Newborn Screening for Methylmalonic Acidemia/propionic Acidemia: Systematic Evaluation of a Two-pronged Approach Based on MS/MS and UPLC-MS/MS

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Abstract

Background: An improved second-tier test is needed to reduce the false-positive rate of newborn screening (NBS) for inborn metabolic disorders in Xuzhou, China.

Methods: We designed an expanded second-tier assay using newborn dried blood spots (DBSs). Analytical and clinical performance were evaluated in 53 newborns with methylmalonic acidemia (MMA) or propionic acidemia (PA) reported by the Xuzhou Maternity and Child Health Care Hospital NBS program. Additionally, we analyzed NBS data regarding seasonal variation of metabolites, birth weight and gestational age to improve the identification of true positive MMA/PA individuals.

Results: Among the 53 MMA/PA individuals assessed, two pathogenic or likely pathogenic (P/LP) variants in an MMA/PA-associated gene were identified in 46 patients, and a pathogenic variant and a variant of unknown significance (VUS) were identified in 7 patients. No such variants were detected in MMA/PA false-positive individuals or healthy controls. Ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)-based analysis of the initial NBS metabolic profile correctly identified MMA/PA individuals and reduced the initial NBS false-positive rate by 98.86%. MMA/PA false-positive infants in Xuzhou, China, were most likely to be summer-born.

Conclusion: We established a two-pronged approach to reduce false positives by nearly 99% and provided a novel NBS strategy. Challenges in neonate metabolic testing and DNA variant interpretation regarding season, birth weight and pregnancy status remain for this Chinese population.

Introduction

Methylmalonic acidurias (MMAs) are characterized by inherited errors of metabolism that are attributed to total or partial deficiency of l-methylmalonyl-CoA mutase activity (the mut^0 and mut^- subtypes, respectively), defects in the synthesis of its cofactor (5-adenosylcobalamin; the cblA , cblB , cblC and cblD MMA subtypes) or methylmalonyl-CoA epimerase deficiency^{1–3}. The diagnosis of MMAs is based on the measurement of abnormal accumulation of MMA accompanied by increases in methylcitric acids and propionylglycine in urine, as well as propionylcarnitine (C3) in blood. Propionic acidemia (PA) is an autosomal recessive disorder with defects in the biotin-dependent enzyme propionyl-CoA carboxylase^{4,5}. Large amounts of urinary 3-hydroxypropionic and methylcitric acids and tiglylglycine propionylglycine and the absence of MMA are required for the diagnosis of PA⁶. Similarly, increased amounts of C3 in the blood are detected. The worldwide prevalence of MMAs/PA varies widely depending on ethnicity and the method of ascertainment. Based on newborn screening (NBS) programs in Europe and the USA, the incidence of MMA deficiency is estimated to be 1:46,000 to 1:200,000 in Europe and the United States⁷. However, the incidence of MMA deficiency in China varies greatly and affects neonates from 1:3220 to 1:21,488 in different reports⁸. Conversely, for PA, the previously reported epidemiological data appears to be slightly lower, with an incidence of PA is a rare organic acidemia with an average estimated incidence of ~ 1:100,000-150,000 in the worldwide^{9,10}.

NBS using tandem mass spectrometry (MS/MS) and dried blood spots (DBSs) is a significant innovation in inherited metabolic disease detection¹¹. Previous studies showed that determination of the C3 analyte content in DBSs, a common specimen collected by heel stick 48 ~ 72 hours after birth, can be used to confirm MMAs and

PA; however, NBS with MS/MS assessment of C3 favors sensitivity over specificity and yields a number of false positive results. Although MS/MS screening is beneficial for maximizing the number of neonates identified (sensitivity), this approach has a slightly high false-positive rate caused by its poor specificity, which results in considerable adverse financial and emotional burden effects given the required follow-up visits and diagnostic delays for true positive infants¹². To reduce the false-positive rate, further evidence is needed for second-tier tests utilizing ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to measure more specific disease markers (e.g., MMA in DBSs) to confirm true positives or reject false positives from the primary screening¹³. Both primary and second-tier screening methods utilize the original DBSs and do not require a new blood draw, minimizing turnaround time.

Here, we adapted a novel validated supplemental UPLC-MS/MS technology using three 3-mm DBS punches and used it to evaluate archived DBSs from newborns with different types of positive screens for MMAs and PAs. C3-associated disorder screening is fraught with false-positive cases that require second-tier confirmation using UPLC-MS/MS, and DNA sequencing is necessary to reach a final diagnosis and to identify which pathogenic genes are responsible and the severity of the specific variant. On the basis of 509,313 newborns born in Xuzhou, China, over a 5-year period between November 2015 and December 2020 C3, C3/C2 and methionine (Met) data measured by first-tier MS/MS technologies, we developed a novel strategy using MMA, methylcitrate (MCA) and homocysteine (HCY) values as second-tier test parameters for differential screening in initially positive samples¹⁴. In addition to the primary MS/MS analytes currently used in NBS for MMAs and PA, our statistical approach utilizes information from the entire MS/MS metabolic profile measured at birth.

