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A North Carolina Newborn Screening Pilot for Mucopolysaccharidosis II: Evaluating Endogenous Non-Reducing End Glycosaminoglycan Analysis and *IDS* Sequencing as Higher-Tier Testing Options

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ABSTRACT

Purpose: Mucopolysaccharidosis II (MPS II, OMIM 309900) is a lysosomal disorder recommended for newborn screening (NBS) in the United States. This study evaluated outcomes of high-throughput NBS for MPS II and use of two reflex testing methods to improve sensitivity and specificity.

Methods: We implemented a first-tier LC-MS/MS laboratory-developed test measuring iduronate-2-sulfatase (I2S) enzyme activity in dried blood spots (DBS). Newborns with activity ≤10% of the daily median underwent reflex testing for endogenous non-reducing end (NRE) glycosaminoglycan (GAG) and *IDS* sequencing. Screen-positive infants were referred for clinical follow-up with urinary GAG testing and repeat DBS I2S activity measurement.

Results: Among approximately 220,000 newborns screened, 33 tested positive with low I2S activity. Two were confirmed with MPS II. Results of the remaining 31 newborns were indicative of pseudodeficiency. The NRE GAG ratios correlated with confirmatory urinary GAG measurements and were elevated exclusively in newborns with MPS II.

Conclusions: NBS for MPS II can be efficiently achieved with LC-MS/MS measuring I2S activity. The NRE GAG biomarker successfully differentiated between newborns with MPS II and those with pseudodeficiency. Utilization of NRE GAG analysis as a 2nd tier test significantly reduces the false-positive rate. *IDS* sequencing provides additional information for clinical evaluation and follow-up.

Keywords: mucopolysaccharidosis II; Hunter syndrome; newborn screening; NRE; IDS; I2S

INTRODUCTION

Mucopolysaccharidosis II (MPS II, Hunter syndrome, OMIM 309900, Hunter syndrome) is an X-linked lysosomal disease caused by pathogenic variants in the iduronate 2-sulfatase (*IDS*) gene (HGNC ID: 5389), resulting in iduronate-2-sulfatase (I2S) enzyme deficiency, and accumulation of the glycosaminoglycans (GAGs), dermatan sulfate (DS) and heparan sulfate (HS) in lysosomes.[1] The primary accumulation of HS and DS results in progressive cellular damage and multisystemic disease manifestations, significantly impacting quality of life and reducing life expectancy.[2]

Although infants with MPS II typically do not have a clinical phenotype at birth, signs and symptoms of the disorder typically emerge during early childhood. MPS II is best described as a continuous spectrum of clinical severity, extending from the severe neuronopathic form to the attenuated non-neuronopathic form, with all forms having somatic involvement.[3, 4] The neuronopathic form, which accounts for approximately two-thirds of cases, is characterized by neurocognitive manifestations that begin in early childhood, followed by a plateau phase and a subsequent neurological decline that typically starts after four to five years of age, ultimately leading to death in teenage years.[5-7] In contrast, the non-neuronopathic form is characterized by milder physical disease progression, with symptoms emerging later in life with minimal to no impact on neurocognitive function. Individuals with this form can survive into their fifth or sixth decade.[5] Upon clinical suspicion, the diagnostic testing includes I2S enzyme activity in blood, GAG levels in urine, and *IDS* analysis. Decreased I2S enzyme activity together with elevated urinary GAGs is required for the diagnosis of MPS II.

While hematopoietic stem cell transplantation is the recommended treatment for Hurler syndrome (MPS I), its efficacy in MPS II is unclear. Therefore, in the United States, current standard of care for MPS II is intravenous (IV) idursulfase enzyme replacement therapy (ERT)

with recombinant human I2S.[8] This recombinant form of human I2S has been shown to stabilize or improve somatic disease, particularly if administered prior to the onset of symptoms,[9] but does not halt the progression of neurocognitive decline in the neuronopathic forms given it does not cross the blood-brain barrier in sufficient amounts. However, new therapeutic approaches targeted to treat the central nervous system are in development for MPS II, including IV enzyme therapy designed to penetrate into the brain, intrathecal gene therapy, and *ex vivo* gene therapy.[10]

Because of the variability of symptoms, rate of progression and rarity, the diagnosis of MPS II is typically delayed and most individuals have irreversible clinical features at the time of diagnosis. Therefore, universal newborn screening (NBS) is essential to equitably identify individuals with MPS II prior to onset of clinical symptoms and provide access to therapeutic interventions before irreversible tissue and organ damage occurs.[11-13] MPS II was added to the Recommended Uniform Screening Panel (RUSP) in the United States in August 2022 based on the success of previous and ongoing small pilot programs, as well as screening efforts in Taiwan,[11] Illinois (US),[14] and Missouri (US).[15] Outcomes from these efforts highlighted the feasibility of implementing NBS and the reported benefit of presymptomatic treatment compared with clinical case detection.[13]

With significant expansion of NBS for MPS II underway, studies have reported varied birth prevalence across cohorts, 1:73,000 in the United States,[14] about twice that in Taiwan, and up to 1:20,000 or more in Japan.[12] Accurate prevalence estimates and diagnosis are further complicated by uncertainties around genotype-phenotype correlations based on I2S enzyme activity and *IDS* sequencing assays during NBS.

NBS programs typically measure I2S enzyme activity via LC-MS/MS as the primary screening method, but interpretation may be complicated by factors such as variants of uncertain

significance (VUS) and pseudodeficiency variants in *IDS*. The term "pseudodeficiency" refers to significantly reduced enzyme activity detected in laboratory tests, without accompanying clinical phenotype of the corresponding lysosomal storage disease.[16] Absent long-term clinical follow-up data, we relied on reduced enzyme activity with normal urinary GAG results and a normal clinical evaluation at birth to define pseudodeficiency for the purposes of diagnosis and variant classification within the context of NBS.

NBS for MPS II has been successful at identifying newborns with MPS II but using 1st-tier enzyme activity alone results in low positive predicted value (PPV) because it cannot fully distinguish among MPS II cases and and individuals with pseudodeficiency alleles. As seen in NBS for MPS I,[17, 18] a successful second-tier method has the ability to significantly improve specificity, thereby decreasing the follow-up burden and unnecessary parent anxiety. GAG and DNA analysis using the original DBS has the potential to significantly improve the PPV and provide important information to support clinical evaluation and follow up.[19] To support the implementation of MPS II screening across NBS programs, our team conducted a prospective newborn screening pilot study for MPS II in North Carolina and evaluated the utility of endogenous non-reducing end glycosaminoglycan analysis and *IDS* sequencing as higher-tier testing options.

MATERIALS AND METHODS

Participants: All newborns with sufficient residual DBS after completion of the standard North Carolina NBS panel were included in the study under a waiver of informed consent protocol (UNC IRB #22-0316). All available residual specimens including repeats were tested. The samples became available for the study 14 days of receipt by the state lab, and MPS II testing was conducted 15-30 days after receipt by the state lab. Newborn DBS cards were stored

at room temperature in airtight bins with desiccant until testing. Quality control (QC) specimens were stored at -20°C. At the time of testing, 3.2 mm diameter disks were punched from residual DBS into 96-well plates and processed.

