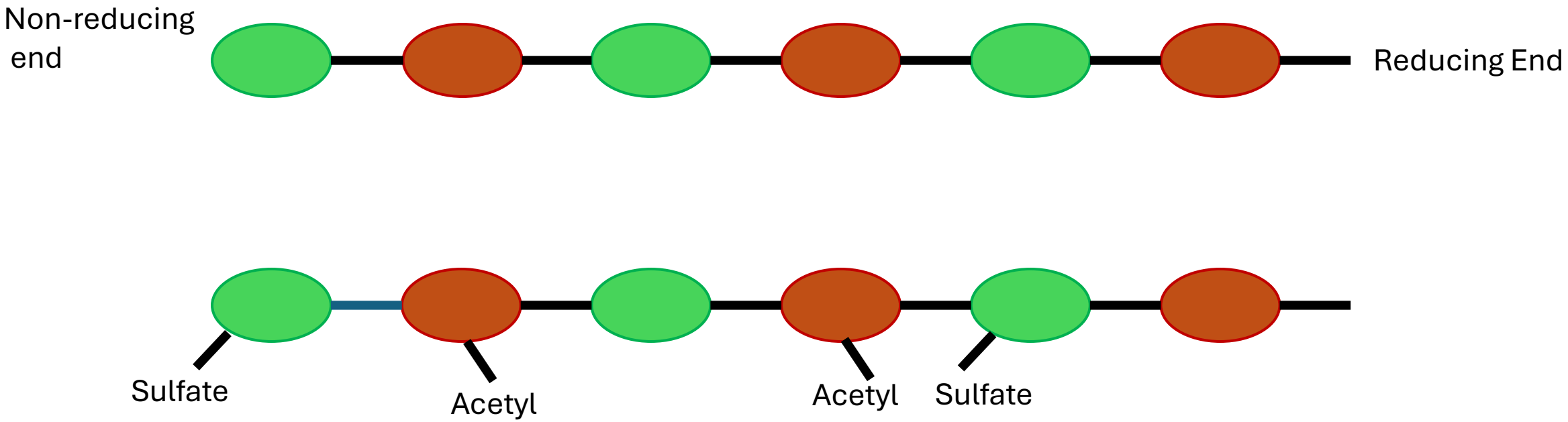
Late stage clinical trials for MPS-IIIA.

Newborn screening for MPS-I and –II is live in several NBS labs across the USA and in a few additional countries.

The newborn screening assays (developed by the Gelb lab at Univ. of Washington) involves first-tier measurement of the activity of the relevant enzyme in dried blood spots. It is now accepted by essentially all experts in the field that second-tier analysis of GAGs in DBS is the method of choice to reduce false positives. This is considered more accurate than genotyping, although genotyping is often done to augment the newborn screening report.

Enzymatic assays for all MPS enzymes in dried blood spots have been published by the Gelb lab. They can all be multiplexed together into a single LC-MS/MS assay (and multiplexed with other lysosomal storage and other diseases).

GAGs are always composed of single sugars (ovals) hooked together into long chains. The GAG class (Heparan Sulfate, Keratan Sulfate, etc…) is determined by the identity of the sugars that form the diad repeat unit.
The polymer chain is polarized (ends are different), one is called the reducing end and the other the non-reducing end.



The GAG chains are "sprinkled" with additional structural elements (sulfation and acetylation), and the location of these elements is thought to be random or semi-random. Also in some GAGs, the identify of the monosaccharide is variable (i.e. iduronic acid and glucuronic acid in the repeating diad unit of Heparan Sulfate). This gives 1000s of different molecular species; thus heparan sulfate for example is a highly complex mixture of polymers.

Why do we measure enzymatic activity first tier and GAGs by second-tier instead of the vice-versa approach?

I have heard some experts suggest we analyze GAGs first. I disagree with this. It should be enzyme first.

Newborn screening for the full set of mucopolysaccharidoses in dried blood spots based on first-tier enzymatic assay followed by second-tier analysis of glycosaminoglycans
Molec. Genet. Metab. (2023) 140(3)

Measuring GAGs first is actually more expensive than measuring enzymes first when you consider all factors.

Costs for enzymatic substrates are coming down due to the multiple companies now providing them rather than just one.

The false positive rate of first-tier GAG analysis is much higher than that for first-tier enzymatic activity assays.

The enzymatic assays are better multiplexed with other LSDs and diseases.

GAG analysis typically requires more sensitive MS/MS instruments and longer analysis times, thus it makes more sense to run GAG analysis on the handful of samples per year per lab that are first-tier, low-enzyme activity positive.

Modern methods are based on tandem mass spectrometry of GAG FRAGMENTS. Analysis of the full length polymer is not possible with mass spec, too many molecular species. Older methods are based on colored dyes bindng to the charged GAG polymers and give a qualitative estimate of the amount of total GAG polymer. The dye binding methods are not specific for the type of GAG.

There are 4 MS/MS GAG methods in play. Two are Non Reducing End (NRE) and two are not:

1. Digest the GAG with bacterial enzymes to make 3-4 different disaccharides that come from the entire length of the polymer (not just from the ends). So 1000s of molecular species become 3-4 disaccharides (depending on where the sulfation and acetylation occurs).

Enzymatic Internal Disaccharide method (used by several reference labs including Mayo). Probably the most commonly used method worldwide.

2. An alternative to the above is to heat the GAGs with methanol causing non-enzymatic cleavage into 3-4 different methyl glycoside-disaccharides that come from the entire length of the polymer

Methanolysis Internal Disaccharide method (used by Greenwood Genetics and Duke)

3. Digest the GAG with bacterial enzymes to make 3-4 different short saccharides that come from the non-reducing end of the polymer

Enzymatic NRE method (also known as Sensi-Pro, used by ARUP). The NRE fragments have a characteristic structural signature that distinguishes them from Internal Disaccharides (that do not come from the NRE).

4. No digestion of the GAG polymer, rather short saccharides from the NRE already present in biological fluids are detected

Endogenous NRE method (used by Fuller lab in Adelaide, Revvity Genomics and other places coming)

All of these methods give rise to ~3-4 different short GAG-derived fragments that can be individually detected by MS/MS.

NOTE: There are TWO NRE methods in play, so not precise to simply say NRE method !!!

There was a satelite mtg associated with the Athens SSIEM mtg about 10 yrs ago organized by BioMarin to discuss GAG methods in certified reference labs worldwide.

A report was published, and the conclusions are that there are 5 methods (1 dye binding, 4 mass spec) in play.

Absolute values of GAGs biomarkers in biological fluids CANNOT be compared across different testing labs. There are no calibration standards to allow for congruence across different testing labs.

Relative changes in GAG levels may be comparable across different testing labs.

For example, the FOLD change in GAG biomarker from a patient prior to initiation of treatment versus time after treatment.

GAG levels are age-dependent, and testing labs have tried to compare GAG levels in treated patients to age-matched levels in non-treated patients.

Two problems were identified at the Athens SSIEM mtg:

1. Some false positives remain with MPS NBS

2. Cannot compare absolute GAG values across different reference labs.

The main point of my talk today is that both problems are now solved.

It is informative to review what has happened with measurement of Psychosine for newborn screening and management of Krabbe disease.

The absolute concentration of psychosine matters. If DBS psychosine > 10 nM it is almost always infantile Krabbe disease and rush to prepration for transplant is the current consensus guideline.

When DBS psychosine is 2-10 nM it is increased risk for late onset Krabbe disease, and this determines the follow-up plan for the newborn.

So the absolute value of psychosine matters, just like the absolute value of cholesterol determines the treatment.

Prior to achieving congruence, multiple psychosine testing labs were not reporting the same concentration of psychosine on the same samples tested across different labs. The only solution is for all labs to use the same set of psychosine calibrators, which are now available. Just like for any test, callibration is required.

Herbst et al. Int J Neonatal Screen. 2020 Jun;6(2):29

As you will now see, the Endogenous NRE GAG analysis method is substantially better than the other methods for second-tier newborn screening of all types of MPS disease.

Newborn screening of MPSs is based on first-tier measurement of the activity of the relevant enzyme in dried blood spots.

False positives are found (~30-50 per 100,000 newborns).

