The follow-up of Chinese patients in mut-type methylmalonic acidemia identified through expanded newborn screening

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Abstract

Background: Isolated methylmalonic acidemia (MMA), an autosomal recessive disorder of propionate metabolism, is usually caused by mutations in the methylmalonyl-CoA mutase gene (mut-type MMA). Because no universal consensus was made on whether mut-type MMA should be included in newborn screening (NBS), we aimed to compare the outcome of this disorder detected by NBS with that detected clinically and investigate the influence of NBS on the disease course. **Methods**: In this study, 168 patients with mut-type MMA diagnosed by NBS were compared to 210 patients diagnosed after disease onset while NBS was not performed. Clinical data of these patients from 7 metabolic centers in China were analyzed retrospectively, including initial manifestations, biochemical metabolites, the responsiveness of vitamin B12 therapy, and gene variation, to explore different factors on the long-term outcome. **Results**: Among patients diagnosed through NBS, 77 patients (45.8%) remained asymptomatic and 87 patients (53.4%) showed favorable neurocognitive outcomes. In contrast with individuals diagnosed clinically, only 30 cases (16.2%) developed healthily. In our comparison of patients whether detected by NBS, the age at diagnosis, the incidence of disease onset, the responsiveness of vitamin B12, age at the start of vitamin B12 treatment, levels of biochemical features before and after treatment, and the long-term prognosis were remarkably different ($P<0.01$). The presence of disease onset and the blood C3/C2 ratio were more associated with poor outcomes of patients whether identified by NBS. Importantly, after considering NBS, the odd ratio of disease onset for poor outcome decreased and the unresponsiveness to vitamin B12 increased. **Conclusion**: Through preventing major disease-related events and allowing an earlier treatment initiation, NBS is beneficial for the prognosis of infants with mut-type MMA.

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Methods : In this study, 168 patients with mut-type MMA diagnosed by NBS were compared to 210 patients diagnosed after disease onset while NBS was not performed. Clinical data of these patients from 7 metabolic centers in China were analyzed retrospectively, including initial manifestations, biochemical metabolites, the responsiveness of vitamin B12 therapy, and gene variation, to explore different factors on the long-term outcome.

Results : Among patients diagnosed through NBS, 77 patients (45.8%) remained asymptomatic and 87 patients (53.4%) showed favorable neurocognitive outcomes. In contrast with individuals diagnosed clinically, only 30 cases (16.2%) developed healthily. In our comparison of patients whether detected by NBS, the age at diagnosis, the incidence of disease onset, the responsiveness of vitamin B12, age at the start of vitamin B12 treatment, levels of biochemical features before and after treatment, and the long-term prognosis were remarkably different (P <0.01). The presence of disease onset and the blood C3/C2 ratio were more associated with poor outcomes of patients whether identified by NBS. Importantly, after considering NBS, the odd ratio of disease onset for poor outcome decreased and the unresponsiveness to vitamin B12 increased.

Conclusion : Through preventing major disease-related events and allowing an earlier treatment initiation, NBS is beneficial for the prognosis of infants with mut-type MMA.

Keywords:

Methylmalonic acidemia, *MMUT* gene, long-term outcome, newborn screening

1. Introduction:

Isolated methylmalonic acidemia (MMA, OMIM # 251000) comprises a wide range of rare inherited metabolic diseases characterized by increased levels of methylmalonic acid in the blood, urine, and other body fluids(Hörster et al., 2021). These disorders result from the disturbed conversion of L-methylmalnoyl-CoA to succinyl-CoA and mostly are caused by a complete or partial defect of methylmalonyl-CoA mutates (MCM), of which the cofactor is adenosylcobalamin(Forny et al., 2016; Fowler, Leonard, & Baumgartner,

2008). MCM deficiency can be divided into mut, cblA, cblB, and cblD-subtypes, which are caused by mutations in*MMUT, MMAA* , *MMAB,* and *MMADHC* genes, respectively. Based on residual MCM activity, *MMUT* mutations are called mut^0 for an indetectable residual activity or mut^- for a low to moderate residual activity responsive to high concentrations of adenosylcobalamin. Isolated MMA occurs in 1:110 000 live births in all regions worldwide. In China, isolated MMA accounts for typically 30% of all types of MMA, and the*MMUT* gene deficiency accounts for 93.5% of the isolated MMA(Hong et al., 2017; Yang et al., 2020; Zhou, Li, Wang, Wang, & Gu, 2018).

The clinical spectrum of mut-type MMA is heterogeneous(Kang et al., 2020). Affected patients could present the first clinical symptoms during the neonatal period, even immediately after birth, but commonly develop within the first year of life(Dionisi-Vici, Deodato, Röschinger, Rhead, & Wilcken, 2006), which are characterized by variable and non-specific clinical signs such as poor feeding, vomiting, drowsiness, coma, hyperammonemia, and metabolic crises(Baumgartner et al., 2014; Liu et al., 2018). If the disorder is not recognized in time or if the patients are already too severely compromised to respond to treatment, they could deteriorate rapidly and progress to coma followed by death. Moreover, patients who survive the initial acute metabolic acidosis usually get into chronic progression and suffer from anemia, renal dysfunction, epilepsy, developmental delay, psychological behavior abnormalities, and other long-term complications(Baumgartner et al., 2014; Liu et al., 2018).