LC-MS/MS method: Two liquid chromatography tandem mass spectrometry (LC-MS/MS) methods were used during the study due to limited availability of the original custom-made formulation. Briefly, Assay 1 was a custom designed multiplex formulation containing substrate and internal standard (S/IS) for iduronate-2-sulfatase (I2S) and an internal enzyme control, alpha-L-iduronidase (IDUA). The I2S/IDUA custom formulation was then replaced with a commercially available reagent formulation for I2S only (Assay 2). In Assay 2 the ammonium acetate (C2H7NO2) in the buffer was substituted for sodium acetate (CH3COONa) and the I2S internal standard concentration changed from 8.08 µM to 5.00 µM. All testing was performed on two QSight® 225 MD UHPLC-MS/MS Screening Systems (Revvity).

Statistical analysis: I2S enzyme activity was calculated using the equation shown in the footnote of Table S1. Descriptive statistics and all plots were generated in Excel or R using ggplot2, tidyverse, e1071, and nortest. Normality of I2S activity distribution was assessed using visualizations (hist), Q-Q plot (qqnorm, qqline), Kolmogorov-Smirnov test (ks.test), skewness, kurtosis (e1071 package), and Anderson-Darling test (ad.test). Z-scores were calculated for the 10^{th} percentile median cutoff values for each subcategory to determine the number of standard deviations away from the mean. Z-scores were converted to percentiles using the pnorm() function to determine where they fall within the distribution in each subset. Empirical cumulative distribution function for the overall 10^{th} percentile was calculated for the full dataset using the ecdf function.

Assay validation: The assay was implemented according to clinical laboratory standards as a laboratory developed test (LDT). Precision was evaluated by replicate testing of QC DBS specimens comprised of four levels of I2S activity (base pool, low, middle, and high) (Revvity) in a 5x5x5 design (5 plates measured over 5 days with 5 replicates per sample) to assess intra-run, inter-run, inter-operator, and inter-instrument variation. Stability was evaluated for reconstituted reagents (6 days), processed specimens (3 days), and DBS card storage conditions (27 days). The percent difference was calculated according to Equation 2, where A_1 is the mean activity at t=0 and A_y is the mean activity of the subsequent processing time points.

$$\%\, difference = \left[\left(\frac{A_y}{A_1}\right) \times 100\right] - 100$$

Equation 2. Calculation of the percent difference in activity of QC DBS between t=0 and subsequent time points.

Screening algorithm: The screening algorithm (Figure 1A) involved an initial screen with a single punch. All specimens ≤10% of the daily median were retested in duplicate. All newborns with at least one retest ≤10% of the daily median were classified as screen-positive and referred to follow up at the Muenzer MPS Research & Treatment Center (Muenzer MPS Center) at the University of North Carolina at Chapel Hill. Supplemental reflex testing was performed by an external commercial laboratory (Revvity Omics) on the same DBS for all screen-positive infants. This testing included NRE GAG biomarker (Fuller Method)[20, 21] and *IDS* sequencing, including deletion/duplication analysis. All variants, regardless of pathogenicity, were reported to inform follow-up. *IDS* sequencing data analyses were based on GRCh37 (hg19).

Follow up algorithm: We created a one-page educational flyer about MPS II for parents (7th grade reading level), and a two-page flyer for providers (Supplementary Files). The flyers

were provided for all positive-screen cases and included basic information about MPS II, the meaning of a positive screen, recommended next steps, and links to online MPS II resources. All positive screens were communicated directly to the families by the Muenzer MPS Center genetic counselor. Newborns whose families consented to follow-up received an evaluation by an MPS specialist, genetic counseling services, and confirmatory testing (Figure 1B). Confirmatory testing was performed at the Duke University Health System (DUHS) Biochemical Genetics Lab and included I2S enzymatic activity using a fluorimetric bench assay on a new DBS sample and urinary quantification GAG analysis via ultraperformance LC-MS/MS (UPLC-MS/MS) internal disaccharide biomarker method.[22, 23] Infants confirmed with MPS II were transitioned to comprehensive clinical care with the Muenzer MPS Center. Additional clinical follow-up of infants with pseudodeficiency was not recommended.

RESULTS

Assay Validation (Assay 1): The average I2S activity of the lower limit of quantitation (LLOQ) was 0.308 µM/hr with a %CV of 14.3%. The limit of blank (LOB) and limit of detection (LOD) were 0.121 µM/hr and 0.166 µM/hr, respectively. The blank response for the I2S product was 14.0% of the LLOQ. Linearity was within the acceptance criteria of correlation coefficient $R \geq 0.980$ on all five testing days with $R \geq 0.999$ (Table S1, Figure S1). DBS storage stability for all QC standards over 27 days was within 19% of t=0. The stability of the reconstituted reagent vials at -20°C for all QC levels over six days was within 13% CV of t=0. The stability of the processed specimens at room temperature over three days was within 6% CV of t=0 (Figure S2).

Assay Validation (Assay 2): The performance of the I2S-only formulation (Assay 2) was comparable to the performance of Assay 1 (data not shown). Shortening the incubation time

from 18-20 hours to 3 hours was considered[24]; however, implementing such workflow was determined to be impractical as the process would require two days to complete regardless of incubation length and the longer overnight incubation was previously shown to result in a more complete substrate conversion and better separation.[24]

Reference range and cutoff: Most newborns with MPS II are expected to have DBS I2S activity results less than 1 to 2% of the population median activity.[14] However, some causal *IDS* variants that impair enzyme processing and typically lack functional enzyme in the lysosome,[12, 14] may retain residual activity. Based on this, the cutoff was conservatively set at ≤10% of the daily median activity to ensure that no affected individuals were missed with the expectation that infants with pseudodeficiency alleles may be detected.

As a part of the LDT validation, a retrospective screen of 5,382 de-identified newborn DBS specimens was performed over 10 days to establish an initial reference range specific to the screened population. Accuracy of NBS with a ≤10% daily median cutoff was confirmed by testing 6 newborn DBS from individuals confirmed with MPS II and 6 other DBS that previously tested ≤10% of the daily median in another NBS laboratory. The ≤10% daily median cutoff was used throughout the study.

No screen positive females were identified with I2S activity below the ≤10% daily median cutoff. Two females had I2S activity at <15% and a total of 4 females had I2S activity <20% of the daily median (Table S2); therefore, unaffected heterozygous females with low I2S activity are unlikely to be identified by the 1st-tier test. Furthermore, heterozygous females would not be expected to have elevated GAGs in DBS; therefore, if used as a 2nd-tier test, the NRE GAG test should effectively eliminate reporting unaffected heterozygous females.

I2S Activity Distribution: A total of 228,259 specimens from 219,757 newborns were screened in the study (Table 1). The I2S activity results were approximately normally distributed (Figures S3 and S4). Descriptive statistics were calculated across all I2S results and for subgroups (Table S3 and Figure S5). The median I2S activity over all tested specimens was 18.74 μM/hr and the mean was 18.99 μM/hr indicating an approximately normally distributed dataset. The overall 10% median corresponds to 0.015 percentile of the total population distribution for all I2S activity results from the study. There were no statistically significant differences in I2S activity between initial and repeat specimens (19.02 μM/hr, $p = .07$). Of the initial specimens tested, 0.66% yielded unsatisfactory results. This dropped to 0.46% for repeat specimens.