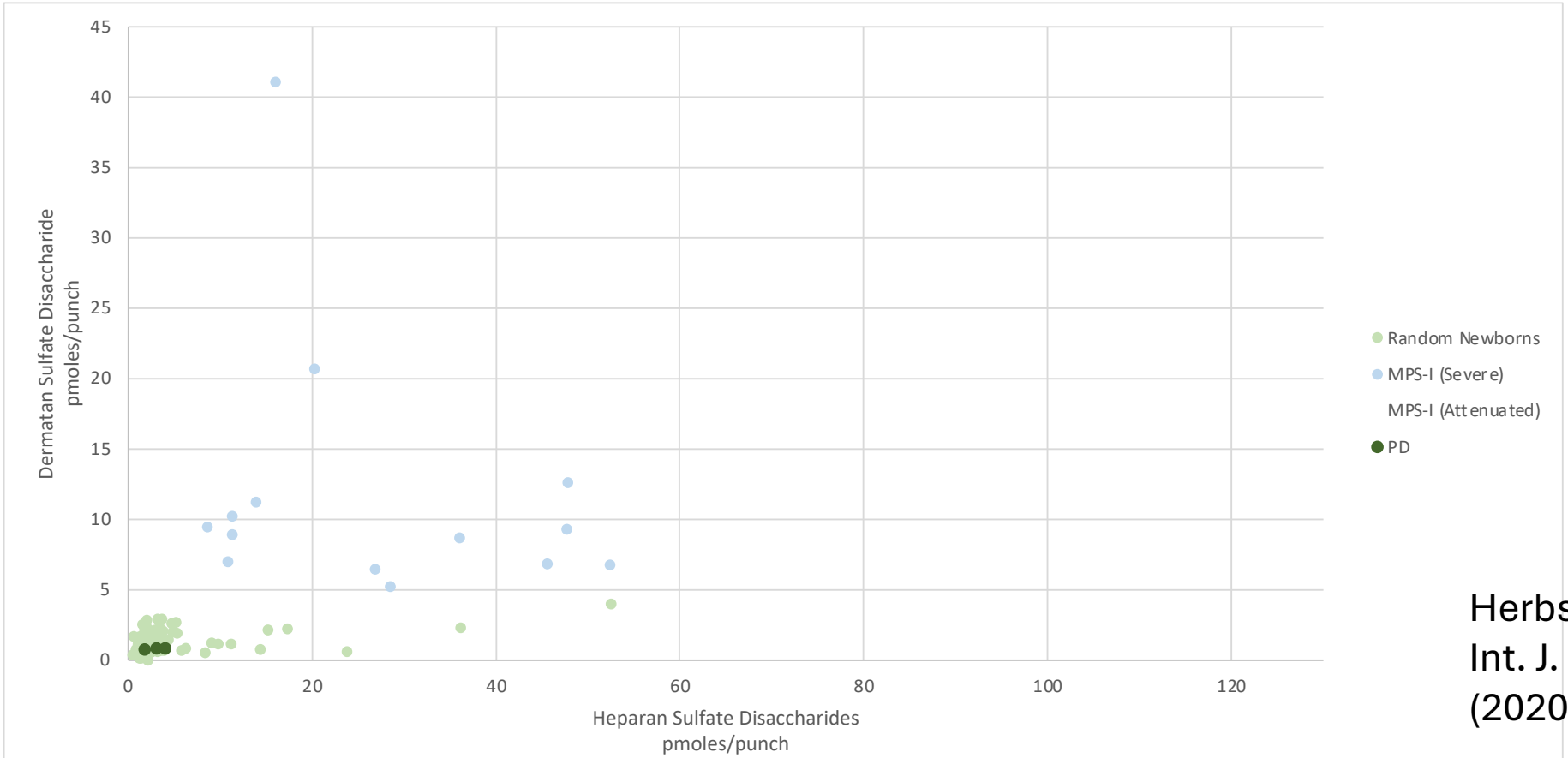
GAG analysis provides a second-tier component of newborn screening that is more powerful than molecular analysis (genotyping) for eliminating false positives (as you will see).

We measure enzyme activity first because it is faster and easier than measurement of GAGs and it works better.

Enzymatic Internal Disaccharide GAG Analysis of Newborn DBS for MPS-I

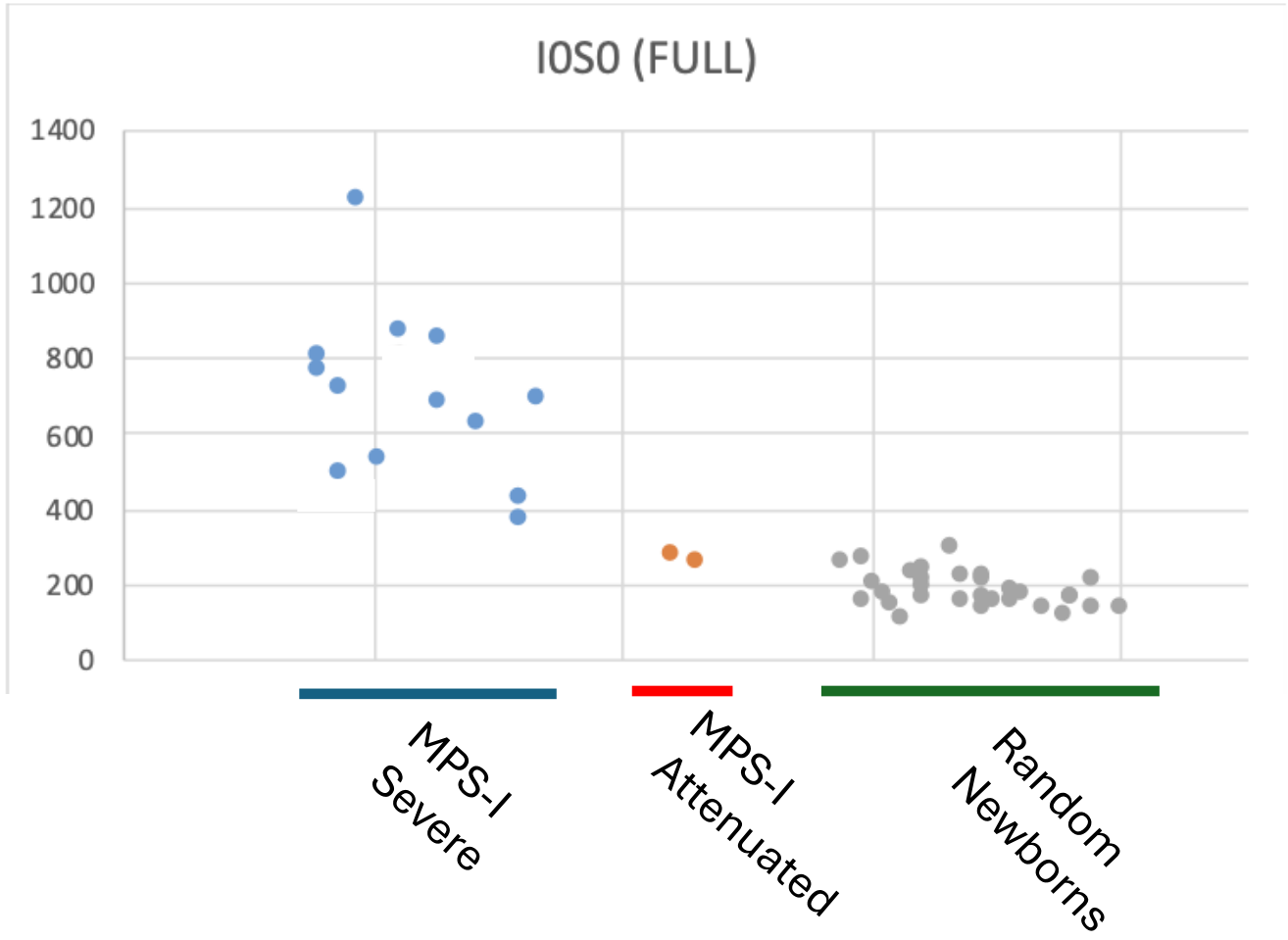
3 GAG fragments measured, 2 are from Heparan sulfate and are summed to give the X-axis. The third is from Dermatan Sulfate and is on the Y-axis. This is what the Mayo clinic reports.

The values below were measured in the Gelb lab using NEWBORN DBS from clinically confirmed MPS-I patients. Of course, only newborn data is required if we are going to use newborn GAG analysis for NBS.



Herbst et al
Int. J. Neonat. Screen (2020)