Materials And Methods

Subjects

In total, 509,313 neonates (270,100 males and 239,213 females) were recruited for NBS with MS/MS at Xuzhou Maternity and Child Health Care Hospital between November 2015 and December 2020. On the basis of C3 levels and relative ratios, 263 suspected positive individuals and 1,830 healthy individuals were subjected to second-tier screening by UPLC-MS/MS between January 2019 and March 2020. Written informed consent was obtained from all the parents of the patients. A flow diagram of the screening inclusion process is shown in Supplementary Figure IA. This study was approved by the Ethical Committee of Xuzhou Maternity and Child Health Care Hospital (protocol ID: 2019. [08]).

Determination of the C3 level in DBSs by MS/MS for NBS

NBS for MMAs and PA was used to detect the C3 level in DBSs. Samples were collected on neonatal screening cards at 36 or 72 hours after birth and dried at room temperature using commercial kits. One DBS of 3 mm in size (equivalent to 3.2 μ L whole blood) was placed in a 96-well U-shaped plate and preprocessed with 100 μ L extraction solution containing internal standard mixture (NeoBase™ Nonderivatized MS/MS Kit, PerkinElmer™, Turku, Finland.). Then the plate was incubated at 45 °C for 45 min, transferred to a 96-well V-shaped plate and covered with aluminum film before detection. After the samples were allowed to rest at room temperature for 2 hours, the concentrations of C3 in the DBSs were quantified by MS/MS (Xevo TQD/TQD, Waters, USA). The data were collected using electrospray positive ion scanning and the multiresponse monitoring mode. The total

determination time was 3 min for each sample with a 15 μ L injection volume. Masslynx V4.1 software was used to analyze the screening data.

Determination of the MMA, MCA and HCY levels in DBSs by second-tier UPLC-MS/MS

The concentrations of MMA, MCA and HCY in 3.2-mm punch-outs from the collected NBS samples were quantified by UPLC-MS/MS (Waters, USA). Stock solutions contained more than 3 different standardized acylcarnitines. Three dried blood ounces ($d = 3.2$ mm) were deposited into a 96-well U-shaped plate. A solution containing the internal standards along with methanol and dithiothreitol (DTT) was added to each well, and the samples were allowed to rest for 20 min at room temperature. Then the supernatant was transferred to a 96-well V-shaped plate and dried at 55 °C covered by aluminum film. Stock solution was incubated at 65 °C for 30 min, and subsequently, the samples were dried with nitrogen at 40 °C and redissolved in distilled water. Finally, the sample was centrifuged for 10 min at a speed of 3,000 rpm at 4 °C. Prepared samples were assessed with a Kinetex 2.6 μ m XB-C18 100A column (Phenomenex, USA) in a Waters ACQUITY UPLC System (Waters, USA) equipped with a Xevo XE tandem mass spectrometer (Waters, USA). m/z 231 $\rightarrow$ 119 and m/z 231 $\rightarrow$ 175 were selected as characteristic qualitative ion pairs of methylmalonic acid by gradient elution, while m/z 234 $\rightarrow$ 122 and m/z 234 $\rightarrow$ 178 were used as the characteristic ion pairs of the internal standard D3-methylmalonic acid. The characteristic qualitative ion pairs of MCA, D3-MCA, HCY and D8-HCY were m/z 375 $\rightarrow$ 119 and m/z 375 $\rightarrow$ 273, m/z 378.2 $\rightarrow$ 202.1, m/z 192 $\rightarrow$ 90.1 and m/z 192 $\rightarrow$ 136.2 and m/z 192 $\rightarrow$ 94.1, respectively. The total run time was 7 min per reaction. The characteristics peak of the mass spectrometer is shown in Supplementary Figure IB.

Molecular Genetics analysis

Mutation analysis was performed for further diagnosis, involving DNA sequencing as described, and “site validation” of variants relevant to the screened disorders was analyzed. Using an ABI-3100 automated sequencer (Applied Biosystems, Foster City, CA, USA), molecular genetics were analyzed with mutation sites in pathogenic genes. The DNA sequencing pipeline developed for screening purposes, which included *MUT*, *MMAB*, *MMACHC*, *PCCA*, and *PCCB* genes as alternative targets, was employed to clinically diagnose individuals positive by MS/MS screening. As shown in supplementary Table 1, DNA panels used in our center included 78 genes which divided into two groups based on evidence for association with Xuzhou area metabolic conditions.

Statistical analysis

NBS metabolic data analysis: We performed a retrospective analysis of NBS data from 509,313 newborns that focused on 53 confirmed individuals' MS/MS analytes. Three analytes, namely, C2, C3 and Met, were interesting parameters in C3-associated disorders. We first compared analyte levels between different MMA/PA subtype individuals and controls (Fig. 1). Analysis of variance (ANOVA) was used to compare the 46 analytes between three specific phenotypic subgroups of 53 MMA patients (5 isolated MMA, 40 cbIC deficiency and 8 PA individuals). As differences in seasons, birth weight and gestational age (GA) may be associated with distinct metabolic profiles, we further stratified newborns into three subgroups: control, MMA/PA-negative (MMA/PA-N) and MMA/PA- true positive (MMA/PA-TP). Of 509,313 newborns, some individuals might have other abnormal metabolic levels that were detected by MS/MS and were removed from analysis. For comparison of distributions of C3, C3/C2, C3/C0 and Met values among neonates with Mann-Whitney U test and Kruskal-Wallis test, a p value < 0.05 was considered significant. Only C3 and C3/C2 assignments from screening samples were used to plot the receiver operating characteristic (ROC) curve (Fig. 3A and B).