Nowadays, the introduction of tandem mass spectrometry (MS/MS) to newborn screening for inborn errors of metabolism has been a great success in the field of public health over the last 20 years("Newborn screening: toward a uniform screening panel and system–executive summary," 2006). In China, MS/MS-based NBS was first applied in Shanghai in 2003. A total of 116000 newborns underwent the NBS from 2003 to 2007, only 3 MMA patients were identified suggesting an incidence of 1:40000(Gu, Wang, Ye, Han, & Qiu, 2008). However, subsequent studies have shown the prevalence of MMA varied considerably in different regions of China, especially between the north and the south(Wang et al., 2019; Yang et al., 2020). In recent years, mut-type MMA is usually discovered through neonatal screening, there is no doubt that NBS could increase the chance of an early diagnosis of MMA patients(Grünert et al., 2012), especially in late-onset cases(Heringer et al., 2016). Although NBS decreased neonatal mortality(Dionisi-Vici et al., 2006), it may not prevent life-threatening metabolic crises during the first days after birth among some patients, potentially due to rapid disease progression. Furthermore, there is limited information available on the long-term outcome of screened individuals. Therefore, in our study, we compared the natural history and prognosis of patients diagnosed through NBS and those diagnosed on clinical bases to illustrate the benefit of NBS programs for individuals with mut-type MMA and to investigate the effect of variable factors on the screened outcome.

2. Methods

2.1 Patients

A total of 378 mut-type MMA patients who were diagnosed and treated at multiple hospitals in China from January 2003 to December 2021 were enrolled. All patients were ascertained through genetic testing and metabolic investigation using MS/MS, and a majority of them received both personalized therapy and regular follow-up. In order to evaluate the effect of NBS on health outcomes of neonatally identified mut-type MMA patients, we divided patients into two cohorts and compared their current health conditions. Patients identified by MS/MS-based NBS (n=168) comprised the first cohort, which is defined as the NBS-detected cohort. The other cohort included patients diagnosed by onset of first symptoms (n=210), for which NBS was not performed. Then patients were divided into two groups, with the normal outcome group living a healthy life and the poor outcome group suffering from different degrees of physical and mental impairment, such as movement disorders, intellectual impairment, developmental delay, renal or cardiac complications, and death. A total of 231 patients with poor outcomes in the whole cohort and 76 patients with poor outcomes in the NBS-detected cohort were respectively analyzed by univariate logistic regression. Factors that included the onset of symptoms, the practice of NBS, unresponsiveness of vitamin B12, nucleotide variants, and biochemical markers before and after treatment were analyzed. The Ethics Committee of Xinhua Hospital approved this study (approval number XHEC-D-2022-062). Written informed consent was

obtained from the parents or legal participants.

2.2 Newborn screening and confirmatory testing

According to the recommended time frame (Mak, Lee, Chan, & Lam, 2013), blood collection from newborns is taken between the ages of 3 to7 days. By using blood filter papers, levels of acylcarnitines in blood, including propionylcarnitine (C3) and acetylcarnitine (C2), were detected by MS/MS (API 4000, American Bio-System Inc). Then the ratio of C3/C2 was calculated. Second blood spots were requested upon abnormal first-screening results(Niu et al., 2010). To further confirm the diagnosis, organic acids in urine, which contain methylmalonic acid (MMA) and methylcitric acid (MCA) were measured by gas chromatography-mass spectrometry (GC-MS) (Shimadzu Limited, QP2010)(Al Dhahouri et al., 2018; Al-Dirbashi et al., 2019).

2.3 Mutation analysis of the *MMUT* gene

Genetic analysis was performed by Sanger sequencing or high-throughput next-generation sequencing. The mutation was identified by the normal human *MMUT* sequence as a reference (GenBank, NC_000006.12). Then the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), the Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php), and the previous literatures were used to identify whether the mutations had been reported. The pathogenicity of novel variants was interpreted according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines(Richards et al., 2015).

2.4 Treatment

For mut-type MMA patients, treatment varies with the difference in vitamin B12 responsiveness and is adjusted depending on personal conditions(Baumgartner et al., 2014). Based on the guideline, for vitamin B12 responsive patients, the long-term therapy involved cobalamin intramuscular injections, oral L-carnitine, and a special diet consisting of low isoleucine, valine, threonine, and methionine, while the main treatment of vitamin B12 unresponsive patients were oral L-carnitine and special diet.(Forny et al., 2021). For these patients, the efficacy of vitamin B12 was evaluated on the vitamin B12 loading test and the therapeutic effect of vitamin B12 during the treatment process. If the blood C3/C2 ratio and urine methylmalonic acid are decreased more than 50% after the vitamin B12 loading test, compared with those before treatment, it is defined as "completely responsive". While a reduction of less than 50% in the blood C3/C2 ratio and urine methylmalonic acid content after the vitamin B12 loading test is deemed to be "partly responsive"(Baumgartner et al., 2014). Then adjustment of treatment is made depending on concentrations of biochemical metabolites. Typically, the first choice of most patients was intramuscular injections of hydroxocobalamin at a dose of 1 to 20 mg each time, once every 1-20 days, and oral L-carnitine at 50–100 mg/kg per day.