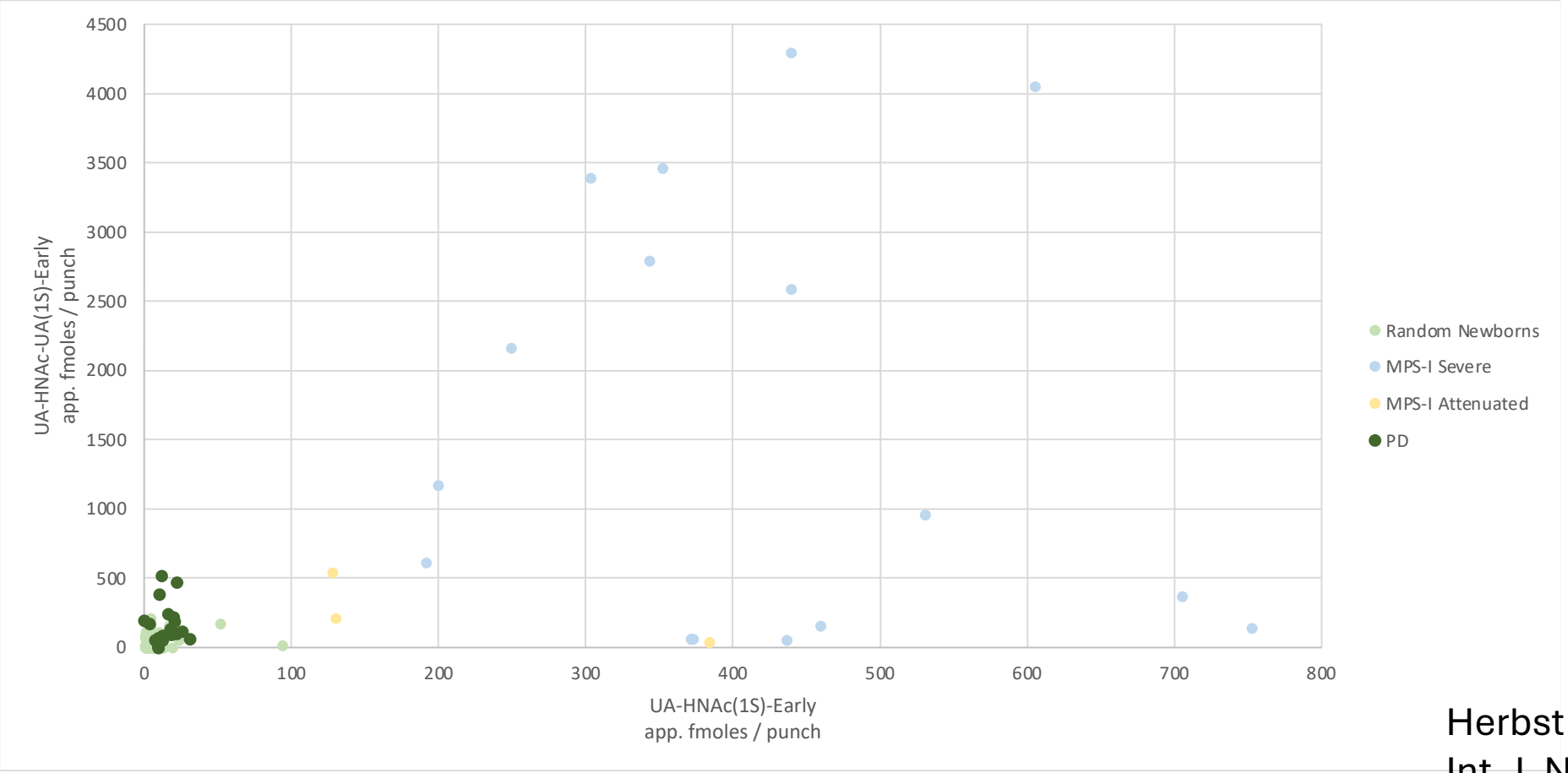
Enzymatic-NRE (aka Sensi-Pro NRE GAG Analysis of Newborn DBS for MPS-I)



Based on this poor performance
and the fact that Sensi-Pro involves a relatively complicated
and lengthly sample prep., we did not continue with Sensi-Pro for other MPSs.

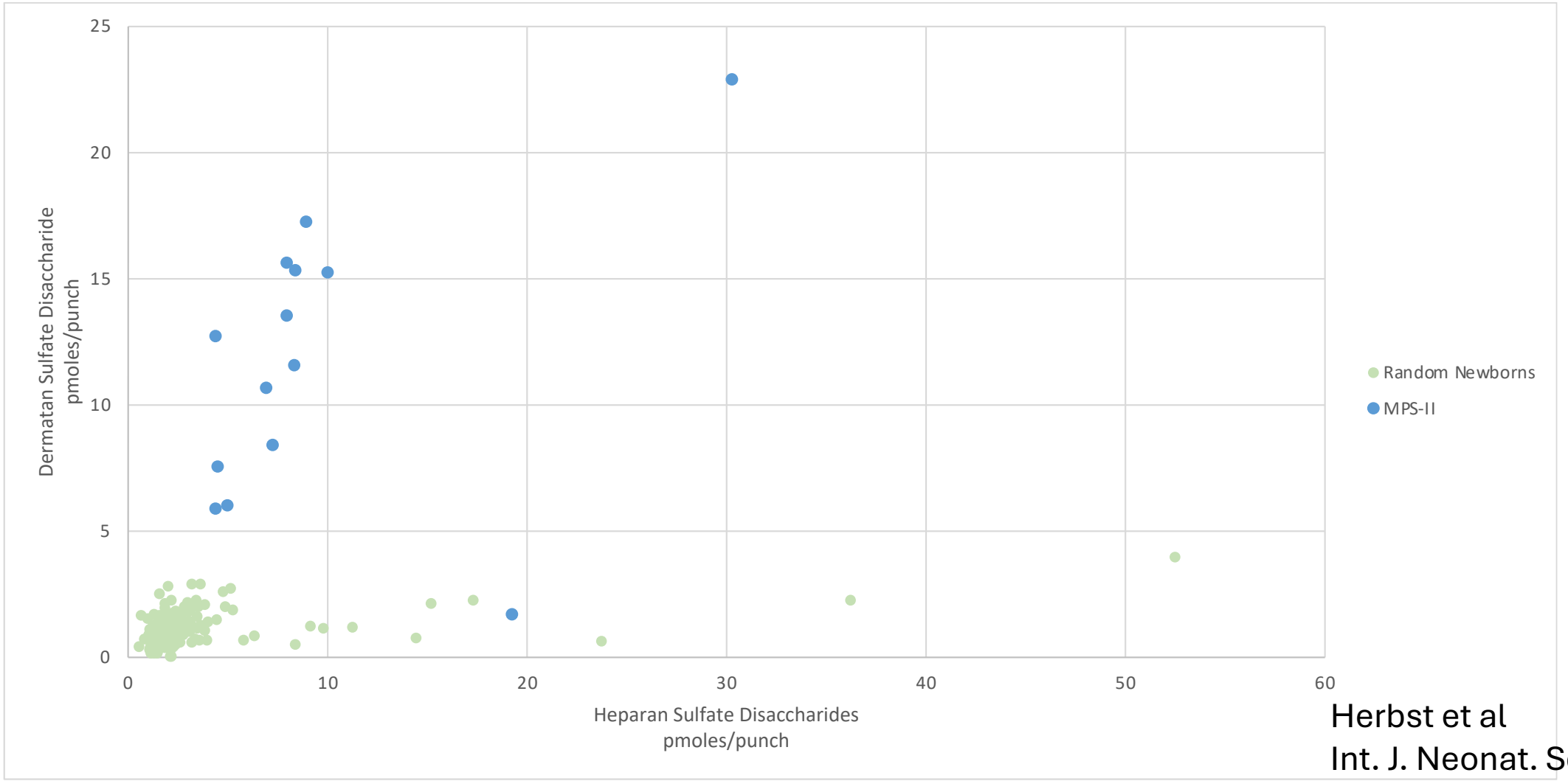
Herbst et al
Int. J. Neonat. Screen (2020)

Endogenous-NRE GAG Analysis of Newborn MPS-I DBS



Herbst et al
Int. J. Neonat. Screen
(2020)

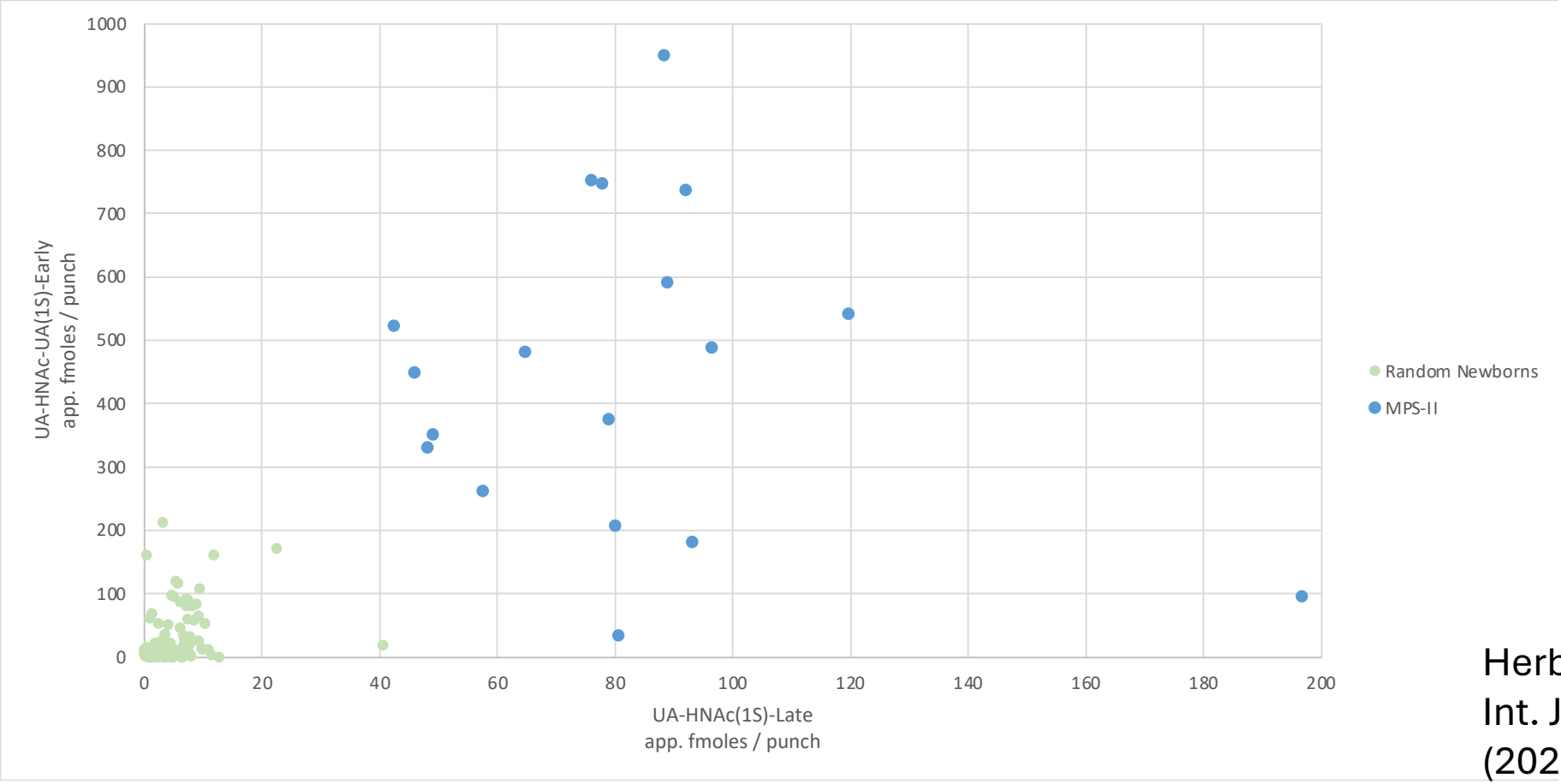
Enzymatic Internal Disaccharide GAG Analysis of Newborn DBS for MPS-II