Screen positive newborns: Of the 219,757 newborns screened for MPS II, 33 screened positive. Two newborns were confirmed with MPS II, and results of the remaining 31 newborns were indicative of pseudodeficiency. The birth prevalence of MPS II in the screened newborn population was 1:109,879, or 1:55,349 males (equivalent to 0.91 per 100,000 infants and 1.81 per 100,000 males). The PPV of the 1st-tier test was 6.1% and false positive rate was 0.014%. No false negative cases have been identified to date, but many more years of clinical surveillance will be needed to determine if any MPS II cases may have been missed by this study.

All 33 male infants with positive first-tier screen had sufficient residual DBS sample for NRE GAG testing and *IDS* sequencing. Thirty-one families consented to confirmatory testing and clinical evaluation; 30 were evaluated at UNC and one at an alternate site in North Carolina due to travel limitations. For the two families who declined follow-up (cases #13 and #23), a letter containing the NBS results and contact information for genetic counseling services was

provided. Both infants had negative DBS GAGs and *IDS* sequencing (Table 2, Figure 2). See Figure S6 for correlation plots of I2S activity, DBS GAGs and urine GAGs.

Confirmed Cases: Two newborns (cases #12 and #27, Table 2) were confirmed to have MPS II, based on elevated NRE GAG biomarker, decreased I2S activity in repeat DBS specimens, elevated urinary HS and DS, and suspected causal *IDS* variants; i.e. both GAG test results and follow up enzymology were clearly suggestive of MPSII.

The NBS I2S activities for the two confirmed cases (#12 and #27) were <5% daily median [#12: 0.2% (0.308 μM/hr) and #27: 4.10% (0.797 μM/hr)]. Notably, the 4.10% daily median result (mean of the duplicate retests) was higher than several values observed in individuals carrying pseudodeficiency variants (Figure 2A and 2B). The initial single-punch result for #27 was 0.5% daily median, more consistent with the expected I2S loss of function (see footnote in Table 2). The reason for the discrepancy between the initial (0.5%) and retest (4.10%) values is unknown.

The first confirmed infant with MPS II (case #12) was identified to have an *IDS* variant, initially classified by the reporting laboratory as a VUS, NM_000202.8 (IDS): c.1439C>T; p.(Pro480Leu) (Table S4). At the time of reporting, ClinVar submissions[25] for this variant were conflicting, with other labs classifying it as pathogenic, likely pathogenic, or a VUS. At the time of publication, all submitters classify this variant as likely pathogenic or pathogenic. This variant has been published in at least one male with attenuated MPS II[26]. The Muenzer MPS Center clinical team is also aware of another adult male (case not published) with the same variant and diagnosed with attenuated MPS II at 18 years of age presenting with joint limitations and managed on standard of care ERT. These two cases suggest this variant is associated with an attenuated phenotype. Targeted parental variant analysis determined that this variant was

maternally inherited in the newborn. There is no family history suggestive of MPS II. The family declined further familial cascade testing. The infant had a normal physical exam at 6 weeks of age and remained asymptomatic at his 11-month virtual check-in and 16-month in-person follow-up appointment with age-appropriate development and normal hearing assessment. Standard of care intravenous ERT was recommended but the family declined treatment and opted for disease monitoring.

The second infant with confirmed MPS II (case #27) was also identified to have a novel *IDS* variant with laboratory classification as a VUS, NM_000202.8 (IDS): c.1562A>G; p.(Glu521Gly) (Table S4). To our knowledge, this variant has not been reported in the literature but different substitutions at the same codon position—p.(Glu521Lys) and p.(Glu521Asp)—have been reported in individuals with MPS II. Functional analysis of one of the variants—p.Glu521Lys—indicates impaired protein processing in vitro[27]. The similar reported missense variants have been associated with variable disease severity including severe/neuronopathic with intellectual disability,[27] as well as intermediate[28] and mild[29] phenotypes. Given the novelty of the identified variant, *SUMF1* gene analysis (sequencing and del/dup) was pursued to rule out the possibility of multiple sulfatase deficiency and this testing was negative. This variant is absent from the Genome Aggregation Database (gnomAD v4.1.0). The in-silico prediction tool REVEL has a score of 0.9, suggestive of a deleterious effect. Given this information, as well as the low enzyme activity and the elevated NRE GAG biomarker observed in this individual, and the presence of other likely pathogenic or pathogenic variants at this same amino acid position, this variant could be reclassified as likely pathogenic.

Targeted variant analysis determined this variant was present in his mother and maternal aunt. The maternal grandfather was found to have atypical zygosity for the familial variant and is

suspected to have mosaicism. His biochemical labs were unremarkable, including normal I2S enzyme activity, NRE GAG biomarker levels, and urinary HS and DS results. The infant had a normal physical exam at 7 weeks of age but at a 2-month follow-up, he was found to have an inguinal hernia with an otherwise unremarkable examination. Hearing assessment and baseline imaging, including abdominal ultrasound and echocardiogram, were normal. Standard of care intravenous ERT was initiated at 3 months of age. The family expressed interest in enrollment in a central nervous system (CNS)-targeted clinical trial; however, the infant did not qualify due to the absence of evidence supporting a severe phenotype. Follow-up evaluations at 9 and 12 months of age remained essentially unremarkable, with no additional somatic manifestations of MPS II and age-appropriate developmental testing to date.

Pseudodeficiency Cases: All 31 newborns with pseudodeficiency had negative NRE GAG biomarker and negative or unremarkable urinary HS and DS (Figure 2D, 2E, and 2F). Of note, 22 newborns had mild isolated elevations of urinary DS that were well below the range observed in those with MPS and were considered nonspecific. These mild elevations of DS were most likely secondary to an interference from hyaluronic acid (HA), which was discovered more recently after completion of the study.[23] Reanalysis of a subset of specimens using a method that separated out this interference showed lower DS values that were within reference limits (Table S5). Of the 31 pseudodeficiency cases, 22 had I2S activity on NBS in the range of 5%-10% of the daily median and nine tested below 5% daily median. All 22 newborns with I2S activity between 5%-10% of the daily median and eight of the newborns with I2S activity below 5% of the daily median had *IDS* variants previously identified by other NBS programs and reported as pseudodeficiency alleles. Confirmatory I2S activity testing for several of those individuals was also positive (Figure 2C) further confirming that enzyme activity testing alone is insufficient for

disease confirmation. One newborn (case #29) had the second lowest NBS enzyme activity (0.7%) among all 33 screen-positive newborns and a novel VUS, NM_000202.8 (IDS): c.1438C>T; p.(Pro480Ser) that has not been previously published by NBS programs (Table 2). The interpretation of this novel variant was determined to be a pseudodeficiency variant based on the normal urine GAG results supported by normal NRE results.