Second-tier test data analysis: Based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (EP28-A3c: Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory, Approved Guideline, 3rd edition), the D/R ratio was $\leq 1/3$, and the two values of MMA and HCY were not outliers. Cases that lacked quantitative data (less than 5000 cases) were assessed using Kolmogorov-Smirnov (K-S) testing for normal distribution analysis. Significant data (p value < 0.05) were not identical to the normal distribution using K-S testing, and the percentile of a nonparametric test was used to establish the cutoff. A nonparametric test requires two values to be calculated, including the minimum reference value r_1 [e.g. if the 2.5th percentile is the corresponding observed value $r_1 = 0.025 (n + 1)$] and the maximum reference value r_2 [e.g. if the 97.5th percentile is the corresponding observed value $r_2 = 0.975 (n + 1)$]. Here, n represents the sampled data measurement.

Results

Determination of metabolites for NBS by MS/MS

Screening for MMAs and PA was performed with a cutoff of C3 acylcarnitine $\geq 4.3 \mu\text{mol/L}$ or C3/C2 ratio ≥ 0.2 , and both parameters were equally important for the initial NBS by MS/MS. From 2015 to 2020, 53 screen-positive individuals benefited from the expanded NBS program in Xuzhou, China, including 45 individuals with MMAs and 8 individuals with PA. Notably, 1 of 45 MMA individuals presented C3 and C3/C2 values below the established thresholds in the recall test and thus was technically a false negative in the NBS by MS/MS. This individual was subjected to second-tier metabolic analyses, and a significantly increased MMA concentration was identified by UPLC-MS/MS, which ultimately resulted in an isolated MMA diagnosis. To investigate MS/MS metabolic patterns, we determined the levels of three C3-associated NBS metabolic analytes in confirmed individuals and healthy infants (Fig. 1). Not unexpectedly, the concentrations of C3 showed a significant difference between the MMA/PA individuals and the control group individuals ($p = 3.1\text{e-}29$), but there was no significant difference between MMA individuals and PA individuals ($p = 0.50$). Significant differences were also identified for the C3/C2 ratio ($p = 8.01\text{e-}21$) between MMA/PA individuals and control group individuals. Met showed significant differences between MMA/PA subtypes, with relatively decreased Met levels in individuals with remethylation defects (cblC type) compared to those in individuals with propionyl-CoA carboxylase deficiency. However, Met levels were not significantly different between the isolated MMA group and the cblC type group (Fig. 1C). Unfortunately, there were almost no differences in other NBS analytes between neonates with MMA/PA and healthy infants, indicating that the other analytes were metabolically similar.

Biochemical and molecular genetic detection of MMA and PA individuals

Of the 53 confirmed individuals who were analyzed, multiple individuals carried compound heterozygous variants in pathogenic genes (Fig. 1D), resulting in a low percentage of homozygotes with only the cblC type in Xuzhou, China. DNA sequence analysis of 86 genes for NBS detected more variants in confirmed MMA/PA cases, in whom five associated genes (*MUT*, *MMAB*, *MMACHC*, *PCCA*, and *PCCB*) were identified. Forty-five MMA individuals (5 of whom had isolated MMA and 40 of which had cblC deficiency) and eight PA individuals had two P/LP variants or one P/LP variant and a variant of unknown significance (VUS) in a pathogenic gene. Eight different known *MUT* variants (c.323G > A, c.441T > A, c.581C > T, c.1106G > A, c.1219A > T, c.1741C > T, c.1677-1G > A and c.1880A > G) were identified in the 5 isolated MMA individuals from 5 unrelated families, and all the variants were located in exons, except for one splicing type variant. In addition, eleven variants were detected in the *PCCA* gene, and four variants were detected in the *PCCB* gene in 8 PA individuals, as shown in

Supplementary Table 1. Indeed, not only isolated MMA individuals but also PA individuals were confirmed to carry compound heterozygous variants. We observed a strikingly high frequency of cblC-type individuals in our cohort (75.47%, $n = 40/53$), in which 80 independent cblC alleles were affected. The sequencing results confirmed the compound heterozygous or homozygous variants in the *MMACHC* gene in 40 infants, of which 7 were homozygous for c.609G > A (71.4%, 5/7), c.658_660del (14.3%, 1/7) and c.80A > G (14.3%, 1/7) (Supplemental Table 1). To calculate metabolite levels, individuals homozygous for the *MMACHC* gene (patients 3, 9, 17, 30, 31, 33 and 40) were placed into one group for further analyses, with the other individuals placed in a second group. The mean C3/C2 ratio of the homozygous group obtained from the initially measured NBS data was 0.926, which was significantly different from that of the compound heterozygous group (C3/C2 ratio = 0.483). Correspondingly, the Met values in the homozygous group with *MMACHC* variants ranged from 3.34 to 11.01 $\mu\text{mol/L}$, with a mean value of 7.03 $\mu\text{mol/L}$, which was near the minimum value of the normal range of Met and lower than that in the compound heterozygous group. However, no significant difference in C3 values for individuals with cblC deficiency was found based on the biochemical data (Fig. 1).