Herbst et al
Int. J. Neonat. Screen
(2020)

Endogenous-NRE GAG Analysis of Newborn MPS-II DBS

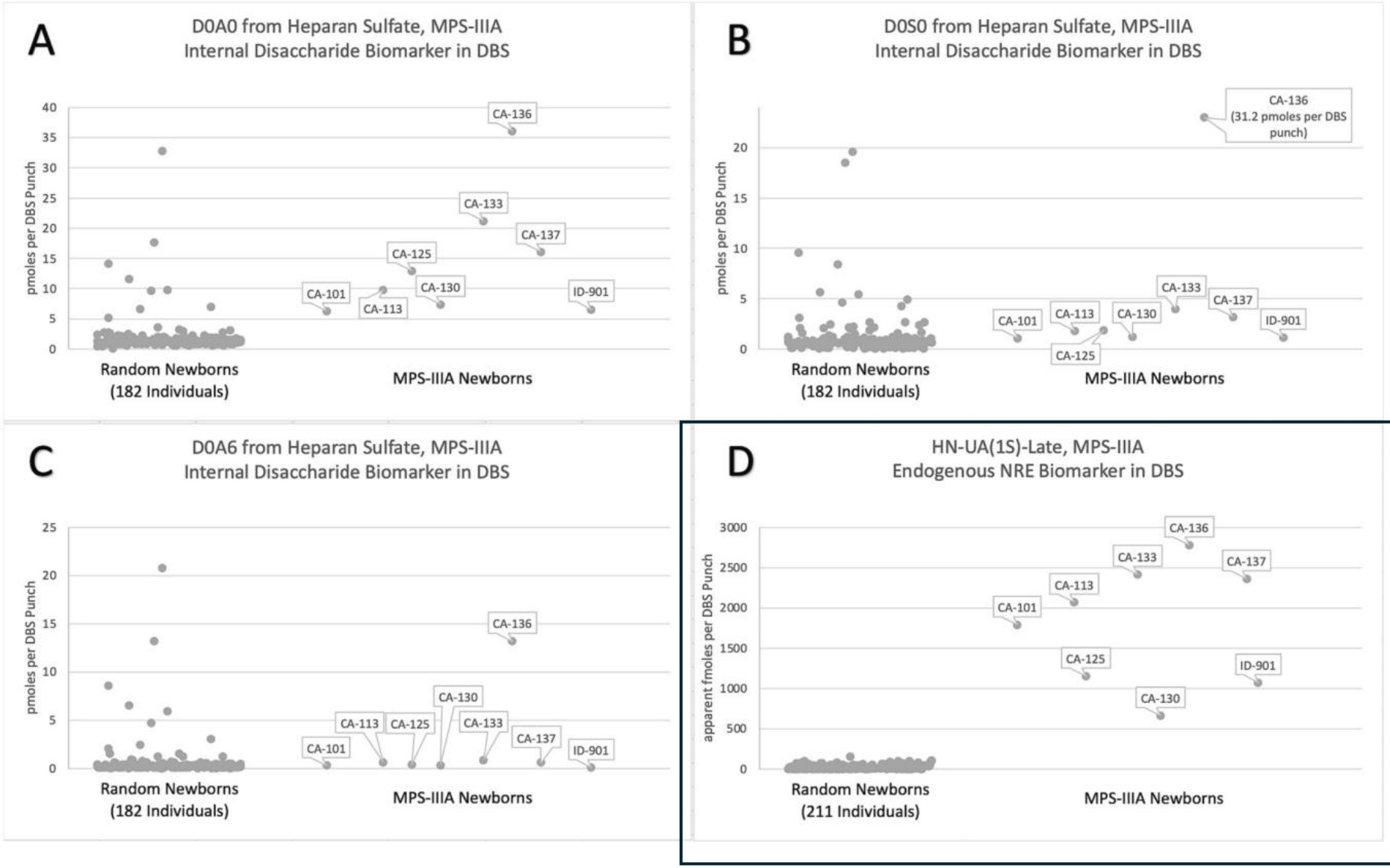
This is a mixture of neuronopathic and non-neuronopathetic samples, so although this method is the best performing in terms of reducing false positives, it is not prognostic for the severity of MPS-II.



Herbst et al
Int. J. Neonat. Screen (2020)

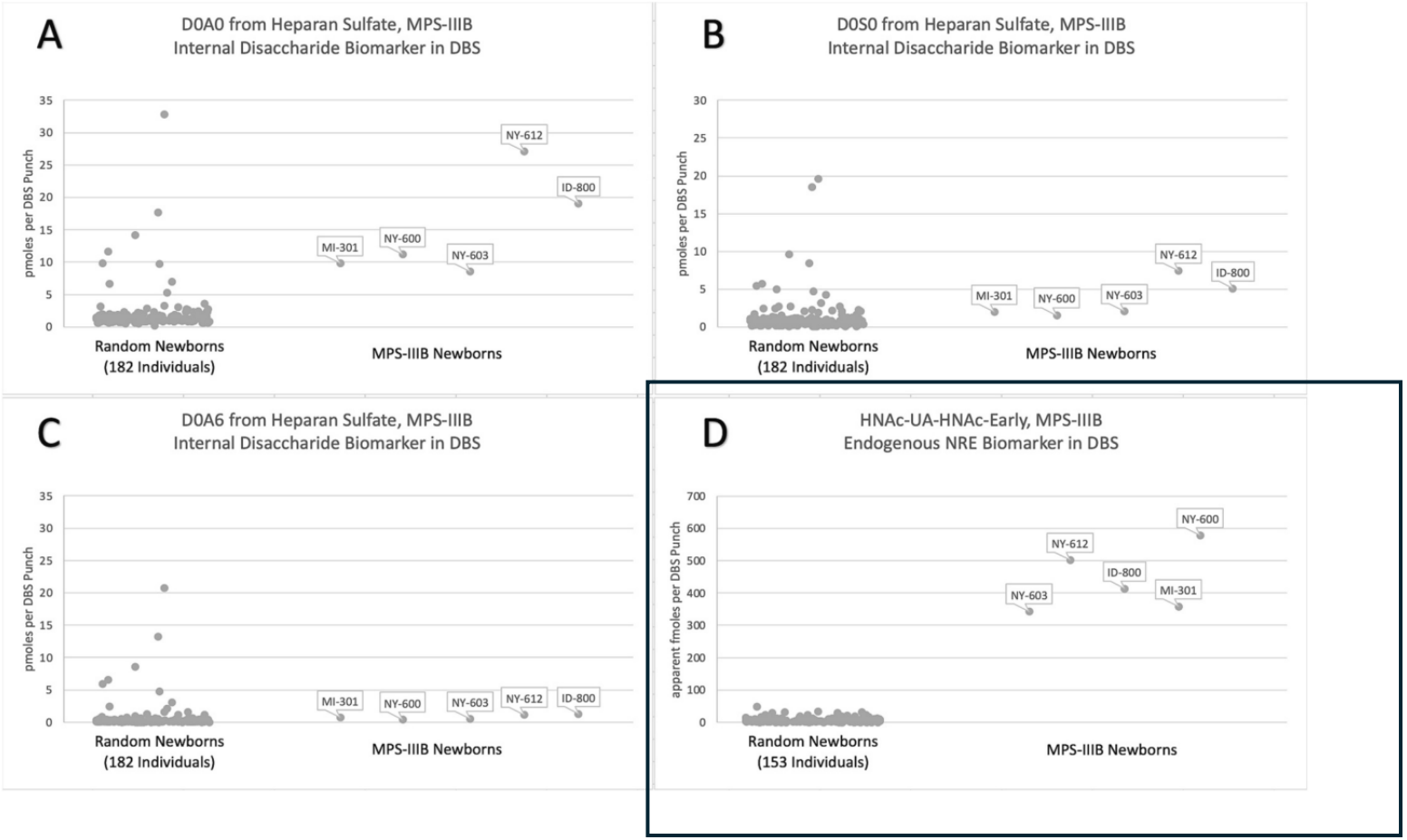
MPS-IIIA

Herbst et al (2024)
Molec. Genet. Metab.