2.5 Statistical analysis

The data were analyzed using SPSS 24.0 (IBM, Chicago, Illinois). Descriptive statistics were expressed as the median and the range for continuous variables and as percentages(%) for categorical variables. Baseline clinical, biochemical, and genetic characteristics were compared between patients whether detected by NBS and patients with different prognoses using a Student independent t-tests, $\chi 2$/Fisher exact test, or Wilcoxon rank-sum test as appropriate. Univariate odds ratios of poor outcomes were assessed using logistic regression. A value of P <0.05 was considered statistically significant.

3. Results

3.1 Patients population

Among all 378 MMA patients attributable to the *MMUT* gene, 168 patients (44.4%) were identified by NBS first, and 210 patients (55.6%) were diagnosed after the manifestation of symptoms while not undergoing NBS by MS/MS. In the NBS-detected cohort (85 males, 83 females), 16 cases (9.5%) died and 5 cases (3.0%) were lost to follow-up. While, in patients identified after disease onset (131 males, 79 females), 46 cases (21.9%) died and 25 cases (11.9%) were lost to follow-up. For almost all patients, information was available

from NBS or before treatment to the last moment of follow-up. A comparison of demographic variables between those identified by NBS with or without poor outcomes was described in Table 1 and a comparison between patients diagnosed by NBS or disease onset was presented in Table 2.

3.2 Age at onset, diagnosis, and the start of vitamin B12 therapy

In patients identified by NBS, the diagnosis was made between 0.7 and 6 months after birth, with a median age of 1 month. In the clinically-diagnosed cohort, the median age at diagnosis was 2.5 months, ranging more widely from 1 month to 8 years. As for the age at the treatment of vitamin B12, the median ages in the two cohorts were 1 month (range 21 days to 7.3 years) and 6 months (range 30 days to 17 years) respectively. Undoubtedly, NBS led to significantly earlier diagnosis as well as earlier treatment (both P <0.01). However, in 54.2% of patients detected by NBS, the first symptoms were already noted between 1 day and 28 months of life. This implies that a proportion of them had already exhibited their first symptoms before receiving NBS results. Indeed, 45 of 168 (26.8%) patients who were identified by NBS had shown clinical symptoms during the newborn period. In the NBS-detected cohort, the age at first symptoms of patients with normal outcome was 90 days (range 3 days to 1.9 years), while 3 days (range 1 day to 2.3 years) in the poor outcome group. Noteworthy, the age at onset was younger in the poor outcome group than in the normal outcome group among patients identified by NBS (P =0.01). Moreover, the age both at diagnosis and therapy of vitamin B12 were consistently lower in the normal outcome group (both P =0.02).

3.3 Initial manifestation

As shown in Table 2, among patients identified by NBS, 91 of 168 (54.2%) developed symptoms eventually, such as feeding refusal, vomiting, lethargy, seizures, psychomotor retardation, and so on. While 77 of 168 (45.8%) have not presented any symptoms so far. A considerable number of patients exhibited clinical manifestations during the first 30 days of life, thus being classified as early-onset patients. Fortunately, 14 cases had disease onset beyond day 30, which all showed normal psychomotor and cognitive development at our last visit. The most common phenotype of both early-onset and late-onset children was metabolic acidosis, such as aversion to eating and vomiting, which were found in 77.9% (60/77) and 57.1% (8/14) of both groups, respectively. Neurological symptoms ranked second, including drowsiness, coma, and seizures that occurred in 52 patients (67.5%) in the early-onset ones and 7 cases (50%) in the late-onset ones, suggesting clinical symptoms were more severe in patients with early-onset. To test for the benefit of an early diagnosis, the clinical picture of all patients identified either by NBS or disease onset was evaluated, and all showed significant differences. In both cohorts, vomiting and drowsiness were the most prominent symptoms. Furthermore, a comparison of the occurrence of different kinds of clinical features was made between individuals with or without normal physical and neurocognitive development in the NBS-detected cohort. Consistently, the incidence of variable initial symptoms was significantly higher in the poor outcome group (P <0.05), indicating the onset of disease might have a negative effect on the long-term prognosis.