Evaluation of NBS for MMAs and PA based on season, gestational age and birth weight

The levels of specific metabolites detectable (e.g., C2) by MS/MS for NBS are known to have seasonal variation. We assessed the seasonal profile of MMA/PA screening parameters (Fig. 2A/C/E) and found a significantly increased percentage of false negative results in newborns caused by low concentrations of C2 and Met in the summer than in other seasons from 2015 to 2020 ($p < 0.05$) (Fig. 2B). The relatively higher temperature and humidity in summer reduced the positive predictive value (PPV) from 3.86–1.83% with numerous false positive cases in Xuzhou, China. A previous study indicated that metabolic profiles may vary by gestational age (GA) or birth weight. We further compared GA between the MMA/PA-positive (MMA/PA-TP) group, MMA/PA-false-positive (MMA/PA-N) group and healthy control group (Fig. 2D). Within the MMA/PA-N group, there was a significantly higher proportion of preterm ($\text{GA} \leq 37$ weeks) than full-term (> 37 weeks) births after analysis with the Mann-Whitney U test. An additional analysis of neonate birth weight (normal: 2,500–4,000 grams) revealed a relatively lower birth weight for MMA screening-positive newborns (both true positives and false positive newborns) than that for the healthy group (Fig. 2E).

To evaluate the sensitivity of MS/MS analytes for MMA/PA screening, receiver operating characteristic (ROC) curves were utilized to distinguish true and false positive MMA/PA individuals. The optimal screening efficiency was determined using a sensitivity of 0.957 and a specificity of 0.972 under the curve area with 0.996 for parameter C3 (Fig. 3A and B). However, the parameter C3/C2 had a better sensitivity and specificity than C3 with ROC analysis. The mean decrease in the accuracy (MDA) index (Fig. 3D) suggested that the C3/C2 ratio was a significant parameter for MMA/PA screening. However, other metabolic analytes, including the C0 and Met levels, showed similar rankings by MS/MS. Of the above several covariates analyzed, seasonal variation played a fundamental role in primary NBS. Ranking analysis of the MS/MS metabolites showed significant differences based on phenotypic subtype. Individuals with cobalamin deficiency might have lower Met levels than those with other phenotypes (Fig. 1C). Moreover, the C2 and Met levels declined sharply during the summer due to the higher air temperature in Xuzhou, and parameter C2 was crucial for identifying MMA/PA in the initial MS/MS screening (Fig. 3E/F).

Generalization of a novel second-tier approach: determination of cutoffs for MMA, MCA and HCY

From January 2019 to March 2020, 263 samples with increased C3 concentrations at the initial NBS test were further assessed by novel second-tier UPLC-MS/MS screening. As the dataset was not symmetrical, a total of 1,830 negative subjects were used to calculate the cutoff value based on the percentile distribution of metabolites. The statistical results of the percentile distribution of the negative controls are shown in Table 1. Notably, the value "4.54" of the MMA metabolite and the value "12.64" of the HCY metabolite were two suspicious outliers. According to the NCCLS C28-A3 file, $D/R (MMA) = (4.54 - 3.96) / (5.55 - 0.00) = 0.10$, while $D/R (HCY) = (12.64 - 10.03) / (17.14 - 0.9) = 0.16$; however, both D/R ratios were $\leq 1/3$, and there was no outlier in any of the three metabolite indexes. Previous literature and clinical research have indicated that sex does not affect the three analytes that were measured with UPLC-MS/MS. Based on the distribution of the histogram chart, the 99.5th percentile and the 99.7th percentile were considered the cutoff values for the second-tier test (Table 1). When using the 99.5th percentile as the cutoff value, the positive rate of MMA/PA detection was estimated to be 0.45%, 0.40% and 0.76%, respectively. When the 99.7th percentile was reached, the positive rate of detection for each disorder was reduced, and the number of recall cases decreased from 43 to 29 (Table 2). A comprehensive analysis of several published laboratory parameters was performed, and as shown in Table 3, the 99.7th percentile was similar to the cutoff values established by the Mayo Clinic, USA, and the 99.5th percentile is used as the cutoff value in Nanjing, China. A strict test of the generalizability of the novel second-tier test should require confirmed MMA/PA individuals to be identified by this screening method. Considering the significantly increased concentrations of MMA, MCA and HCY in patients, the cutoff values for MMA, MCA and HCY for the second-tier test were 3.13, 0.29 and 9.77 $\mu\text{mol/L}$ (the 99.7th percentile), respectively.