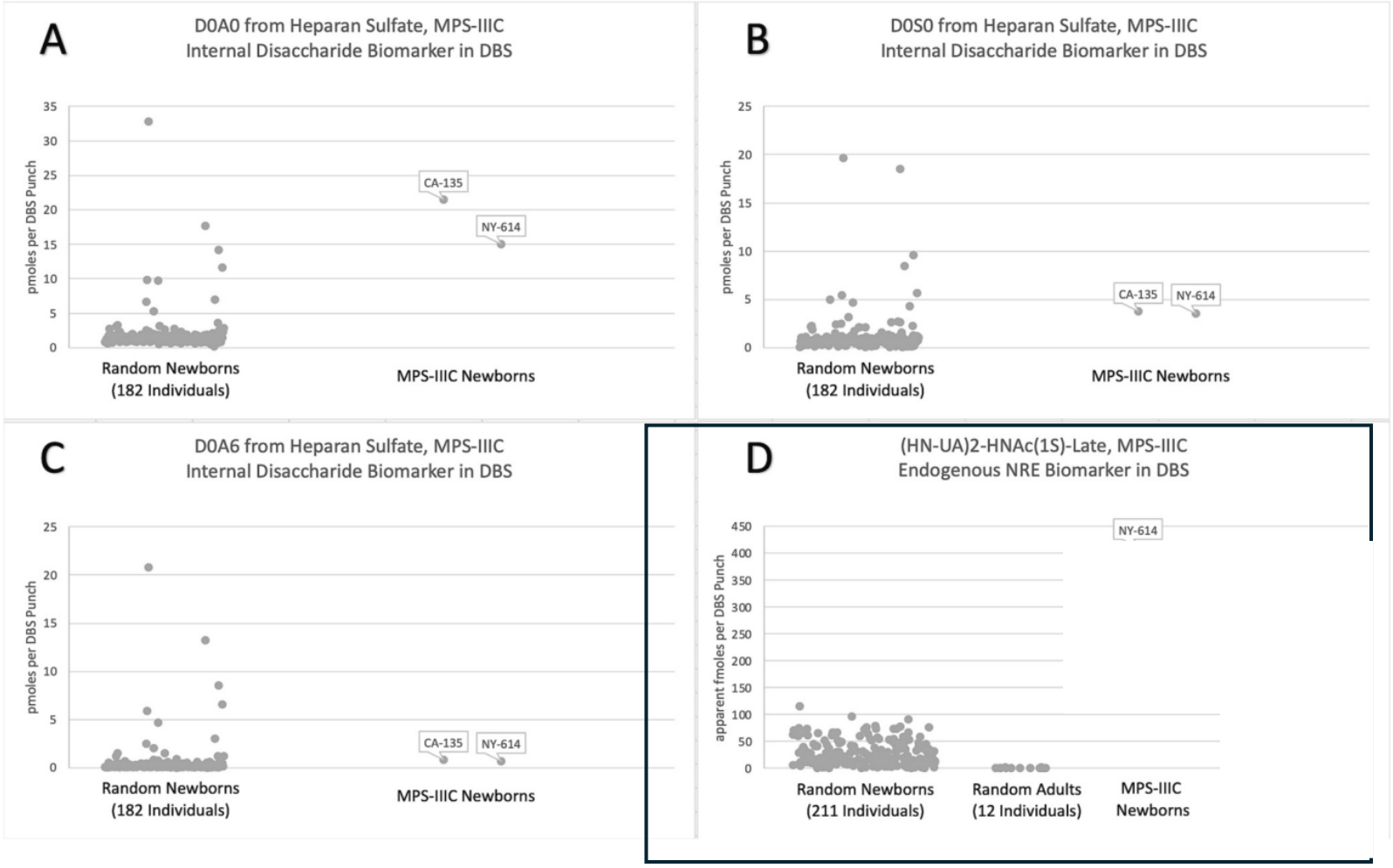
MPS-IIIB

Herbst et al (2024)
Molec. Genet. Metab.



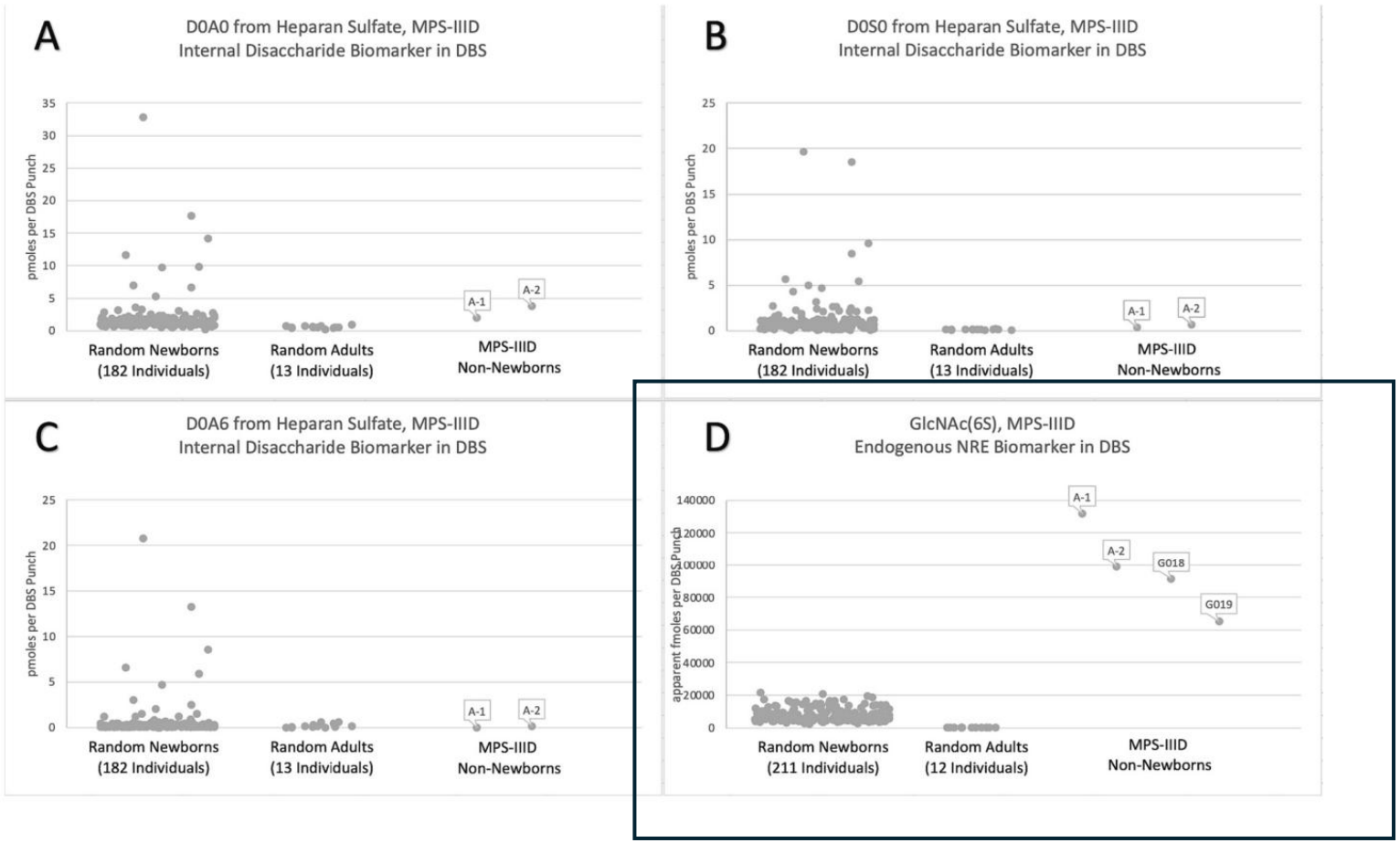
MPS-IIIC

Herbst et al (2024)
Molec. Genet. Metab.



Herbst et al (2024)
Molec. Genet. Metab.