3.4 Biochemical features

The biochemical markers before and after treatment in the normal outcome group and poor outcome group among patients detected by NBS are present in Table 1. Before treatment, the poor outcome group showed higher levels of C3, C3/C2, MMA and MCA in the NBS sample than the normal group (all P <0.01). Additionally, these metabolites after treatment varied in individuals with different outcomes, in which all of them were much lower in the normal outcome group than those in the poor outcome group, with significant statistical differences (all$P$ <0.01). In the NBS-detected cohort, 5 cases showed C3/C2 ratios within the normal range, but their blood C3 levels were above the cutoff, thus being screened positive. These 5 cases all showed normal development. Meanwhile, only 9 newborns had blood C3 levels under 4 μmol/L, whereas the ratio of C3/C2 was over 0.2. Among them, only one patient whose ratio of C3/C2 was 1.15 showed developmental delay, while the blood C3/C2 ratios of the other eight children were a little higher than the cutoff. At the same time, all 8 patients remained asymptomatic and showed normal outcomes during the follow-up (Supplementary Table 3). As is shown in Table 2, there were remarkable differences in biochemical data that included the blood C3, C3/C2 ratio, urinary MMA, and MCA between patients identified by NBS

and clinical symptoms. However, no significant statistical differences in urinary MCA were observed at the last available visit between the NBS-detected cohort and the clinically-diagnosed cohort (P =0.21).

3.5 Gene analysis

As is shown in Supplementary Table 3, in the NBS-detected cohort,*MMUT* mutations were observed on both alleles in 161 of 168 cases (95.8%), whereas in 7 cases (4.2%) a single mutation on one allele was detected. Sequence analysis identified 107 mutations in the *MMUT*gene, including 64 missense mutations, 19 nonsense mutations, 3 duplications, 11 deletions, 4 insertions, and 6 splice-site mutations. Among them, the most frequent mutations were c.729_730insTT (p.D244Lfs*39), c.1663G>A (p.A555T), c.1106G>A (p.R369H), c.323G>A (p.R108H), and c.914T>C (p.L305S), which accounted for 10.9% (36 of 330), 10.6% (35 of 330), 7.3% (24 0f 330), 4.9% (16 of 330) and 3.6% (12 of 330), respectively. Between groups with or without good outcomes in the NBS-detected cohorts or cohorts whether patients were identified by NBS or clinical symptoms, only the variant of c.1663G>A(p.A555T) strongly differed (P <0.01). For patients who had a favorable long-term outcome, the variant c.1663G>A(p.A555T) seemed more common (37.9% versus 2.6%). In analogy, the variant of c.1663G>A(p.A555T) in *MMUT* was increasingly observed in the NBS-detected cohort than in the clinically-diagnosed cohort. Additionally, we noted that patients carrying the variant of c.753+3A>G had milder clinical phenotype, lower levels of biochemical metabolites and even merely receiving vitamin B12 orally could have a better prognosis. But this mutation in the *MMUT* gene is relatively rare, only 3 cases were found in our study. Therefore, through NBS receiving disease-specific therapy and preventing disease progression, patients carrying the above-mentioned variants in our cohort seemed to have a better prognosis.

3.6 Long-term clinical outcomes

According to Tables 1 and 2, in the NBS-detected cohort, 87 patients (53.4%) showed normal physical and neurocognitive development, and 76 patients (46.6%) had poor outcomes, in which 16 patients died of recurrent and severe metabolic crises. In the clinically-diagnosed cohort, only 30 cases (25.64%) were with normal outcomes, and 155 cases (83.78%) showed different degrees of developmental delay, including 46 deceased cases. And a statistical trend (P <0.01) was found for normal development in the NBS-detected group in comparison with the clinically-diagnosed group. In addition, the mortality rate was much lower (9.5% versus 21.9%) in the NBS-detected group compared to the individuals diagnosed after disease onset (P <0.01). Among them, the age at death was nearly all during the initial stage of the disease onset in the neonatal period, except for 2 cases in the clinically-diagnosed cohort, who died at the age of 2 and 5 months separately due to poor treatment compliance. It is worth noting that 1 patient who didn't receive neonatal screening died of kidney dysfunction at the age of 4 days. And a total of 5 patients underwent liver transplantation, in which two of them were identified by NBS while the other was identified by the occurrence of metabolic crisis. After transplantation, only one patient who was also detected by NBS developed healthy, three clinically diagnosed patients still showed developmental delay or intellectual impairment, and one patient was lost to follow-up.

3.5 Factors affecting poor outcomes

In the univariate analysis for the whole cohort which included NBS-detected group and clinically-diagnosed group, the onset of disease (odds ratio, 140.88; 95% confidence interval, 42.40-468.04; P=0.00), presence of c.1106G>A (odds ratio, 2.85; 95% confidence interval, 1.29-6.32; P =0.00), unresponsiveness of vitamin B12 (odds ratio, 6.78; 95% confidence interval, 3.96-11.61;P =0.00), blood C3/C2 (odds ratio, 9.48; 95% confidence interval, 4.19-21.46; P =0.00; odds ratio, 58.30; 95% confidence interval, 22.05-154.15; P =0.00; respectively) before and after treatment were more likely to predict poor outcomes (Table 5). In contrast, the practice of NBS (odds ratio, 0.17; 95% confidence interval, 0.10-0.28;P =0.00) and presence of c.1663G>A (odds ratio, 0.04; 95% confidence interval, 0.01-0.12; P =0.00) were associated with more favorable outcomes.