Here, we developed a following nonderivatized approach using UPLC-MS/MS instead of primal derivatized testing. Despite there being only 207 negative controls for nonderivatized detection for the second-tier test, the new cutoffs for MMA and HCY showed significantly increased performance (Supplementary Table 2 and Supplementary Figure II). For comparison of the distribution characteristics of MMA, MCA and HCY values before and after modification, the Mann-Whitney test was performed. As shown in Fig. 2, there were significant differences among the three indexes before and after experimental improvement. First, we compared the 10th, 25th, 75th and 90th percentile MMA, MCA and HCY values. The 75th percentile values of MMA and HCY with primal derivatized detection were similar to the 25th percentile values with the nonderivatized approach, and the difference between the 10th and 90th percentiles was greater than 30%; there were only small differences in the MCA values between the 10th and 90th percentiles for the second-tier test. Taken together, these data show that the distribution of MCA values from the derivatized and nonderivatized tests was similar, which allowed us to conclude that the previous cutoff values could be generalized. In a specific test of the generalizability of the cutoff values for MMA and HCY, however, the previous cutoff values were not equal to the novel nonderivatized test values.

Comparison of the second-tier test with NBS analysis for newborns suspected to have MMA/PA disorders

Thirteen individuals with confirmed MMA/PA disorders were identified by second-tier screening from January 2019 to March 2020, and their disease was confirmed by urinary GC/MS and DNA sequencing. As shown in Fig. 1C, the individuals were identified with a one-step second-tier test before genetic analysis. The C3 level, C3/C2 ratio and Met level were analyzed in patients grouped based on the initial MS/MS NBS results: the isolated MMA group ($n = 1$), the Cbl group ($n = 6$), the PA group ($n = 2$) and the healthy group ($n = 1,830$). As shown in Fig. 1A, C3, Met and C3/C2 were generally increased in the three groups compared to the healthy group, while the C3/Met ratio was not significantly different. Unfortunately, none of the differences between the three disease groups

were significant. However, the second-tier UPLC-MS/MS test could directly distinguish these groups, and further statistical analysis of the parameters revealed significant differences (Fig. 4B, C). The mass spectrum peaks (shown in Fig. 4) revealed that a solely increased MMA peak likely indicated isolated MMA, an increased MMA peak combined with an increased HCY peak likely indicated Cbl deficiency, an increased MCA peak likely indicated PA, and other patterns likely indicated none of these disorders. Patients who fall within the uncertain category in initial NBS would be differentially screened using a second-tier test, as confirmation via genetic analysis and clinical follow-up is required, which means that this second-tier test can reduce the initial recall rate of patients suspected of having these disorders on NBS. The consideration of MMA, MCA and HCY as markers for MMA/PA would have eliminated 88.97% (234/263) of unnecessary referrals and recall tests.

Discussion

NBS for MMAs/PA is performed by measuring C3 concentrations in DBSs, which is considered a poor discriminator. Although MS/MS-based testing might identify numerous neonates with propionate metabolic disorders, the exact number of false positive cases appears to be affected by the unfavorable balance between screening specificity and sensitivity^{15–17}. Combined consideration of the C3/C2 ratio and Met improves the PPV, but the false positive rate for C3-associated disorders remains high without further differentiation¹⁸. This shortcoming necessitates further diagnostic workup and may lead to an unbalanced cost-benefit ratio of NBS, in addition to placing unnecessary emotional burden on the affected families. Here, we expanded a novel second-tier test based on UPLC-MS/MS specifically for the analysis of three specific metabolites (MMA, MCA and HCY¹⁹), which also provides useful information for differential screening for MMAs/PA, as these metabolites are currently recognized as candidate biomarkers for MMAs and PA. All metabolites and ratios calculated in the initial MS/MS screening or from the second-tier test, if altered, still require urinary GC/MS testing and DNA sequencing to confirm the suspected diagnosis¹¹.

Analysis of the regional season profiles of metabolites described in our center revealed that MMA/PA false positive cases were most increased in the summer in Xuzhou, China, during which the heat and humid environment accelerated the degradation of C2 before measurement^{20, 21}. While the birth prevalence for several disorders is known to vary among different GA groups, the finding of a higher MMA/PA false positive rate in groups of individuals with inadequate duration of pregnancy and low birth weight was surprising^{22, 23}. MMAs/PA are detected with NBS based on an elevated C3 level and/or C3/C2 ratio. Our first hypothesis was that summer-born infants might have a naturally lower level of C2 or a slightly higher C3/C2 ratio, which could directly result in an increased rate of MMA/PA false positives compared to that in non-summer-born infants. Another hypothesis was that neonates who are born prematurely might have higher levels of C3 or a higher C3/C2 ratio. The data suggest that preterm infants are very similar with respect to metabolite concentrations regardless of whether they have MMAs/PA; as such, it is difficult to reduce the false positive rate of NBS. However, the current limited results did not support a substantially higher preterm birth rate in summer-born newborns. Thus, further study is needed in a large-scale newborn population to test these hypotheses.

Interestingly, molecular genetic analysis for individuals with MMAs/PA is strongly recommended for cases in which the plasma Met and C3/C2 values are significantly lower (as seen with mild alterations with compound heterozygotes compared to homozygotes in cblC deficiency²⁴). Moreover, the detection of C3 is now well recognized as a complicating factor in initial MS/MS screening, primarily because most cases of MMA/PA

deficiency may present with elevated C3 concentrations. However, distinguishing features that limit the specificity of MS/MS screening for MMA/PA are lacking^{25, 26}.