MPS-IIID



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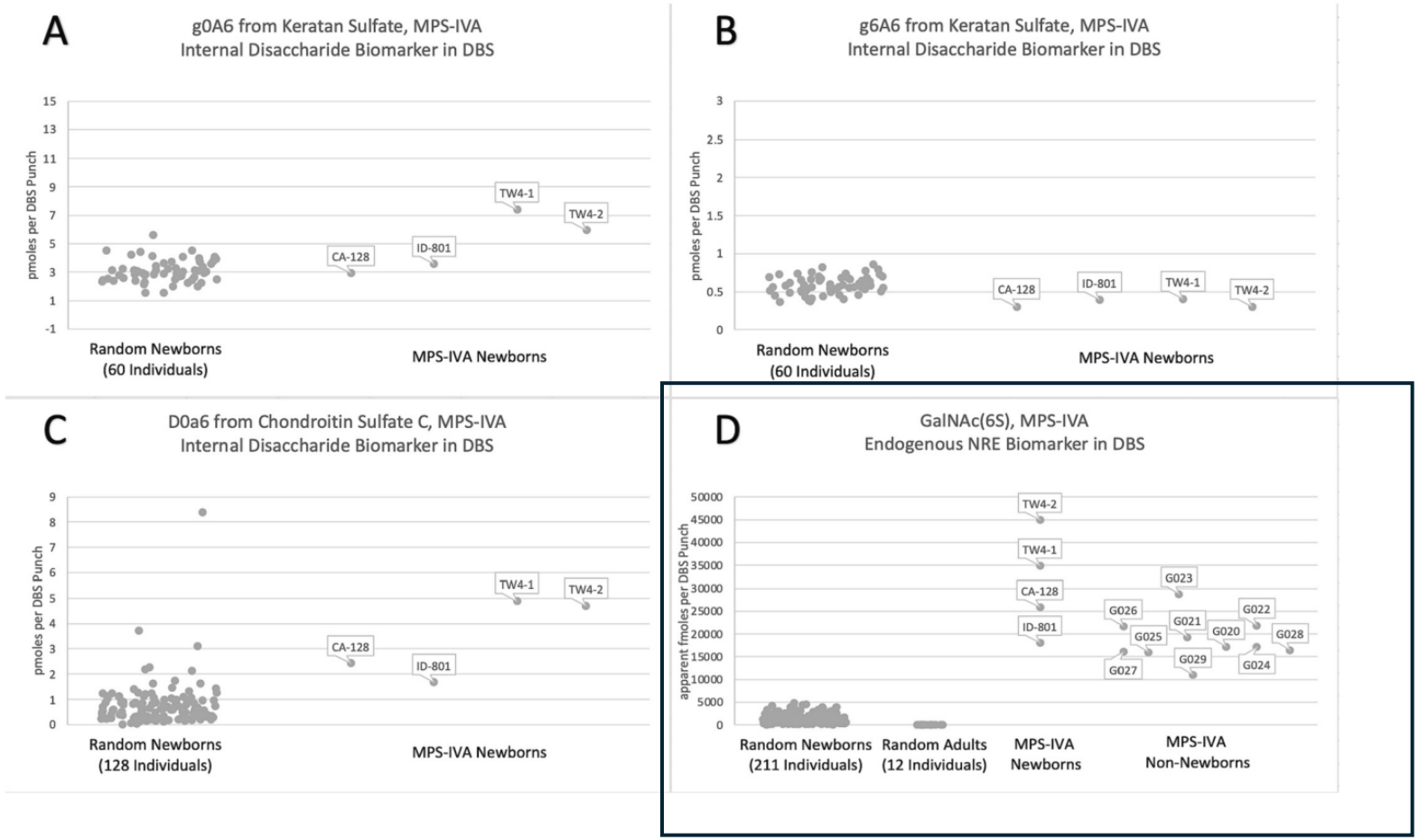
MPS-IVA



Herbst et al (2024)
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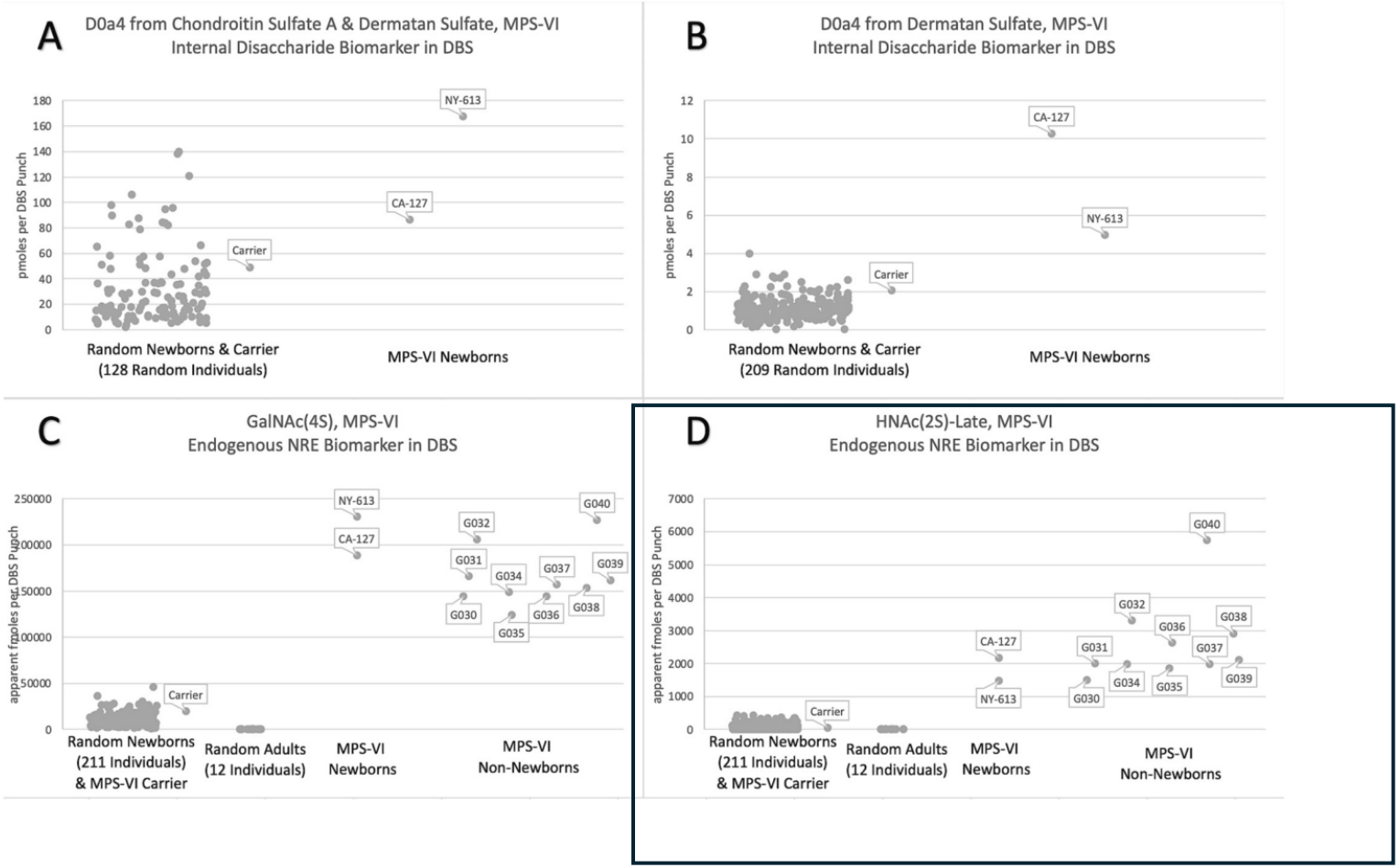
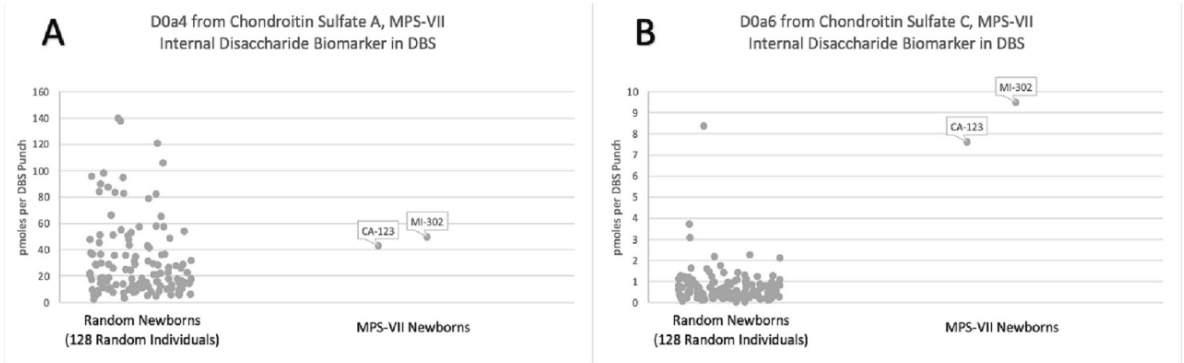
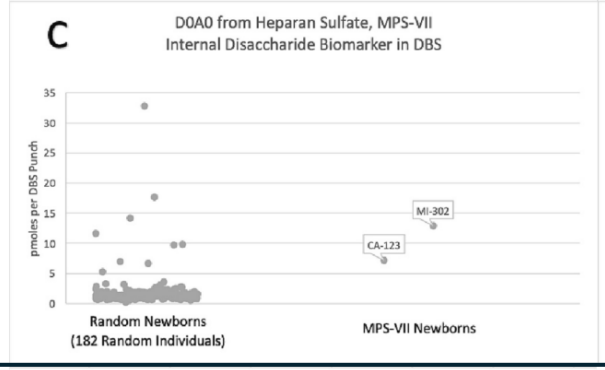
MPS-VI

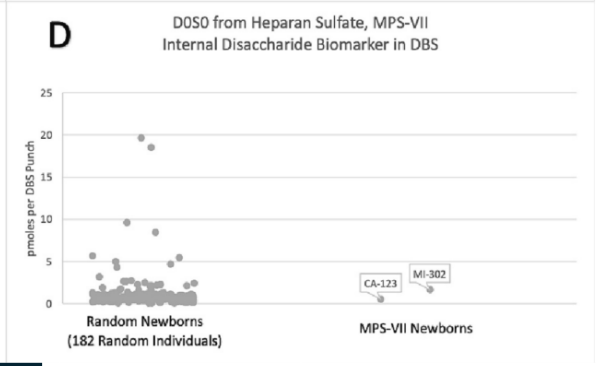


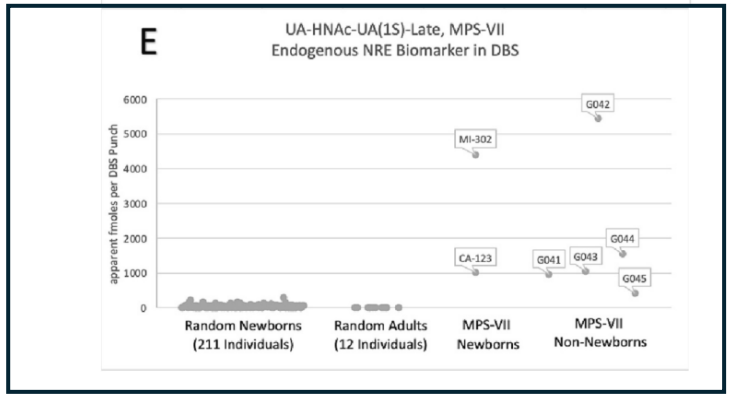
MPS-VII

Herbst et al (2024)
Molec. Genet. Metab.