In subjects with poor outcomes and also underwent through NBS, the univariate analysis showed that onset of symptoms (odds ratio, 126.88; 95% confidence interval, 34.98-460.22; P =0.00) , the mutation of c.1106G>A in the *MMUT* gene (odds ratio, 4.19; 95% confidence interval, 1.57-11.20; P =0.00), unresponsiveness of

vitamin B12 (odds ratio, 18.02; 95% confidence interval, 7.92-40.99; P =0.00) and blood C3/C2 (odds ratio, 7.23; 95% confidence interval, 2.70-19.35; P =0.00; odds ratio, 61.50; 95% confidence interval, 17.02-222.20; P =0.00; respectively) before and after treatment were correlated with the outcome of intellectual impairment(Table 4). Interestingly, when we took NBS into consideration, the odds ratio of disease onset decreased and the unresponsiveness to vitamin B12 increased, which illustrates that NBS could prevent disease course by allowing treatment started as soon as possible.

4. Discussion

Previous NBS programs for inherited metabolic diseases such as PKU greatly improved the health conditions of affected children (Lüders et al., 2021; Mütze et al., 2020), facilitating the development of NBS disease panels. From this experience, it seems that overall MS/MS-based NBS has substantially improved outcomes of various metabolic disorders in general, but also that benefit is not uniform. Moreover, the long-term clinical benefits of NBS programs to screen infants with mut-type MMA remain unclear. Here, we conducted an extensive, nationwide retrospective study including 168 NBS-detected individuals and 210 clinically-diagnosed ones with confirmed mut-type MMA to evaluate their long-term outcomes and to determine the major predictors of the disease course.

4.1 NBS leads to earlier diagnosis and treatment

In line with the previous studies(Heringer et al., 2018; Mütze et al., 2020), the number of neonatally symptomatic patients increases with every day of life and the severity of neonatal metabolic decompensation is a predictor of impaired physical and neurocognitive development. This emphasized the need for timely diagnosis during NBS process to that the results of NBS are available earlier to avoid severe metabolic impairment which results in long-term complications or death. We clearly demonstrated that NBS shortened the time to diagnosis for mut-type MMA, especially for individuals with favorable clinical and cognitive long-term outcomes. Major arguments against the inclusion of mut-type MMA in NBS program are that propionylcarnitine with a low specificity should not be considered as a screening marker for MMA and the early onset of symptoms. However, our results indicated that a significant proportion of mut-type MMA infants could be detected by NBS meanwhile remaining asymptomatic, thereby preventing a later metabolic decompensation by early diagnosis and therapeutic interventions. Importantly, once positive NBS results had been reported at birth, the normal group seemed to take action of treatment more promptly, underlining the importance of NBS programs to prevent severe organ damage and death by ideally allowing pre-symptomatic treatment to be initiated.

4.2 NBS, avoiding metabolic decompensation in half of the screened infants, predicts favorable long-term outcomes.

In the majority of mut-type MMA patients who carry a life-long risk of metabolic decompensations(Hörster et al., 2021), these potentially life-threatening episodes were prevented by early diagnosis and timely initiation of therapy. Compared to clinically-diagnosed individuals, only a minority of screened individuals presented with clinical symptoms at a high risk of neonatal metabolic acidosis and death. Despite metabolic crises, the overall health outcome remained favorable in screened individuals, which is confirmed by the high survival rate (90.5%), and the less occurrence of persisting clinical signs (16.1%). It has become evident that the odds ratio of disease onset decreased if NBS is included, suggesting NBS could prevent neonatal metabolic crisis in about half of screened individuals. Importantly, mut-type MMA is usually with a high frequency of severe and potentially life-threatening neonatal decompensations that often occurred during the first days of life, but can be avoided in a large number of screened individuals who adhere to medical recommendations timely and properly. Early diagnosis, the timely start of therapy, and the prevention of severe complications were beneficial for long-term prognosis.

4.3 Diagnostic mode and onset of disease are important effects on long-term outcomes.

Early diagnosis serves as a prerequisite for early therapeutic intervention. In accordance with the previous studies(Lindner et al., 2011; Lund et al., 2012; Wilcken et al., 2009), patients in the NBS-detected cohort

are more likely to have a stable clinical course with less frequent recurrences of metabolic decompensation in comparison with those diagnosed clinically. Consistent with earlier reports(Han et al., 2015; Kang et al., 2020; Worgan et al., 2006), milk refusal, vomiting, and drowsiness were the three most common manifestations of the initial metabolic crisis in our patients. Severe neurological manifestations such as seizures and coma tended to be more frequent in the clinically-diagnosed cohort than in the NBS-detected cohort. Similar observations have been reported that patients detected by NBS were usually with symptoms at diagnosis being less severe(Dionisi-Vici et al., 2006). Follow-up in this study also confirmed that clinically-diagnosed patients had poorer outcomes than those identified by NBS, including death, organ transplantation, poor developmental outcomes, and mental retardation. In addition, in the normal outcome group, a majority of patients remained asymptomatic, which illustrated that a later metabolic decompensation could be avoidable by early diagnosis. Therefore, a slightly earlier diagnosis by NBS is related to a milder clinical course and better outcome.

