Measuring of Non-reducing Terminal Glycosaminoglycan Fragments increases specificity and differentiates Mucopolysaccharidosis Type I (MPS I) from Mucopolysaccharidosis Type II (MPS II)

Taraka Donti[1], Sara E. Smith[1], Sasha Chernenkoff[1], Madeline Ellgass[1], Madhuri Hedge[1]

1. PerkinElmer Genomics, Pittsburgh, PA 15275

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INTRODUCTION

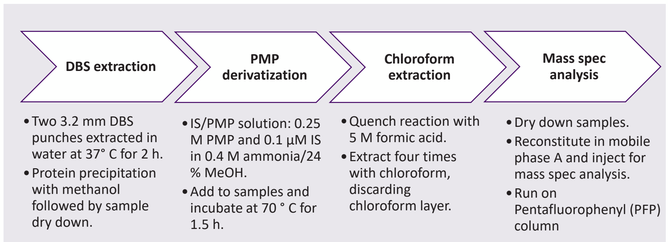
- Mucopolysaccharidosis (MPS) disorders are a category of lysosomal storage diseases that result in a disruption of the catabolism of glycosaminoglycans (GAGs), macromolecules consisting of long polysaccharide chains. There are a total of seven MPS disorders, with MPS I and II on the (Recommended Uniform Screening Panel) RUSP.
- Primary screening for MPS diseases is accomplished through enzyme activity testing[1], however, additional methods are required to identify false positives.
- Second tier tests have traditionally relied on elevations of broad classes of GAGs such as dermatan sulfate and keratan sulfate.
  - This approach cannot differentiate between MPS I and MPS II disorders.
- Recent discovery of terminal non-reducing fragments cleaved from GAGs within affected patients[2] presented us with an opportunity to investigate the utility of measuring these markers in specific MPS disease subtype.

MATERIALS AND METHODS

- MPS I
  - Whole blood DBS enriched with fibroblast generated MPS I biomarker served as the positive control (GelbChem)
  - Presumed normal whole blood DBS served as the negative control
- MPS II
  - MPS II positive patient urine served as the positive control during validation studies carried out and shown in Figure 1.
  - Whole blood DBS enriched with fibroblast generated MPS II biomarker was implemented as the positive control for clinical samples. (GelbChem)
  - Presumed normal whole blood DBS served as the negative control.
- Both biomarkers used Chondroitin disaccharide-d4 as the IS (Cayman Chemical) and 1-phenyl-3-methyl-5-pyrazolone (PMP) (Sigma-Aldrich) as the derivatizing agent.

MPS I Patient set
- 25 apparently normal
- 27 MPS I pseudo-deficient or carrier
- 4 MPS I VUS
- 3 known MPS I positive

MPS II Patient set
- 25 apparently normal
- 4 known MPS II positive in treatment
- 3 known MPS II positive not in treatment



Sample preparation methods and instrument parameters were designed from methods previously reported by Herbst *et* al.[3,4]

MPS I MARKER

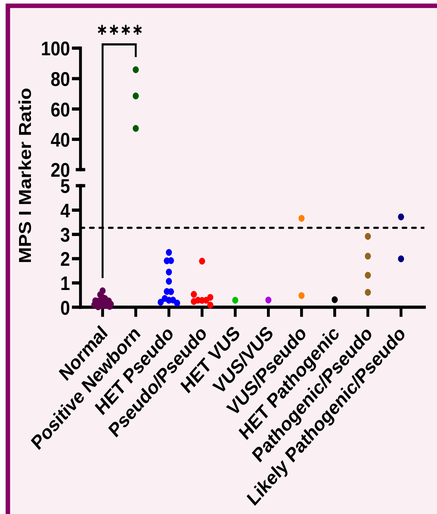


Figure 1. MPS I marker ratio values for all samples analyzed. Genotyped specimens are categorized according to their determined allele variants.

- This assay is semi-quantitative due to the inability to create a calibration curve of neat standards.
- A reference range was created for both MPS I and MPS II using ± 3 SD from the mean MPS marker ratio of all normal/unaffected samples.
- An initial cutoff value was set as the limit of semi-quantitation for both assays, based on the expectation that the marker would only be present in affected patients. Once additional data has been collected, this cutoff will be re-evaluated.
- The established cutoff ratio for the MPS I marker was determined to be 3.27, shown by the black dotted line in Figure 1.
- The statistical differences amongst groups for Figures 1 and 2 were determined using one-way ANOVA.
- MPS I Positive newborn patients report a significant difference ($p < 0.0001$) when compared to normal patients.
- Out of the normal and unaffected samples tested, only 2 reported marker ratios above the 3.27 cutoff. This is a clinical specificity of 96.4%.