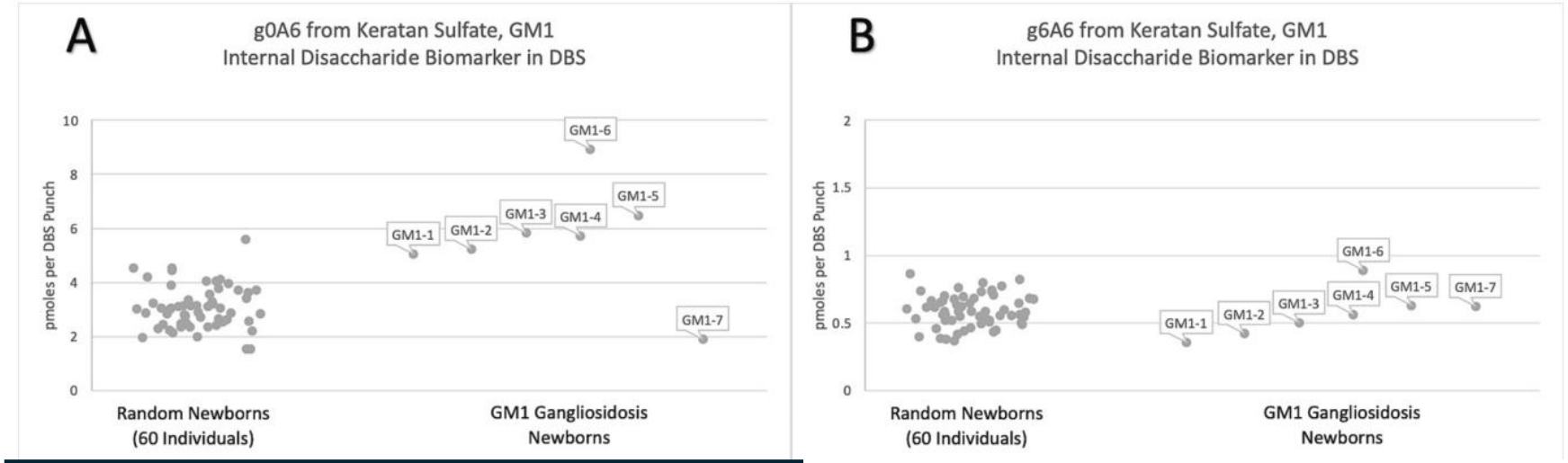


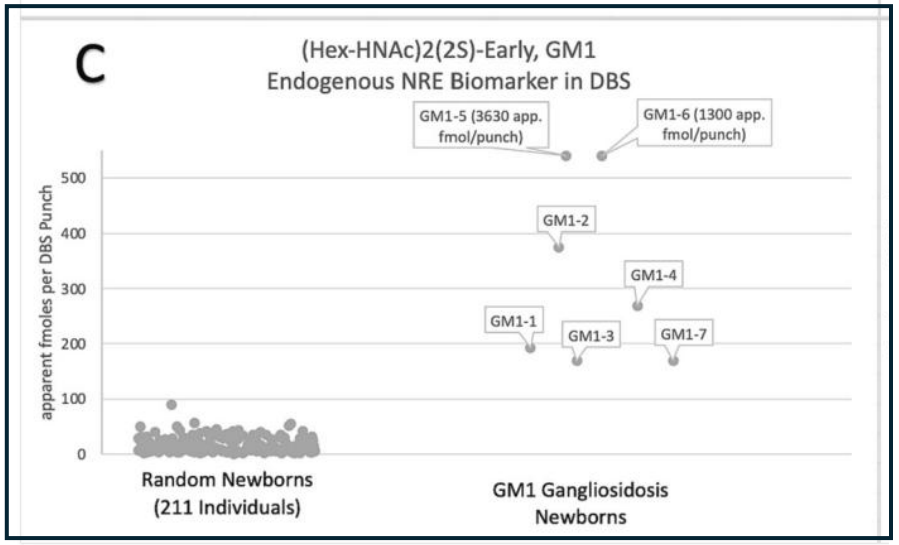


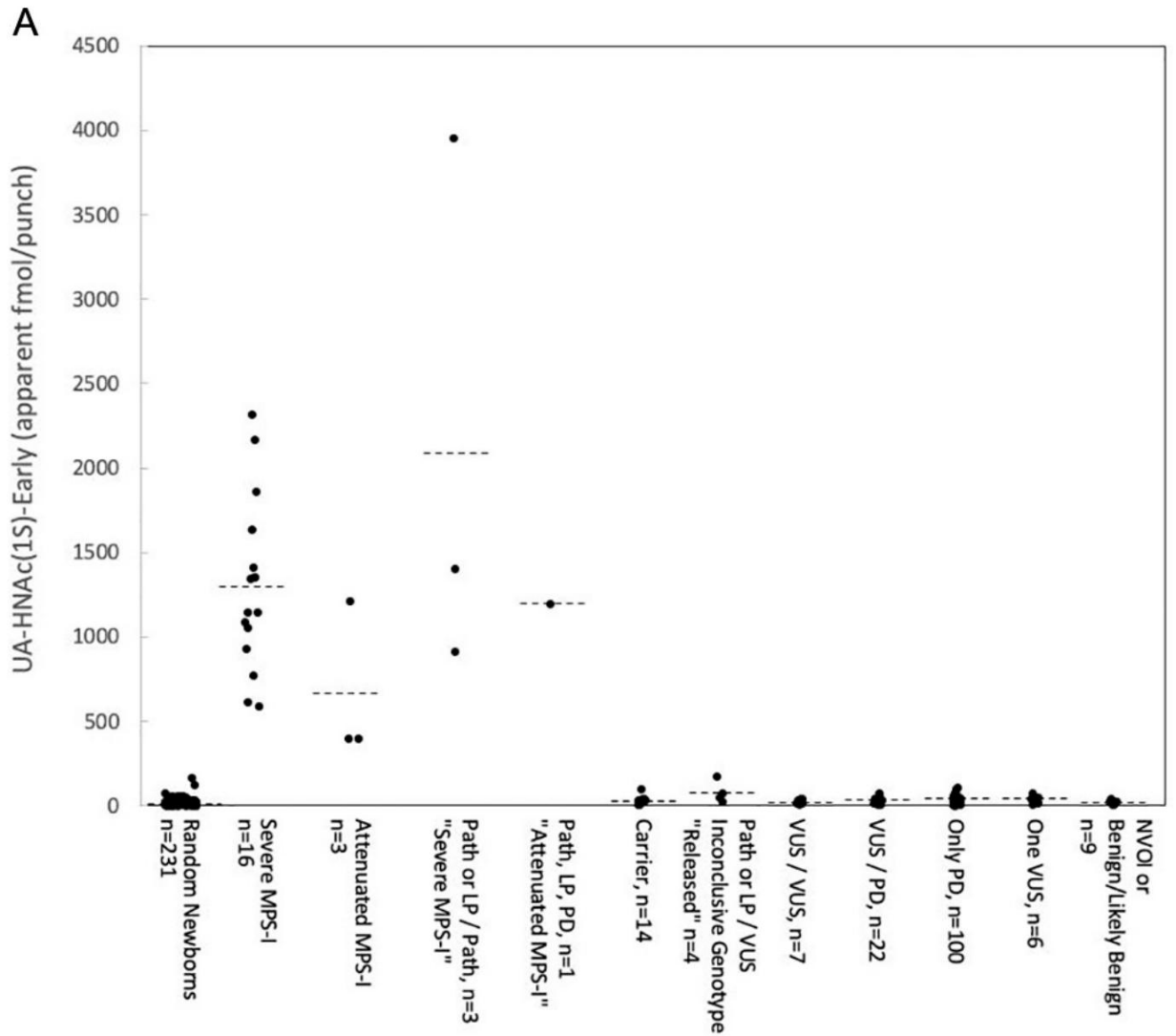


Herbst et al (2024)
Molec. Genet. Metab.

GM1-Gangliosidosis







~250,000 newborns screened for MPS-I in Ontario, CA.
All low IDUA samples submitted to genotype and Endogenous NRE GAG analyses.

11 cases of inconclusive genotypes (VUS), all resolved by endogenous NRE GAG analysis.