It is worth noting that the utility of the second-tier test (which assesses DBSs via UPLC-MS/MS) highlights the importance of specific metabolites for differential disorder screening. Second-tier tests have been available for MMA/PA disorder screening to enable comprehensive detection in a time- and cost-effective manner. To facilitate broader application of the second-tier assay, we first established regional reference intervals for the clinical parameters. In the present study, when the 99.7th percentile MMA, MCA and HCY values (3.13, 0.29 and 9.77 $\mu\text{mol/L}$, respectively) were used as cutoffs, 234 out of 263 (88.97%) false positive babies from the initial MS/MS screening were excluded without recall testing. An HCY cutoff value of 9.77 $\mu\text{mol/L}$ has been proposed to yield higher sensitivity, and the second-tier test of HCY seems to separate the clbC type from isolated MMAs or PA. The detection of MCA, which is a specific metabolic analyte for PA disorders, can be expected to influence the proportion of mixed cases found on initial MS/MS screening. Newborns with elevated MMA alone might also have significantly lower MCA and HCY levels than clbC subtype newborns or newborns with PA disorders^{27, 28}. Overall, following initial MS/MS screening, a novel approach with a second-tier test effectively reduced the false positive rate and avoided unnecessary recall detection.

Conclusion

In summary, MS/MS-based NBS can reflect toxic metabolite alterations in MMA/PA cases as a first-tier test in neonates²⁹, but it results in numerous false positives. However, second-tier UPLC-MS/MS testing reduced the false positive rate by nearly 88.97% without resampling^{12, 30}. Applying this second-tier test, which enables direct and specific analyte analysis, could partly avoid false positives and help focus efforts on true positive individuals who require follow-up testing. The second-tier testing strategy enables the detection of a wide range of metabolic disorders with a single laboratory test and significantly reduces the incidence of morbidity and mortality in infants. Collectively, the combination of such a second-tier approach with initial MS/MS screening would provide more specific metabolic information for the physician, while subsequent molecular genetic validation could be implemented for rapid and inexpensive screening to benefit newborns with MMAs/PA and even other disorders.

Declarations

Authors' contributions

1. Conception and design of study: Wei Zhou and Maosheng Gu
2. Acquisition of data: Huizhong Li, Jinxiu Song and Xin Yin
3. Analysis and/or interpretation of data: Wei Zhou, Huizhong Li and Jinxiu Song
4. Drafting the manuscript: Wei Zhou and Xin Yin
5. Revising the manuscript critically for important intellectual content: Wei Zhou and Zhe Ji.

Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any

product, service and/or company that could be construed as influencing the position presented in the manuscript entitled, 'Newborn screening for methylmalonic acidemia/propionic acidemia: systematic evaluation of a two-pronged approach based on MS/MS and UPLC-MS/MS'.

Acknowledgments and Declaration

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Ethics approval and consent to participate

The protocol was reviewed and approved by the Ethics committee of the Affiliated Xuzhou Maternity and Child Health Care Hospital of Xuzhou Medical University (the committee's reference number: [2019] No.8).

Consent for publication

Informed and written consent was obtained from the guardians of all the patients before clinical testing.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

References

1. Rashka C, Hergalant S, Dreumont N, Oussalah A, Camadro JM, Marchand V, et al. Analysis of fibroblasts from patients with cblC and cblG genetic defects of cobalamin metabolism reveals global dysregulation of alternative splicing. *Hum Mol Genet.* 2020;29:1969-85.
2. Şeker Yilmaz B, KÖr D, Bulut FD, Kilavuz S, Ceylaner S, Önenli Mungan HN. Clinical and molecular findings in 37 Turkish patients with isolated methylmalonic acidemia. *Turkish journal of medical sciences.* 2021.
3. Wajner M. Neurological manifestations of organic acidurias. *Nature reviews Neurology.* 2019;15:253-71.
4. Shchelochkov OA, Carrillo N, Venditti C. Propionic Acidemia. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Mirzaa G, et al., editors. *GeneReviews*(®). Seattle (WA): University of Washington, Seattle Copyright © 1993-2021, University of Washington, Seattle. *GeneReviews* is a registered trademark of the University of Washington, Seattle. All rights reserved.; 1993.
5. Park KC, Krywawych S, Richard E, Desviat LR, Swietach P. Cardiac Complications of Propionic and Other Inherited Organic Acidemias. *Frontiers in cardiovascular medicine.* 2020;7:617451.
6. Wongkittichote P, Ah Mew N, Chapman KA. Propionyl-CoA carboxylase - A review. *Mol Genet Metab.* 2017;122:145-52.