Repeat enzyme testing on the DBS samples obtained at the time of the confirmatory evaluation using the NBS LC-MS/MS assay, revealed higher I2S activity than what was reported using the NBS specimen for 79% (22/28) of newborns with pseudodeficiency alleles. I2S activity remained low in both confirmed cases (Figure S7).

DISCUSSION

This pilot study demonstrated that the LC-MS/MS method for I2S activity measurement in DBS is an effective high-throughput strategy for MPS II NBS. The population distribution indicated a clear separation of affected from unaffected newborns at the 10% daily median. Therefore, when used with the implemented ≤10% daily median cutoff, the assay has a sufficient sensitivity to detect neonates with MPS II. Although differences were detectable in the large population dataset for age, weight, sex, gestational age, and race and ethnicity, implementing different cutoffs was not necessary for any of the groups. The ≤10% daily median cutoff performed equally well across all subgroups. Furthermore, both newborns confirmed with MPS II had I2S activity ≤5% daily median and all individuals with enzyme activity between 5% and 10% were confirmed to have pseudodeficiency alleles, with no molecular or biochemical evidence suggestive of MPS II disease, indicating that the 10% daily median provides a sufficient margin to identify at risk cases. However, as observed in this pilot study, first-tier

enzyme activity values alone cannot reliably differentiate between newborns confirmed with MPS II and those with pseudodeficiency.

Although *IDS* genotyping provided valuable supplementary information to support clinical decision-making, utilization of molecular analysis was complicated by the presence of variants with conflicting or uncertain interpretations in both males confirmed with MPS II and pseudodeficiency cases. Implementation of second-tier molecular analysis that classified newborns with benign or likely benign *IDS* variants as negative NBS results would have improved the PPV from 6.1% to 30.3%. However, incorporating NRE GAG biomarker testing as a second-tier method would have been an effective strategy to increase the PPV from 6.1% to 100% for this cohort.

Studies have demonstrated that the underlying pathological process of GAG accumulation in MPS II is established during fetal development and that the disease process of GAG accumulation begins in utero.[30, 31] Therefore, the biochemical phenotype, as determined through enzyme activity and GAG accumulation, remains the primary and most reliable criterion for confirming MPS II cases initially identified through NBS. In this study, the NRE GAG biomarker ratios and confirmatory urinary GAG results were concordant and abnormal exclusively in newborns confirmed with MPS II, whereas all cases with *IDS* pseudodeficiency alleles had normal NRE GAG ratios (Figure 2D).

Although most newborns with pseudodeficiency variants exhibited mild isolated elevations in urinary DS (Figure 2F) with normal urinary HS (Figure 2E) in all but one, their DS and HS levels remained substantially below the typical range observed in patients with MPS II. Using an improved LC-MS/MS method for DS analysis[23] on an available subset of confirmatory specimens from individuals with pseudodeficiency alleles (Table S5), demonstrated that the mild

DS elevations were attributed to interference in the DS measurement from HA in urine. This observation was discovered after the completion of the original analyses. The normal levels of DS in the reanalyzed samples provide additional support that I2S pseudodeficiency variants are not associated with disease phenotypes at birth.

Studies have shown that second-tier GAG testing in newborn DBS significantly reduces the false positive rate compared to measuring enzyme activity alone,[20, 32] and may be preferable to molecular sequencing, which can introduce uncertainty due to VUS. Two general methods exist for measuring DBS GAGs. One approach is the internal disaccharide method that cleaves GAG polymers with bacterial enzymes to produce quantifiable disaccharides and the other is the NRE method such as the endogenous NRE biomarker, used in this study, which measures naturally occurring NRE fragments without *in vitro* digestion.[33] Although the endogenous NRE biomarker method has been utilized less frequently by NBS programs, this and other studies support its use as a powerful second-tier method for MPS NBS.[32]

In this study, second-tier NRE GAG biomarker results clearly distinguished newborns with confirmed MPS II, who require intervention and comprehensive clinical care, from those with pseudodeficiency, who do not require medical care nor long-term surveillance. In a real-world setting, incorporating the NRE GAG biomarker into the NBS algorithm is expected to reduce clinical burden and follow-up referrals, while minimizing parental anxiety and avoiding unnecessary medicalization for newborns with pseudodeficiency variants.

Conclusion: This study successfully piloted population-wide NBS for MPS II in North Carolina, demonstrating feasibility through semi-automated LC-MS/MS analysis of I2S enzyme activity. While first-tier I2S activity can differentiate newborns with low enzyme activity from the population distribution, our findings demonstrate that a second-tier testing strategy is needed

to address the high false-positive rate observed and improve NBS specificity. The NRE GAG biomarker analysis utilized in this study clearly distinguished between newborns with MPS II and those with pseudodeficiency variants, while molecular analysis failed to provide definitive evidence in both affected males. Our results provide evidence-based guidance for optimizing MPS II NBS algorithms through implementation of a two-tier strategy combining I2S enzyme activity with NRE GAG biomarker in newborn DBS. This newborn screening approach enhances the precision of MPS II identification in newborns while minimizing unnecessary anxiety and follow-up burden associated with false-positive results. Molecular *IDS* analysis, whether included in the NBS algorithm or completed as a part of confirmatory testing, provides additional information for clinical evaluation and follow-up.

DATA AVAILABILITY

All available data are provided in supplemental files. No additional or individual level data are available for privacy reasons.

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AUTHOR CONTRIBUTIONS

Conceptualization: KSK, LMG, SMS, EJ, DB, SPY, CR, MR, JM; Data curation: KC, SB, VC, BM, LT, JC, JA, KT, ET; Funding Acquisition: KSK, LMG, MR, JM; Investigation: KSK, KC, SLB, EJ, DB, SPY, JM; Methodology: SLB, KC; Project Administration: KSK, LG, MR; Resources: SMS, Supervision: KSK, SMS, CR; Visualization: KSK, SLB; Writing-original draft preparation: KSK, KC, LG; Writing-review and editing: SB, JC, JA, KT, ET, SMS, EJ, DB, SY, CR, VC, BM, LT, MR, KC, JM

ETHICS DECLARATION

This study was performed under a waiver of informed consent approved by the University of North Carolina at Chapel Hill IRB (#22-0316).

CONFLICT OF INTEREST

THE AUTHORS DECLARE NO CONFLICTS OF INTEREST.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process: During the preparation of this work the author(s) used ChatGPT (open AI) for context and reference searches and for flow and grammar reviews. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

SUPPLEMENTARY FILES

Supplementary File 1: Figures

Supplementary File 2: Tables

Supplementary File 3: Parents Fact Sheet

Supplementary File 4: Provider Fact Sheet

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FIGURES AND TABLES:

Table 1: Study participant demographics and NBS results

Table 2: Screen positive results

Figure 1: Screening algorithm (A), and short-term follow-up algorithm (B)

Figure 2: NBS I2S activity and reflex and confirmatory results by genotype. (A) NBS I2S activity % daily median, (B) NBS I2S activity, (C) confirmatory I2S activity in repeat blood sample, (D) NRE GAG biomarker in the newborn DBS, (E) confirmatory heparan sulfate in urine, (F) confirmatory dermatan sulfate in urine, (G) confirmatory chondroitin sulfate in urine. Cut-offs for each assay (where applicable) are indicated by a horizontal line. The order of genotypes indicated in panel (A) corresponds to the order in all other panels. The number of individuals with the same genotype is indicated at the top of each box plot in panel (A). The causal variants in the two confirmed positive cases are highlighted in gray in panel (A). Results for the two confirmed positive cases are highlighted by gray shading in each panel.