Herbst et al (2024) Mol. Genet. Metab.

So it is clear and published that the Endogenous NRE GAG method is by far the best method for newborn screening of all MPS disorders and used second-tier to first-tier enzymatic activity testing.

Note that a multiplex assay is available for first-tier enzymatic activity testing of all forms of MPS using LC-MS/MS (~2 min per newborn), and this can be multiplexed with all other lysosomal storage diseases now screened in the USA and worldwide. It will also accomodate new diseases that are likely coming in the next few yrs (MLD, CTX), and it also accomodates galactosemia, biotinidase, and X-ALD.

Khaledi, Gelb (2020) Anal. Chem.
Hong, Gelb (2020) Genet. Med.

Availability of the Endogenous NRE Method in CLIA Labs

1. The Calif. NBS Program is setting up the Endogenous NRE method in DBS to be run internally.
2. Revvity Genomics offers the Endogenous NRE method for MPS-I and MPS-II
3. Greenwood Genetics is going to offer the Endogenous NRE method soon.
4. The Mayo clinic is bringing on the Endogenous-NRE GAG method to replace their Internal Disaccharide method.

I suggest that all reference labs across Europe adopt the Endogenous-NRE GAG method.

Back to the challenges from the Athens SSIEM meeting

1. The Endogenous NRE method solves the false positive problem. Available evidence shows that so far the FP problem is eliminated.

2. Let's now turn to the absolute quantification of GAGs in a way that the results are not dependent on which CLIA lab you use.

For this we need calibrators and we need GAG materials that have been properly quantified!!!

The Enzymatic and Methanolysis Internal Disaccharide methods have been carried out with faulty calibration methods.

They are based on use of commercially available GAG polymers (i.e. Heparan Sulfate) isolated from animal sources, and they are not certified, and they are not pure by weight. Yet CLIA labs weigh out GAGs to make stock solutions for generation of standard curves. This does not work reliably.

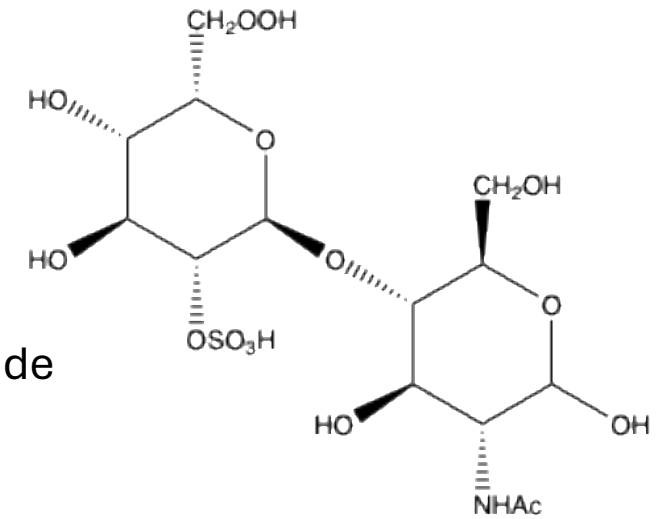
For example, the method using methanolysis to break GAG polymers into short methyl-glycosides:

Sugar1-Sugar2-OCH_3. Yes you can use deuterated methanol to make a chemically-identical, isotopic internal standard used for MS/MS assays Sugar1-Sugar2-OCD_3 but you don't know how many moles of GAG polymer you used in the reaction!

Very recently we have generated synthetic Endogenous NRE GAG standards. Now commercially available for MPS-II and MPS-IIIA (MPS-I coming soon, and the others a bit later).

These are quantified by qNMR, which is really the only analytical technique that can accurately determine the actual number of GAG molecules in the vial even if the compound is not pure by weight!

As an example, the Endogenous-NRE GAG marker for MPS-II was reported as UA-HNAc(1S) based on its mass (it contains 1 uronic acid (iduronic or glucuronic), 1 N-acetyl-hexosamine, and 1 sulfate). Comparison of authentic standards made by total synthesis shows that the structure is

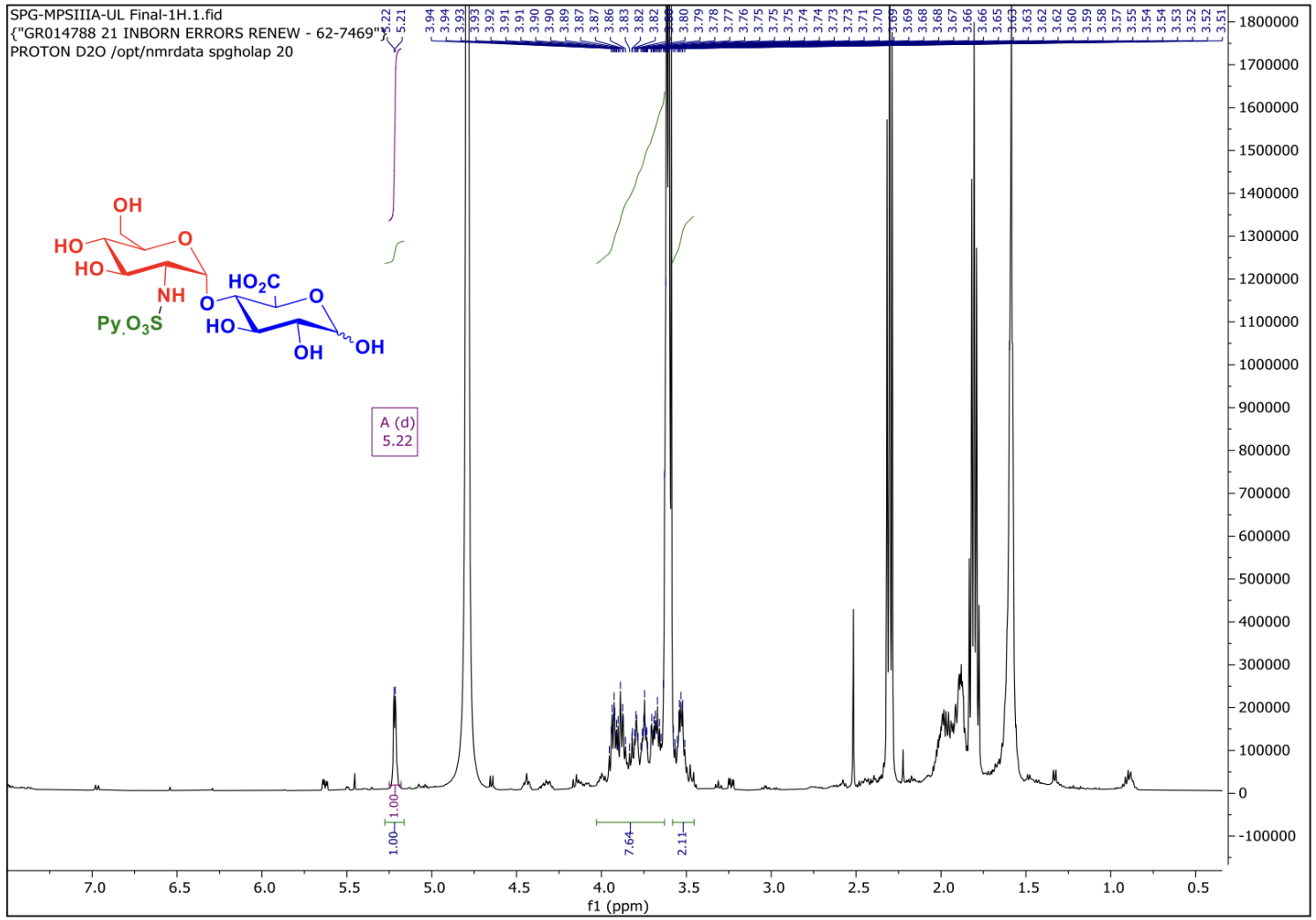


QC DBS are made by spiking blood with true moles of analyte.

MS/MS internal standards are made using ^{13}C-labeled sugars.

It is thus derived from Heparan Sulfate (not Dermatan Sulfate) and contains Iduronate not Glucuronate, and the Sulfate is at the 2-position as expected since the iduronate-2-sulfatase is deficient in MPS-II

Quantitative NMR (qNMR) was used with certified internal standards (DMF, Maleic Acid) to determine the MOLES of disaccharide in the tube. This is a molecule counting method!!!



Total synthesis of the Endogenous NRE standards is no picnic, ~15 steps.

Let’s do the numbers. Say $50,000 to prepare 5 mg.

Need 0.1 nmole int std per assay. So 5 mg is enough for ~100,000 assays at a cost of about $0.5 per assay.

So it is commercially viable to make these reference materials, and they are now available commercially.

Summary

1. NBS for all MPS types should be done by first-tier measurement of the relevant enzymatic activity followed by second-tier measurement of GAGs using the Endogenous NRE method.

2. All labs running the Endogenous NRE method should calibrate their assays by using a set of QCs in which the true moles of Endogenous NRE GAG are known. This is possible only with the use of QCs made from synthetic standards that have been analyzed by qNMR.

3. Also available now are chemically identical, isotopically-distinguished Endogenous NRE GAGs that can be used as Internal Standards to measure GAGs in patient samples. This is more reliable than using surrogate internal standards (i.e. those that are chemically similar but not identical to the analyte of interest).

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