7. Weisfeld-Adams JD, Morrissey MA, Kirmse BM, Salveson BR, Wasserstein MP, McGuire PJ, et al. Newborn screening and early biochemical follow-up in combined methylmalonic aciduria and homocystinuria, cblC type, and utility of methionine as a secondary screening analyte. *Mol Genet Metab.* 2010;99:116-23.
8. Wang C, Li D, Cai F, Zhang X, Xu X, Liu X, et al. Mutation spectrum of MMACHC in Chinese pediatric patients with cobalamin C disease: A case series and literature review. *European journal of medical genetics.* 2019;62:103713.
9. Dai M, Xiao B, Zhang H, Ye J, Qiu W, Zhu H, et al. Biochemical and genetic approaches to the prenatal diagnosis of propionic acidemia in 78 pregnancies. *Orphanet J Rare Dis.* 2020;15:276.
10. Almási T, Guey LT, Lukacs C, Csetneki K, Vokó Z, Zelei T. Systematic literature review and meta-analysis on the epidemiology of propionic acidemia. *Orphanet J Rare Dis.* 2019;14:40.
11. Peng G, Shen P, Gandotra N, Le A, Fung E, Jelliffe-Pawłowski L, et al. Combining newborn metabolic and DNA analysis for second-tier testing of methylmalonic acidemia. *Genet Med.* 2019;21:896-903.
12. Rossi C, Cicalini I, Rizzo C, Zucchelli M, Consalvo A, Valentinuzzi S, et al. A False-Positive Case of Methylmalonic Aciduria by Tandem Mass Spectrometry Newborn Screening Dependent on Maternal Malnutrition in Pregnancy. *International journal of environmental research and public health.* 2020;17.
13. Peng G, Tang Y, Cowan TM, Enns GM, Zhao H, Scharfe C. Reducing False-Positive Results in Newborn Screening Using Machine Learning. *International journal of neonatal screening.* 2020;6.
14. Weiss KJ, Röschinger W, Blessing H, Lotz-Havla AS, Schiergens KA, Maier EM. Diagnostic Challenges Using a 2-Tier Strategy for Methylmalonic Acidurias: Data from 1.2 Million Dried Blood Spots. *Annals of nutrition & metabolism.* 2020;76:268-76.
15. Arunkumar N, Langan TJ, Stapleton M, Kubaski F, Mason RW, Singh R, et al. Newborn screening of mucopolysaccharidoses: past, present, and future. *J Hum Genet.* 2020;65:557-67.
16. Lampret BR, Remec Ž I, Torkar AD, Tanšek M, Šmon A, Koračin V, et al. Expanded Newborn Screening Program in Slovenia using Tandem Mass Spectrometry and Confirmatory Next Generation Sequencing Genetic Testing. *Zdravstveno varstvo.* 2020;59:256-63.
17. Wang Y, Sun Y, Jiang T. Clinical Application of LC-MS/MS in the Follow-Up for Treatment of Children with Methylmalonic Aciduria. *Adv Ther.* 2019;36:1304-13.
18. Okun JG, Gan-Schreier H, Ben-Omran T, Schmidt KV, Fang-Hoffmann J, Gramer G, et al. Newborn Screening for Vitamin B(6) Non-responsive Classical Homocystinuria: Systematical Evaluation of a Two-Tier Strategy. *JIMD Rep.* 2017;32:87-94.
19. Chen T, Liang L, Zhang H, Ye J, Qiu W, Xiao B, et al. Value of amniotic fluid homocysteine assay in prenatal diagnosis of combined methylmalonic acidemia and homocystinuria, cobalamin C type. *Orphanet J Rare Dis.* 2021;16:125.
20. Yao Y, Huang Z, Xie P, Wu L, Ma L, Li T, et al. High temperature shockwave stabilized single atoms. *Nature nanotechnology.* 2019;14:851-7.
21. Ji ES, Lee HK, Park GW, Kim KH, Kim JY, Yoo JS. Isomer separation of sialylated O- and N-linked glycopeptides using reversed-phase LC-MS/MS at high temperature. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2019;1110-1111:101-7.
22. Tan A, Sinclair G, Mattman A, Vallance HD, Lamers Y. Maternal vitamin B(12) status in early pregnancy and its association with birth outcomes in Canadian mother-newborn Dyads. *The British journal of nutrition.*

2021;1-9.

23. Goto E. Ultrasound as a primary screening tool for detecting low birthweight newborns: A meta-analysis. *Medicine (Baltimore)*. 2016;95:e4750.
24. Han L, Chen C, Guo F, Ye J, Peng Z, Qiu W, et al. Noninvasive prenatal diagnosis of cobalamin C (cblC) deficiency through target region sequencing of cell-free DNA in maternal plasma. *Prenatal diagnosis*. 2020;40:324-32.
25. Adhikari AN, Gallagher RC, Wang Y, Currier RJ, Amatuni G, Bassaganyas L, et al. The role of exome sequencing in newborn screening for inborn errors of metabolism. *Nature medicine*. 2020;26:1392-7.
26. Li X, Wang W, Chen J. Recent progress in mass spectrometry proteomics for biomedical research. *Sci China Life Sci*. 2017;60:1093-113.
27. Yap S, Vara R, Morais A. Post-transplantation Outcomes in Patients with PA or MMA: A Review of the Literature. *Adv Ther*. 2020;37:1866-96.
28. Nashabat M, Obaid A, Al Mutairi F, Saleh M, Elamin M, Ahmed H, et al. Evaluation of long-term effectiveness of the use of carglumic acid in patients with propionic acidemia (PA) or methylmalonic acidemia (MMA): study protocol for a randomized controlled trial. *BMC pediatrics*. 2019;19:195.
29. Hu Z, Yang J, Lin Y, Wang J, Hu L, Zhang C, et al. Determination of methylmalonic acid, 2-methylcitric acid, and total homocysteine in dried blood spots by liquid chromatography-tandem mass spectrometry: A reliable follow-up method for propionylcarnitine-related disorders in newborn screening. *Journal of medical screening*. 2021;28:93-9.
30. Al-Dirbashi OY, McIntosh N, Chakraborty P. Quantification of 2-methylcitric acid in dried blood spots improves newborn screening for propionic and methylmalonic acidemias. *Journal of medical screening*. 2017;24:58-61.