Table 1: Study participant demographics and NBS results

Category[1]	Count (%) all screened	Count (%) abnormal I2S
Total Specimens	228,259 (N/A)	N/A
Total Infants	219,757 (100)	33 (100)
Sex		
Male	110,697 (50.4)	32 (97.0)
Female	105,537 (48.0)	0
Ambiguous	3,523 (1.60)	1[2] (3.03)
Birthweight (grams)		
Normal (>2500)	198,532 (90.3)	31 (93.9)
Low (>1500 and ≤2500)	17,694 (8.05)	2 (6.06)
Extremely low (≤1500)	3,531 (1.61)	0
Age at DBS collection (hours)		
<24	4,097 (1.86)	1 (3.03)
≥24 and <48	199,321 (91.9)	31 (93.9)
≥48 and <72	7,414 (4.04)	1 (3.03)
≥72	8,925 (4.06)	0
Gestation (weeks)		
Term (>37)	166,357 (75.7)	21 (63.6)
Premature (>28 and ≤37)	51,502 (23.4)	12 (36.4)
Extremely premature (≤28)	1,898(0.864)	0
Race (DBS card)		
American Indian/Alaska Native	2,241 (1.02)	0
Asian	6,360 (2.89)	6 (18.2)
Black	42,411 (19.3)	3 (9.09)
Native Hawaiian/Pacific Islander	339 (0.154)	0
White	112,802 (51.3)	19 (57.6)
Multiple	5,419 (2.47)	1 (3.03)
Unknown	50,185 (22.8)	4 (12.1)
Hispanic or Latino Origin		
Yes	28,213 (12.8)	1 (3.03)
Unknown	191,544 (87.2)	32 (97)
NICU		
Yes	19,365 (8.81)	3 (9.09)
No	200,392 (91.2)	30 (90.9)
Twins	3562 (1.62)	1 (3.03)
Triplets	78 (<0.1)	0
Other multiple	4 (<0.1)	0

[1]All demographic data were collected from the newborn screening cards

[2]The sex of the screen-positive newborn with abnormal I2S and "ambiguous" sex determination on the NBS card was later confirmed to be a male.

Table 2: Screen positive results

	I2S %Activity µM/hr	IDUA[1] %Activity µM/hr	DBS NRE GAG Ratios Ref. Range ≤1.84	Genomic variant (GRCh38; NC_000023.10)	Coding variant (*IDS*; NM_000202.8)	Predicted protein variant (I2S; NP_000193.1)	HS[2]	DS[2]	CS[2]	Urine GAGs Interpretation	Blood I2S Ref. Range >2.5 pmol/punch/h	Maternal Ethnicity	Age at Positive Screen (Days)
1	7.7% (1.22)	89.5% (0.92)	1.216 normal	g.148582549G>A g.148578727A>G g.148564431G>A	c.438C>T c.709-680T>C c.1499C>T	p.(Thr146=) p.(Thr500Ile)	2.5	8.5	19.6	negative	3.6 negative	White	40
2	7.1% (1.20)	108.8% (1.27)	0.682 normal	g.148578727A>G g.148564431G>A	c.709-680T>C c.1499C>T	 p.(Thr500Ile)	2.1	11.4	21.5	negative	1.4 positive	Asian China	36
3	6.7% (1.08)	112.5% (3.74)	1.004 normal	g.148582549G>A g.148578727A>G g.148564431G>A	c.438C>T c.709-680T>C c.1499C>T	p.(Thr146=) p.(Thr500Ile)	4.1	8.3	25.1	negative	3.1 negative	White	33
4	8.0% (1.602)	106.7% (3.16)	0.428 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	4.2	15.7	33.7	negative	2.1 positive	White Scotch-Irish	33
5	7.9% (1.676)	72.60% (0.894)	0.284 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	7.6	15.7	24.3	negative	1.8 positive	White	34
6	8.9% (1.954)	123.5% (1.33)	0.36 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	4.1	13.6	24.9	negative	3.5 negative	White	29
7	5.2% (0.913)	90.3% (2.79)	1.22 normal	g.148571961C>T	c.890G>A	p.(Arg297His)	8.3	10.7	22	negative	0.9 positive	White	30
8	9.6% (1.712)	133.7% (1.52)	0.42 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	7.4	13.7	32.6	negative	3.1 negative	White	32
9	8.6% (1.599)	113.0% (4.33)	0.28 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	4	23.4	22.8	negative	1.7 positive	White	28
10	3.2% (0.569)	114.4% (3.65)	0.34 normal	g.148579662T>C g.148577905G>A	c.684A>G c.851C>T	p.(Pro228=) p.(Pro284Leu)	3.8	7.7	25.1	negative	0.6 positive	Asian Korea	36
11	7.3% (1.389)	72.5% (1.39)	0.16 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	2.6	10.1	21.4	negative	2.0 positive	White	28
12	0.2% (0.029)	38.4% (3.05)	4.1 abnormal	g.148564491G>A	c.1439C>T[3]	p.(Pro480Leu)	108.4	28.9	38.9	Consistent with MPS II diagnosis	0.0 positive	White	26
13	7.4% (1.375)	127.7% (4.34)	0.68 normal	g.148582549G>A g.148579705G>A g.148578002C>A	c.438C>T c.641C>T c.754G>T	p.(Thr146=) p.(Thr214Met) p.(Asp252Tyr)	not done	not done	not done	not done	not done	African American	24
14	4.2% (0.731)	77.6% (0.82)	0.40 normal	g.148579662T>C g.148577905G>A	c.684A>G c.851C>T	p.(Pro228=) p.(Pro284Leu)	8.3	14.6	24.3	negative	0.0 positive	Asian Taiwan/China	25
15	8.7% (1.499)	75.2% (2.60)	0.27 normal	g.148564431G>A	c.1499C>T	p.(Thr500Ile)	5.7	14.2	26.4	negative	3.2 negative	Asian South Korea	25
16	9.0% (1.503)	78.7% (2.62)	0.22 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	3.9	15.2	25.6	negative	3.5 negative	White	28
17	9.0% (1.539)	79.5% (2.81)	0.62 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	7.6	17.4	24.8	negative	1.0 positive	White	28