MPS II MARKER

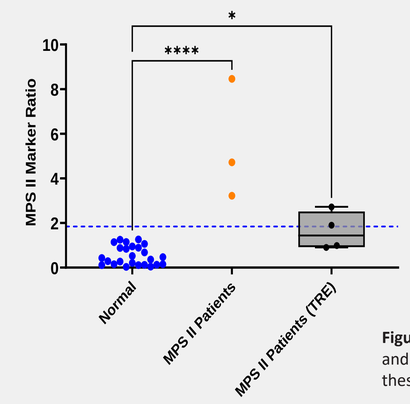


Figure 2. MPS II Marker Ratios of normal patients and MPS II positive patients. Note "TRE" indicates these patients are in treatment for MPS II.

- The established cutoff ratio for the MPS II marker was determined to be 1.84, shown by the blue dotted line in Figure 2.
- Both MPS II positive patients as well as patients in treatment show a statistically significant difference from normal patients.
  - The difference in marker ratio between positive patients and normal patients report a p value of <0.0001.

ACKNOWLEDGEMENTS

We would like to thank GelbChem for the supplied DBS control and the support of Michael Gelb, Zackary Herbst, and Hamid Khaledi throughout this project.

1. Peck *et* al., *Int. J. Neonatal Screen*, **2020**, *6* (1), 10.
2. Saville *et* al., *Genetics in Medicine*, **2019**, *21 (3)I,* 753-757.
3. Herbst *et* al., *Int J. Neonatal Screen*, **2020**, *26 (6)*, 69.
4. Herbst *et* al., *Int J. Neonatal Screen*, **2022**, *21 (8)*, 9

SPECIFICITY OF MPS I & II MARKERS

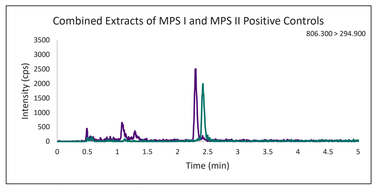


Figure 3. Chromatogram of combined positive control extracts showing the separation of isobaric MPS I and MPS II markers.

The markers of interest in this study are isobaric species of unknown structure. Extracts of positive controls containing each marker were combined to optimize chromatography conditions and achieve separation.

Consistent with previous reports, the markers are not present within healthy newborns. This is an additional advantage over traditional GAGs testing.

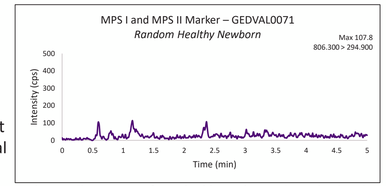


Figure 4. Chromatogram from a healthy newborn DBS sample. There is no detectable MPS I or MPS II marker present.

- MPS I positive newborn samples display a large response for the MPS I marker. We have consistently observed a small signal at the MPS II marker retention time within MPS I positive patients, however this peak is resolved from the true MPS I marker peak and is well below the threshold for the MPS II marker.
- We have not observed the presence of any signal at the MPS I retention time within MPS II affected patients.

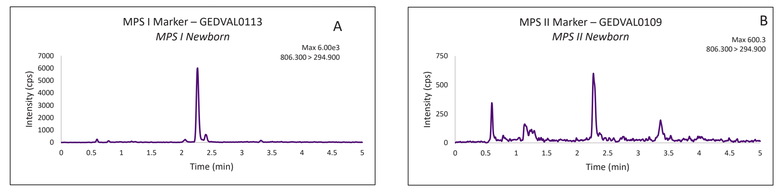


Figure 5. Chromatograms from newborns affected by (A) MPS I and (B) MPS II.

CONTROL STABILITY

MPS Control	Timepoint	Marker Ratio	% Difference from t = 0	Pass/Fail
MPS I Positive Control -80 °C	t = 0	18.84		
	t = 3 weeks	19.95	5.87	Pass
	t = 1 month	21.08	11.89	Pass
	t = 2 months	17.16	8.94	Pass
	t = 3 months	16.41	12.92	Pass
	t = 6 months	15.50	17.77	Pass
MPS II Positive Control -80 °C	t = 0	31.90		
	t = 3 weeks	31.90	0.00	Pass
	t = 1 month	28.20	11.60	Pass
	t = 2 months	24.60	22.90	Pass
	t = 3 months	25.20	21.00	Pass
	t = 6 months	30.10	5.6	Pass

Table 1. Shows MPS I and MPS II control long-term stability data collected thus far. Samples were considered passing if the calculated marker ratio was < 25% difference from time = 0.

CONCLUSION AND NEXT STEPS

- Our validated methods allow for increased specificity between MPS I and MPS II disease subtypes from measuring a characteristic fragment of accumulated GAGs compound.
- The MPS I marker test was performed retroactively on a set of samples that tested low for IDUA during primary newborn screening yet were confirmed unaffected by gene sequencing. The MPS I marker was found to be within normal limits for all 28 samples tested in this set, further supporting the benefit of performing this second-tier test as part of a newborn screening algorithm.
- Continuation of the MPS I allele study to further elucidate any possible correlation with genotype and MPS I marker ratio values.
- An allele and biomarker correlation study for MPS II.
- Validation of MPS I and MPS II biomarkers in urine samples.