4.4 Quality of therapy is a strong predictor of prognosis in a screened population

It is widely acknowledged that mut-type MMA could be divided into two subcategories ($mut^0$and mut^-)(Willard & Rosenberg, 1980), due to the absence (mut^0) or presence (mut^-) of residual enzyme activity in the patient's fibroblasts by the PI assay to supplementation with hydrocobalamin(Forny et al., 2016). Almost all cblA, one-third of cblB, and cblD subtypes patients as well as mut^- patients usually have a better response to treatment of vitamin B12 (Fowler et al., 2008; Tanpaiboon, 2005; Willard & Rosenberg, 1980). Patients with mut-type MMA usually received the therapy combined a low protein diet, hydrocobalamin supplementation (for responsive patients), and other oral medications in order to reduce the accumulation of toxic metabolites that eventually lead to metabolic decompensations and long-term complications(Baumgartner et al., 2014). In the present cohort, there is a significant interaction between cobalamin responsiveness and enzymatic subgroup on the probability of a poor outcome, with vitamin B12 unresponsiveness being the third important modulator of poor outcome. Therefore, vitamin loading tests to evaluate the response should be applied in every mut-type MMA patient, and for responders, vitamin B12 should be recommended as a long-term treatment.

4.5 The identification of patients carrying the variants with mild phenotypes and favorable outcomes should be based on both C3 and C3/ C2 results

On NBS, elevations in the blood C3 level and the C3/C2 ratio are hallmarks of disorders in propionate metabolism including mut-type MMA. Confirmatory testing including the detection of acylcarnitine profile, as well as measurements of urinary MMA and MCA is helpful to determine an individual's specific diagnosis. Due to the wide spectrum of clinical features of mut-type MMA, it is possible that some cases could be missed on NBS, in particular for those carrying the variants with mild phenotypes. In our study, a few mildly affected patients who remained asymptomatic had normal levels of C3, while the blood C3/C2 ratio concentration was beyond the normal range. Meanwhile, the blood C3/C2 ratio concentration of several patients didn't reach the cutoff, but the level of C3 was elevated thus being considered positive. Among these patients who had either normal C3 levels or the C3/C2 ratio, we found most of them carried at least one likely pathogenic mutation according to ACMG criteria. Based on previous reports(Forny, Froese, Suormala, Yue, & Baumgartner, 2014; Forny et al., 2016; Liang et al., 2021), c.323G>A, c.2206C>T, and c.1663G>A were associated with mut^-subclass, thereby being more responsive to vitamin B12 supplementation, and having milder phenotypes as well as better prognosis. Additionally, we know that subjects with c.1663G>A could be asymptomatic or could develop late adulthood symptoms. Similarly, all patients with c.1663G>A in our study were normal with early treatment and no organ damage occurred, except for 4 cases. Among them, 2 cases were diagnosed by NBS but because of refusal to treatment or poor treatment compliance, both exhibited severe physical disabilities and intellectual impairments later. The other two individuals were not subjected to NBS and were misdiagnosed as autistic spectrum disorders, presenting progressive developmental delay due to disease onset and untimely treatment. As a major advantage of NBS would be observed in patients with milder phenotypes and later clinical presentation, there is a need to refine NBS programs to better identify these individuals.

In conclusion, it is highly successful to apply the MS/MS-based NBS for mut-type MMA, allowing an early diagnosis and specialized metabolic therapy promptly. NBS, allowing for early diagnosis and timely initiation of therapy, is beneficial for a favorable long-term outcome. This is confirmed by low frequencies of cognitive disabilities and premature mortalities in screened children. NBS can prevent disease manifestation among almost half of the screened children, but the onset of the disease is still the strongest factor for poor outcomes, there is a need for a safer and more effective treatment strategy for future research.

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Conflict of interest

The authors declare that they have no potential conflict of interest.

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Data availability statement

All data generated or analyzed during this study are included in the article, further inquiries can be directed to the corresponding author.

Ethics statement

This study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Approval number: XHEC-D-2022-062). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients or their legal guardians for being included in the study.