	I2S %Activity µM/hr	IDUA[1] %Activity µM/hr	DBS NRE GAG Ratios Ref. Range ≤1.84	Genomic variant (GRCh38; NC_000023.10)	Coding variant (*IDS*; NM_000202.8)	Predicted protein variant (I2S; NP_000193.1)	HS[2]	DS[2]	CS[2]	Urine GAGs Interpretation	Blood I2S Ref. Range >2.5 pmol/punch/h	Maternal Ethnicity	Age at Positive Screen (Days)
18	4.7% (0.832)	136.2% (3.73)	1.23 normal	g.148579662T>C g.148577905G>A	c.684A>G c.851C>T	p.(Pro228=) p.(Pro284Leu)	7.5	18.1	26.5	negative	0.0 positive	Asian China	31
19	3.7% (0.614)	107.6% (3.53)	1.48 normal	g.148577971A>T	c.785T>A	p.(Val262Glu)	4.7	20.5	31	negative	0.0 positive	White	29
20	2.9% (0.435)	93.2% (1.09)	0.87 normal	g.148564521G>A	c.1409C>T	p.(Ser470Leu)	2.6	10.8	15.7	negative	0.8 positive	African American	26
21	6.8% (0.977)	88.6% (2.77)	0.78 normal	g.148579662T>C g.148577905G>A	c.684A>G c.851C>T	p.(Pro228=) p.(Pro284Leu)	6.8	21.2	28.2	negative	0.4 positive	Asian Korea	28
22	3.9% (0.568)	83.9% (2.62)	1.00 normal	g.148579662T>C g.148577905G>A	c.684A>G c.851C>T	p.(Pro228=) p.(Pro284Leu)	4.9	20	27.3	negative	0.0 positive	Asian Korea	28
23	5.1% (0.947)	102.4% (2.99)	0.68 normal	g.148579662T>C g.148577905G>A	c.684A>G c.851C>T	p.(Pro228=) p.(Pro284Leu)	not done	not done	not done	not done	not done	unknown	29
24	6.0% (1.210)	96% (0.91)	0.34 normal	g.148564452C>T	c.1478G>A	p.(Arg493His)	4.4	18	25.3	negative	0.4 positive	Hispanic Guatemala / El Salvador	27
25	9.5% (1.768)	77.6% (2.61)	0.49 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	6.1	20.3	26.5	negative	2.5 positive	White	32
26	4.70% (0.962)	101.1% (1.18)	0.68 normal	g.148582549G>A g.148577905G>A	c.438C>T c.851C>T	p.(Thr146=) p.(Pro284Leu)	3.7	14.3	25.9	negative	2.4 positive	White	32
27	4.10%[4] (0.797)	88.64% (4.97)	9.86 abnormal	g.148582549G>A g.148564368T>C	c.438C>T c.1562A>G	p.(Thr146=) p.(Glu521Gly)	344	66.1	47.1	Consistent with MPS II diagnosis	0.0 positive	White	30
28	9.10% (2.171)	99.70% (4.75)	1.09 normal	g.148582549G>A g.148579705G>A g.148578002C>A	c.438C>T c.641C>T c.754G>T	p.(Thr146=) p.(Thr214Met) p.(Asp252Tyr)	6.1	24.7	27.1	negative	3.0 negative	African American	30
29	0.70% (0.131)	80.80% (1.10)	0.76 normal	g.148564492G>A	c.1438C>T	p.(Pro480Ser)	7.6	22.4	21.2	negative	0.0 positive	Nepali	26
30	9.97% (2.106)	NA	0.64 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	3.2	19.5	27.3	negative	3.6 negative	White	30
31	7.7% (1.789)	NA	0.19 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	3.9	21.7	29.9	negative	1.0 positive	White	28
32	8.8% (1.84)	NA	0.31 normal	g.148582549G>A g.148564431G>A	c.438C>T c.1499C>T	p.(Thr146=) p.(Thr500Ile)	2.5	10.7	23.1	negative	1.6 positive	White	35
33	2.4% (0.499)	NA	0.87 normal	g.148577971A>T	c.785T>A	p.(Val262Glu)	3.0	11.1	31.6	negative	0.0 positive	White	27

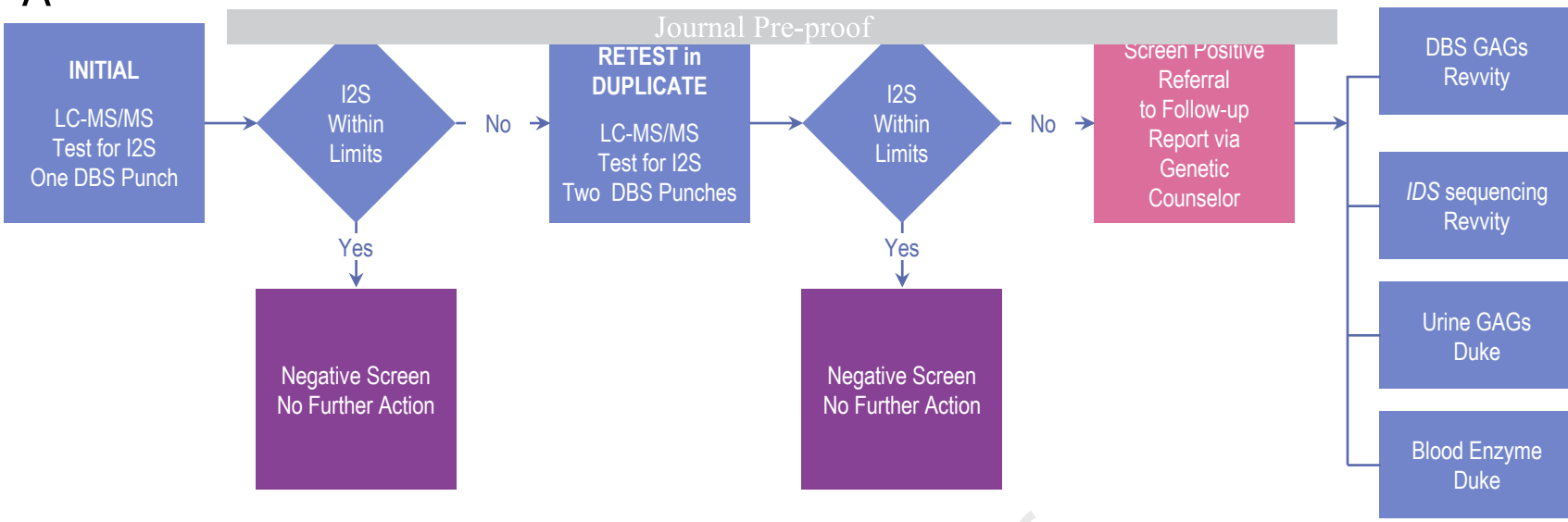
[1]Semiquantitative results. Not measured for cases 30-33.

[2]Heparan sulfate (HS) Ref. range <8.3 g/mol creatinine, dermatan sulfate (DS) Ref. range <10.7 g/mol creatinine, and chondroitin sulfate (CS) Ref. range <55.6 g/mol creatinine.

[3]Maternally inherited, additional familial cascade testing deferred.

[4]Initial NBS result was 0.5% of the daily median (0.109 µM/hr) and the reported mean of the repeat in duplicate results was 4.1 % of daily median (0.797 µM/hr). The duplicate repeat results were concordant [4.35% (0.845 µM/hr) and 3.85% (0.749 µM/hr)].