Tables

Table 1
Statistical percentile of IVG concentrations by UPLC-MS/MS for negative samples (μmol/L)

	Number	Mean ± SD	Max	P50	P95	P97.5	P98	P99	P99.5	P99.7	Normal distribution
MMA	1830	0.51 ± 0.33	5.55	0.46	0.87	1.19	1.3	1.73	2.47	3.13	No
MCA	1830	0.11 ± 0.04	0.37	0.1	0.19	0.21	0.22	0.25	0.27	0.29	No
HCY	1830	2.76 ± 1.15	17.14	2.5	4.79	5.41	5.55	6.33	8.66	9.77	No

Table 2

The positive rate of second-tier test for different MMA/PA subtypes with the 99.5th and 99.7th percentiles

Type	P99.5		P99.7	
	Positive cases	Positive rate	Positive cases	Positive rate
cbIC MMA	10	0.45%	5	0.23%
Isolated MMA	9	0.40%	8	0.36%
PA	17	0.76%	11	0.50%

Table 3

Comparison of cutoff values for second-tier test in different regions

Region	Time	Numbers	MMA	MCA	HCY
Nangjing, China	2016.11 ~ 2017.12	423	0.70	0.18	3.04
Nangjing, China	2017.01 ~ 2018.08	1016	2.37	0.65	7.73
Beijing, China	2017.02 ~ 2017.06	140	-	-	8.02
Mayo, USA	2004 ~ 2007	30000	5	1	15
Xuzhou, China (P99)	2019.01 ~ 2019.09	914	2.34	0.27	6.26
Xuzhou, China (P99.5)	2019.06 ~ 2019.11	1536	3.29	0.28	9.43
Xuzhou, China (P99.7)	2019.06 ~ 2019.12	1871	3.64	0.29	9.97
Xuzhou, China (P99.7)	2019.01 ~ 2020.03	2210	3.13	0.29	9.77

Figures

Figure 1

Metabolite characteristics of MMA/PAs with parameters detected by MS/MS and UPLC/MS/MS among second-tier test samples. (A-C) The levels of C3, and Met and other relative C3/C2 ratio detected by MS/MS in DBSs for initial NBS between patients and healthy individuals. (D) The percentage of homozygote/heterozygote analysis in individuals confirmed with MMA/PA. (E-G) Metabolite measurement comparison of parameters by MS/MS between the homozygote group and heterozygote group with cbIC deficiency. Isolated MMA: n=1; MMACHC: n=13; PA: n=2 Normal: n=2210.

Figure 2

Newborns screened and MMA/PA screen-positive individuals by season/birth weight/gestational age. (A, C, E) Distribution of season/birth weight/gestational age for the NBS program in Xuzhou, China from 2015 to 2020. (B) Season in months and (D) Birth weight in grams and (E) Gestational age (GA) in days for MMA/PA screen positive cases and matched healthy controls with analysis of several parameters by MS/MS (i.e., C3, C3/C2, C3/C0 and Met). The p value of a t test shows a statistically significant difference between group pairs, with a tendency for MMA.FP to be season premature, and an overall higher temperature in summer of all MMA screen-positive newborns. PS: False-positive MMA cases (MMA.FP) and true-positive MMA patients (MMA.TP).

Figure 3

Newborn metabolic pattern analysis with initial MS/MS parameters. (A and B) Receiver operating characteristic (ROC) curve analysis for newborns with and without a confirmed MMA/PA diagnosis using MS/MS. The sensitivity of MMA screening based on the primary newborn screening (NBS) analyte was not altered by 96.1%. (C) C3/C2 and C3 distribution for initial MS/MS screening in Xuzhou, China. Green stars indicate the control, Blue plus signs indicate the negative individuals, and red stars indicate true positive individuals with MMA/PA. (D) The mean decrease in accuracy was used to measure the contribution of individual metabolic analytes. (E) The median fluctuation of C2 and Met between 2016 and 2020. (F) The fluctuation of C2, Met and temperature in

different months. The relative importance of analytes for MMA/PA metabolic pattern recognition is ranked from top to bottom with primary marker C3/C2 in DBSs. AUC area under the curve.

Figure 4

Biochemical, molecular and statistical characteristics of MMA/PAs in Xuzhou, China. (A) The analysis of such parameters by initial MS/MS (i.e. C3, C3/C2, C3/C0 and Met) between MMA/PA individuals and the healthy individuals. (B) Metabolite measurements in second-tier screening by UPLC-MS/MS in DBSs including MMA, MCA and HCY. (C) Chromatographic separation of MMA-associated acylcarnitines detected by UPLC-MS/MS for differential diagnosis. The right three chromatograms are representative differential diagnoses by UPLC-MS/MS.

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