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Table1 Comparison of baseline demographic, clinical, and biochemical characteristics between patients with good and poor outcomes

Characteristics	Normal outcome (n=87)	Poor outcome (n=76)	P value
Age at diagnosis (months), median (range)	1 (0.7-3.5 months)	1.44 (1-6 months)	0.021
Age during follow-up (years), median (range)	5.52 (1.75-18.21 years)	4.78 (2.32-13.98 years)	0.137
Gender, n (%)			0.876
Male	44(50.57%)	37(48.68%)	
Female	43(49.43%)	39(51.32%)	
Onset of first symptoms	14 (16.09%)	73 (96.05%)	0.000
Age at onset of first symptoms (days), median (range)	90 (3 days-1.9 years)	3 (1 day-2.33 years)	0.013
Initial symptoms, n (%)			
Milk refusal	2(2.3%)	37(48.68%)	0.000
Vomiting	10 (11.49%)	31 (40.79%)	0.000
Drowsiness	7 (8.05%)	33 (43.42%)	0.000
Seizures	4 (4.6%)	12 (15.79%)	0.019
Coma	2 (2.3%)	18 (23.68%)	0.000
Dyskinesia	3 (3.45%)	18(23.68%)	0.000
Progressively developmental delay	1 (1.15%)	13 (17.11%)	0.000
Vitamin B12 responsiveness, n (%)	75 (86.21%)	17 (25.76%)	0.000
Age at treatment of vitamin B12 (months), median (range)	1 (0.7-4.7 months)	1.32 (0.7-87.3 months)	0.023
Biochemical features at NBS, median (range)			
Blood C3 level (μmol/L)	6.3(2.11-40.42)	9.91 (3.25-54.85)	0.002
Blood C3/C2 ratio	0.45 (0.12-1.72)	0.70 (0.26-4.30)	0.000
Urinary MMA (mmol/mol Cr)	104.3 (4.62-921)	272.9 (14.85-5915)	0.000
Urinary MCA (mmol/mol Cr)	3.48 (0.3-39.32)	12.30 (0.8-90.88)	0.002
Biochemical features during follow-up			
Blood C3 level (μmol/L)	5.00 (0.83-55.36)	24.44 (2.22-68.3)	0.000
Blood C3/C2 ratio	0.22 (0.03-1.4)	0.89 (0.06-1.67)	0.000
Urinary MMA (mmol/mol Cr)	19.75 (0-928.7)	352.2 (0-2685)	0.001
Urinary MCA(mmol/mol Cr)	0.96 (0-53)	4.5 (0-60.23)	0.000
Nucleotide variant, n (%)			
c.729_730insTT	17 (19.54%)	17 (22.37%)	0.702
c.1663G>A	33 (37.93%)	2 (2.63%)	0.000

Characteristics	Normal outcome (n=87)	Poor outcome (n=76)	P value
c.1106G>A	6 (6.9%)	18 (23.68%)	0.004
c.323G>A	8 (9.2%)	7 (9.21%)	1.000
c.914T>C	3 (3.45%)	9 (11.8%)	0.068
c.424A>G	4 (4.6%)	5 (6.58%)	0.735
c.755dupA	5 (5.75%)	3 (3.95%)	0.725
c.599T>C	6 (6.9%)	8 (2.63%)	0.286
c.1677-1G>A	3 (3.45%)	4 (5.26%)	0.706
c.1280G>A	1 (1.16%)	5 (6.58%)	0.100
c.2080C>T	4 (4.6%)	2 (2.63%)	0.686
c.1630_1631GG>TA	4 (4.6%)	1 (1.32%)	0.373
others	59 (67.82%)	58 (76.32%)	0.295

Normal reference range of blood C3: 0.5-4.0μmol/L; Normal reference range of blood C3/C2 ratio: 0.04-0.2; Normal reference range of urinary MMA: 0.2-3.6mmol/mol Cr; Normal reference range of urinary MCA:0-0.8mmol/mol Cr.

Table 2 Comparison of baseline demographic, clinical, and biochemical characteristics between patients identified by NBS or clinical manifestations

Characteristics	NBS-detected cohort (n=168)	Clinically-diagnosed cohort (n=210)	P value
Age at diagnosis (months), median (range)	1 (0.7-6 months)	2.5 (1 month-8 years)	0.000
Age during follow-up (years), median (range)	5.03 (1.75-18.21 years)	8.5 (2.49-35.06 years)	0.000
Gender, n (%)			0.028
Male	85 (50.6%)	131 (62.38%)	
Female	83 (49.4%)	79 (37.62%)	
Onset of first symptoms	91 (54.17%)	210 (100%)	0.000
Initial symptoms, n (%)			
Milk refusal	41 (24.4%)	119 (56.67%)	0.000
Vomiting	43 (25.6%)	121 (57.62%)	0.000
Drowsiness	42 (25%)	107 (50.95%)	0.000
Seizures	16 (9.52%)	44 (20.95%)	0.003
Coma	21 (12.5%)	44 (20.95%)	0.031
Dyskinesia	22 (13.1%)	63 (30%)	0.000
Progressively developmental delay	15 (8.93%)	59 (28.1%)	0.000
Vitamin B12 responsiveness, n (%)	92 (56.79%)	70 (42.42%)	0.005
Age at treatment of vitamin B12 (months), median (range)	1 (21 days-7.33 years)	6 (30 days-17 years)	0.000
Biochemical features at baseline , median (range)			