A



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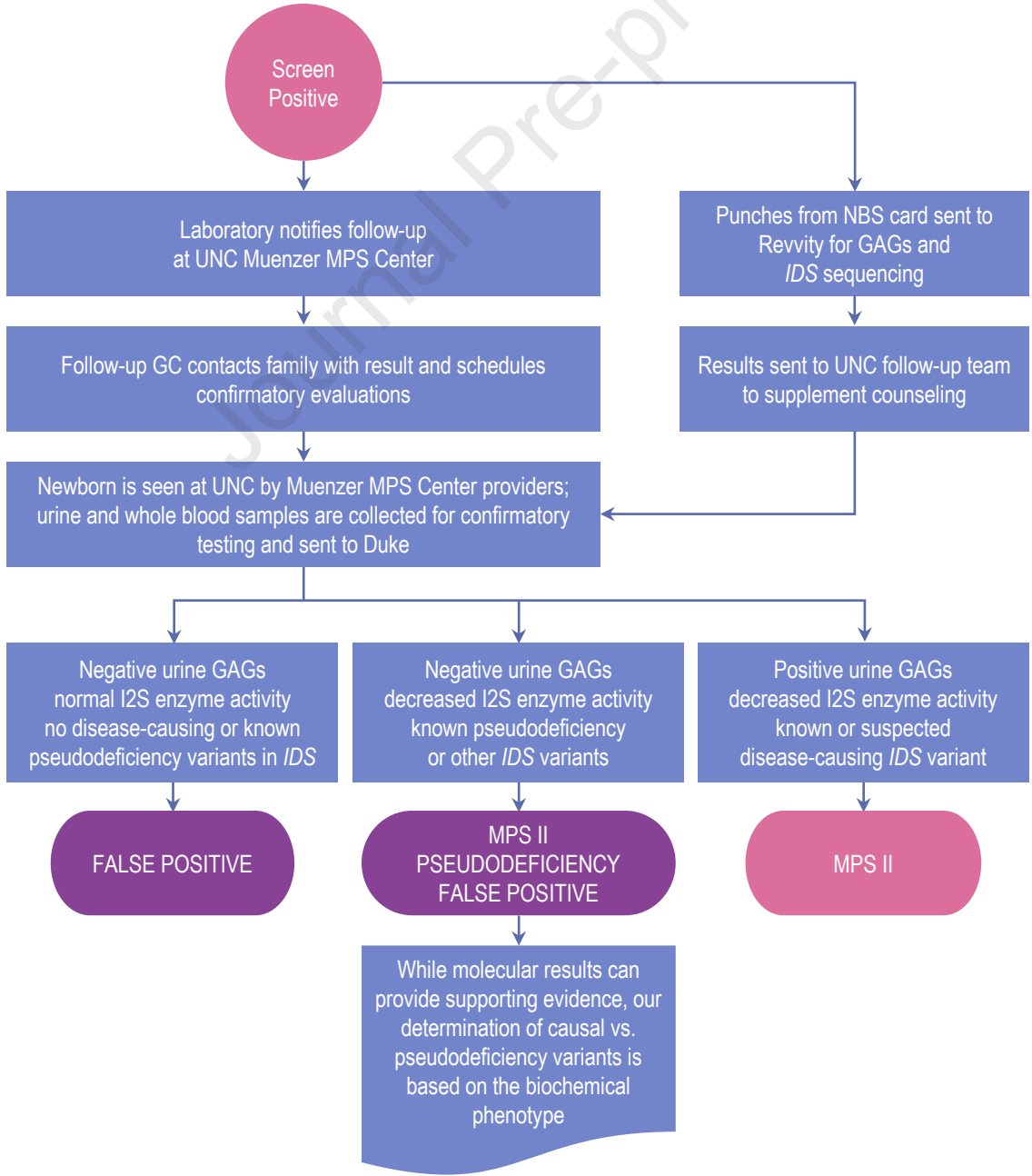

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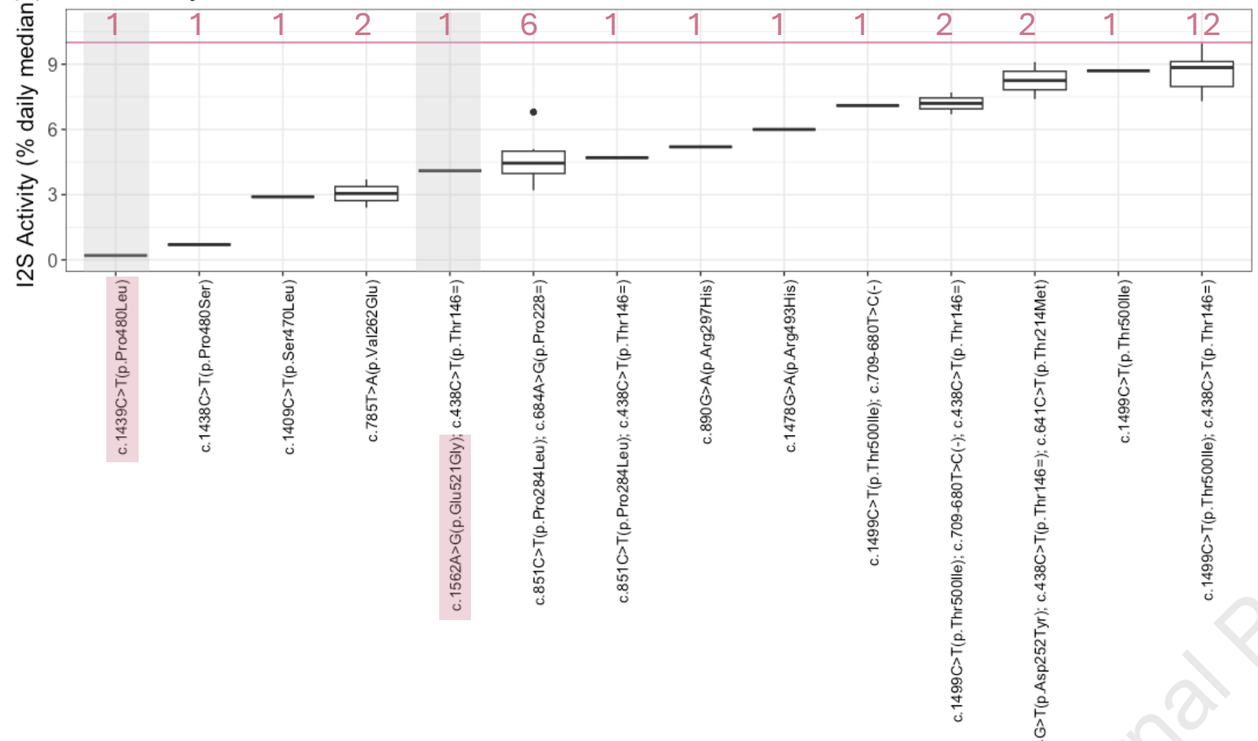

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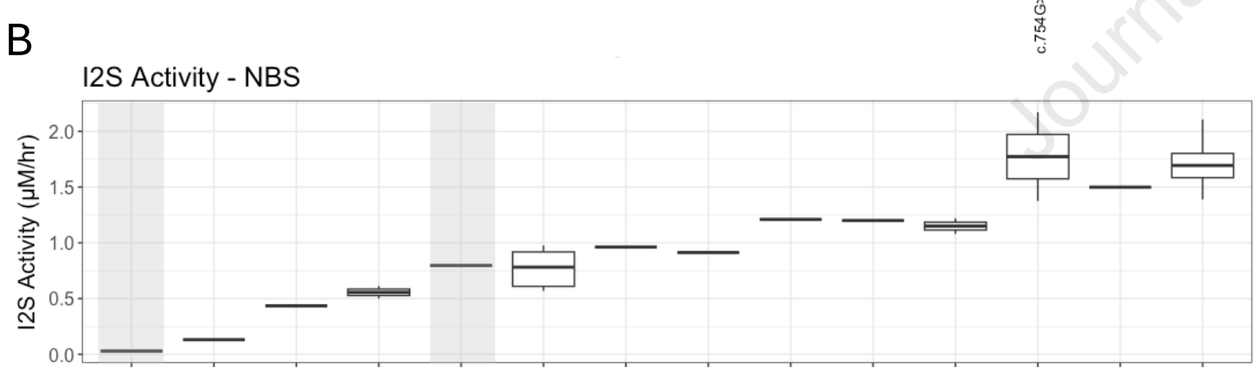

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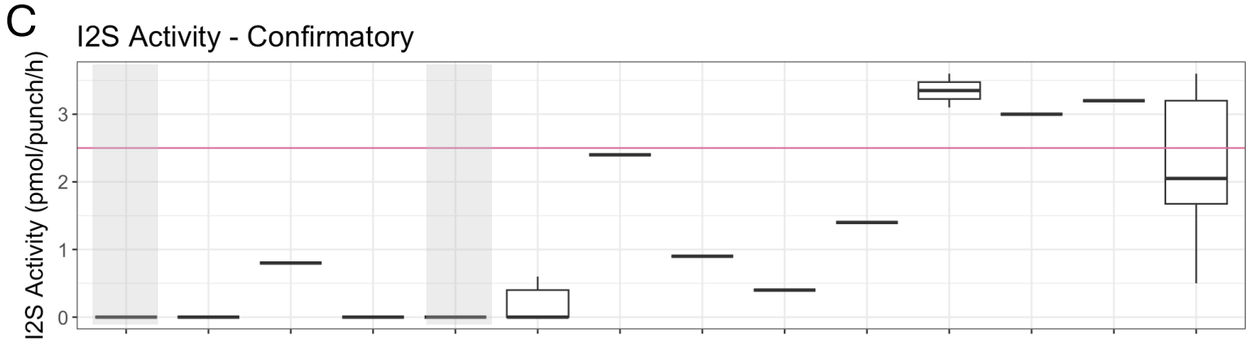

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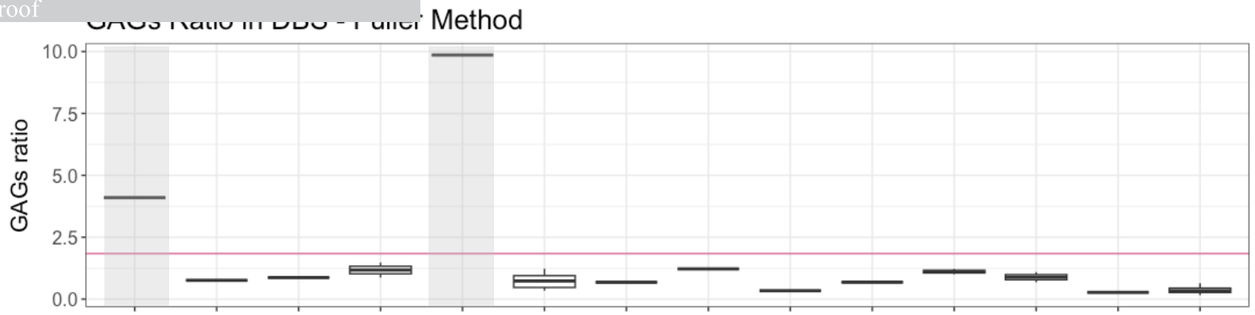

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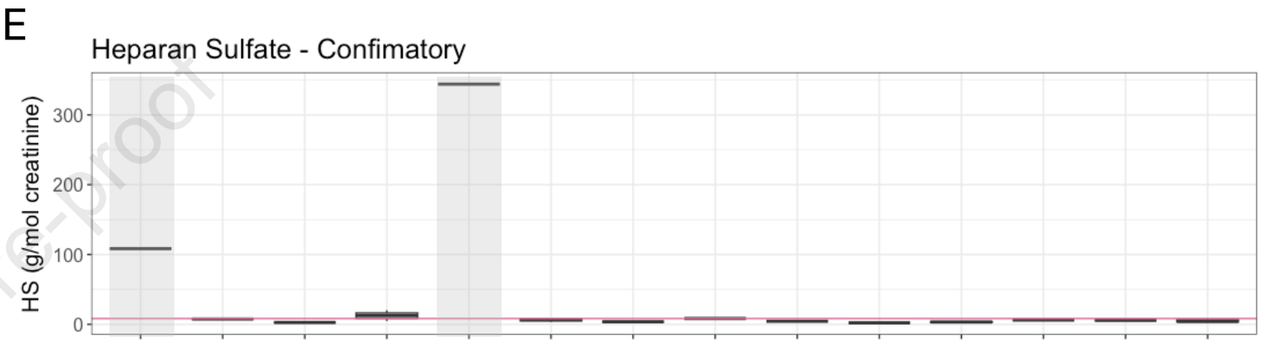

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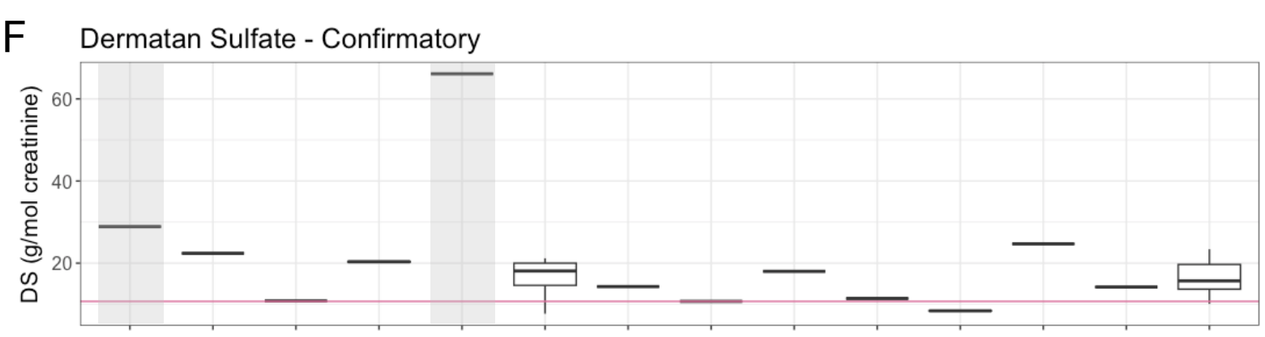

G