Characteristics	NBS-detected cohort (n=168)	Clinically-diagnosed cohort (n=210)	P value
Blood C3 level (μmol/L)	7.88(2.11-54.85)	11.49(2.19-81.13)	0.000
Blood C3/C2 ratio	0.58(0.12-4.3)	0.72(0.19-4.51)	0.013
Urinary MMA (mmol/mol Cr)	160.9(4.62-5915)	366(6.2-15038)	0.004
Urinary MCA (mmol/mol Cr)	6.29(0.3-90.88)	9.05(0.3-250.8)	0.024
Biochemical features during follow-up			
Blood C3 level (μmol/L)	7.79(0.83-68.3)	19.61(0.95-84.22)	0.000
Blood C3/C2 ratio	0.38(0.03-1.67)	0.78(0.06-2)	0.000
Urinary MMA (mmol/mol Cr)	67.56(0-2685)	242(0-3632)	0.006
Urinary MCA (mmol/mol Cr)	1.87(0-103.6)	3.8(0-89.35)	0.207
Nucleotide variant, n (%)			
c.729_730insTT	34 (20.24%)	46 (21.9%)	0.905
c.1663G>A	35 (20.83%)	6 (2.86%)	0.000
c.1106G>A	24 (14.29%)	28 (13.33%)	0.881
c.323G>A	16 (9.52%)	29 (13.81%)	0.263
c.914T>C	12 (7.14%)	21 (10%)	0.364
c.424A>G	10 (5.95%)	9 (4.29%)	0.486
c.755dupA	9 (5.36%)	11 (5.24%)	1.000
c.599T>C	8 (4.76%)	3 (1.43%)	0.068
c.1677-1G>A	8 (4.76%)	17 (8.1%)	0.218
c.1280G>A	6 (3.57%)	18 (8.57%)	0.056
Outcome at follow-up, n (%)			
Normal outcome	87 (53.37%)	30 (16.22%)	0.000
Poor outcome	76 (46.63%)	155 (83.78%)	

Normal reference range of blood C3: 0.5-4.0μmol/L; Normal reference range of blood C3/C2 ratio: 0.04-0.2; Normal reference range of urinary MMA: 0.2-3.6mmol/mol Cr; Normal reference range of urinary MCA:0-0.8mmol/mol Cr.

Table 4 Univariate analysis of predictors for poor outcome in NBS-detected cohort

	Univariate analysis	Univariate analysis	Univariate analysis
	Odds Ratio	95% Confidence Interval	P
Onset of first symptoms	126.881	34.981-460.221	0.000
Biochemical features at baseline			
Blood C3 level (μmol/L)	1.07	1.02-1.123	0.006
Blood C3/C2 ratio	7.228	2.699-19.354	0.000
Urinary MMA (mmol/mol Cr)	1.003	1.001-1.004	0.001
Urinary MCA (mmol/mol Cr)	1.066	1.026-1.107	0.001
Biochemical features at follow-up			

	Univariate analysis	Univariate analysis	Univariate analysis
Blood C3 level (μmol/L)	1.125	1.081-1.171	0.000
Blood C3/C2 ratio	61.499	17.021-222.202	0.000
Urinary MMA (mmol/mol Cr)	1.005	1.003-1.007	0.000
Urinary MCA (mmol/mol Cr)	1.099	1.028-1.175	0.006
Vitamin B12 unresponsiveness	18.015	7.918-40.985	0.000
Nucleotide variant			
c.729_730insTT	1.186	0.557-2.527	0.658
c.1663G>A	0.044	0.01-0.192	0.000
c.1106G>A	4.19	1.567-11.203	0.004
c.323G>A	1.002	0.346-2.905	0.997
c.2080C>T	0.561	0.1-3.151	0.511

Table 5 Univariate analysis of predictors for poor outcome in the whole cohort

	Univariate analysis	Univariate analysis	Univariate analysis
	Odds Ratio	95% Confidence Interval	P
Onset of first symptoms	140.878	42.404-468.038	0.000
NBS	0.169	0.103-0.278	0.000
Biochemical features at baseline			
Blood C3 level (μmol/L)	1.063	1.028-1.099	0.000
Blood C3/C2 ratio	9.478	4.186-21.458	0.000
Urinary MMA (mmol/mol Cr)	1.001	1-1.001	0.007
Urinary MCA (mmol/mol Cr)	1.073	1.038-1.11	0.000
Biochemical features at follow-up			
Blood C3 level (μmol/L)	1.118	1.086-1.152	0.000
Blood C3/C2 ratio	58.295	22.046-154.148	0.000
Urinary MMA (mmol/mol Cr)	1.003	1.002-1.004	0.000
Urinary MCA (mmol/mol Cr)	1.1	1.042-1.16	0.000
Vitamin B12 unresponsiveness	6.776	3.956-11.605	0.000
Nucleotide variant			
c.729_730insTT	1.317	0.756-2.295	0.330
c.1663G>A	0.04	0.014-0.115	0.000
c.1106G>A	2.853	1.289-6.317	0.001
c.323G>A	2.034	0.901-4.59	0.087
c.2080C>T	1.013	0.299-3.437	0.983