Spectrum analysis of inborn errors of metabolism for expanded newborn screening in a northwestern Chinese population

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Expanded newborn screening facilitates early identification and intervention of patients with inborn errors of metabolism (IEMs). There is a lack of disease spectrum data for many areas in China. To determine the disease spectrum and genetic characteristics of IEMs in Xi’an city of Shaanxi province in northwest China, 146152 newborns were screening by MSMS from January 2014 to December 2019 and 61 patients were referred to genetic analysis by next generation sequencing (NGS) and validated by Sanger sequencing. Seventy-five newborns and two mothers were diagnosed with IEMs, with an overall incidence of 1:1898 (1:1949 without mothers). There were 35 newborns with amino acidemias (45.45%, 1:4176), 28 newborns with organic acidurias (36.36%, 1:5220), and 12 newborns and two mothers with FAO disorders (18.18%; 1:10439 or 1:12179 without mothers). Phenylketonuria and methylmalonic acidemia were the two most common disorders, accounting for 65.33% (49/75) of all confirmed newborn. Some hotspot mutations were observed for several IEMs, including PAH gene c.728G>A for phenylketonuria; MMACHC gene c.609G>A and c.567dupT, MMUT gene c.323G>A for methylmalonic acidemia and SLC25A13 gene c.852_855del for citrin deficiency. Our study provides effective clinical guidance for the popularization and application of expanded newborn screening, genetic screening, and genetic counseling of IEMs in this region.

Inborn errors of metabolism (IEMs) are a phenotypically and genetically heterogeneous group of disorders caused by defective enzymes, cofactors, and transporters in metabolic pathways, leading to the accumulation of toxic substrates, the production of by-products, and reduced levels of products, which result in a series of clinical manifestations¹. Since Garrod first proposed the concept of “inborn errors of metabolism” in 1909, more than 1000 different IEMs have been identified. While individual rare, the overall incidence has been shown to be upwards of 1 in 800², IEMs can occur in any ethnic group and across every age. Clinical manifestations are complex and often non-specific, including acute metabolic crisis, epilepsy, metabolic acidosis, severe low blood sugar, high blood ammonia, and multiple organ damage. Some of them are amenable to treatment, if not treated timely, may cause irreversible physical disabilities, mental retardation, and even early death³.

Newborn screening (NBS) for IEMs can improve their prognosis and quality of life through early diagnosis and early intervention, recognizing as a huge success in field of public health of the last 50 years⁴. In the early 2000s, tandem mass spectrometry (MSMS) was introduced into many newborn screening programs, this technology allows inexpensive simultaneous detect dozens of amino acids and acylcarnitine profile in a single test, with commendable analytical accuracy and precision. Consequently, MS/MS has been applied wildly for the screening of IEMs that include most amino acid disorders, organic acidemias, and fatty acid oxidation defects¹.⁵

In mainland China, MS/MS-based NBS was first piloted in Shanghai in 2003. There were 116000 newborns screened from 2003 to 2007, twenty IEMs were identified yielding an incidence of 1:5800⁶. However, subsequent

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studies have shown that the incidence, disease spectrum, and genetic characteristics of IEMs varied greatly in different regions of China, especially between the north and south. Furthermore, although MS/MS has been applied in China for many years, there are neither corresponding technical specifications in China nor expanded NBS data for many areas. Therefore, it is necessary to accumulate data on the clinical practice of MSMS and to study the IEMs disease spectrum and gene mutations in different regions to guide expanded NBS.

Expanded NBS program was begun in 2014 in Xi'an city, Shaanxi province, as an important economic and transportation hub city in northwest China. In this study, we present almost six years of experience with expanded NBS of 146152 newborns in the Northwest Women's and Children's Hospital, which is one of the largest tertiary hospitals for maternity and child care in northwest China, including the incidence, disease spectrum, and genetic characteristics of IEMs. These findings will lay the foundation for the establishment of expanded newborn screening systems with MSMS in this region.

Results
Performance of expanded newborn screening. Over 6 years, a total of 146152 newborns were screening by MSMS, consisting of 75672 males, 70463 females, and 17 infants with unknown sex. The rate of premature infants and low-birth-weight infants was 7.90% (11544/146152) and 6.63% (9683/146152), respectively. Initial screening was performed at an average age of 7.25 days at birth. The baseline characteristics of all the screened infants is shown in Table 1. After initial screening by MSMS, 3387 infants received positive results, yielding a positive rate of 2.32%. However, only 2729 infants (80.57%) were successfully recalled for retesting, and 305 cases still received positive. These infants were referred to specialists for differential diagnosis and genetic testing. In total, 75 infants and two mothers confirmed to have inborn errors of metabolism, with an overall incidence of 1:1898 via MS/MS newborn screening. The incidence of amino acid disorders, organic acid disorders, and fatty acid oxidation disorders was 1:4176, 1:5220, and 1:10439 (including two mothers), respectively. The overall positive predictive value (PPV) was 25.25%, and the overall false positive rate (FPR) was 0.16%. During the entire period reported, false-negative results were not observed, with overall sensitivity of 100% and specificity of 99.84%. The parameters of the study project are shown in Table 2.

Incidence and disease spectrum of IEMs. In this study, we identified a total of 14 types of IEMs, consisting of five amino acid disorders, five organic acidemias, and four fatty acid oxidation defects, which were confirmed in 35 newborns (45.45%), 28 newborns (36.36%), and 12 newborns and 2 mothers (18.18%), respectively. The highest incidence was phenylketonuria (PKU, 1:5220), accounting for 37.33% of confirmed newborns, the mean concentration of phenylalanine at initial screening was 1085.14 (Rang: 347.93–2766.42) μmol/L. Methylmalonic acidemia (MMA, 1:6960) was the next most common disorder, accounting for 28.00% of confirmed newborns, including 10 patients with combined MMA with homocysteinemia and 11 patients with isolated MMA. Citrin deficiency (CD, 1:36538) was a common amino acid disorder except PKU. Three infants each of 3-methyl-butanoyl CoA dehydrogenase deficiency (3MCCD), primary carnitine deficiency (PCD), short-chain acyl CoA dehydrogenase deficiency (SCADD), medium-chain acyl CoA dehydrogenase deficiency (MCADD), and very long-chain acyl CoA dehydrogenase deficiency (VLCADD) were identified, with an incidence of

| Category                  | Newborns without targeted IEMs | Confirmed IEM patients |
|---------------------------|---------------------------------|------------------------|
| Number                    | 146077                          | 75                     |
| Age at initial screening (days, mean ± SD) | 7.04 ± 5.63 | 7.63 ± 3.79 | 0.418 |
| Gender                    |                                 |                        |
| Male                      | 75633                           | 39                     | 0.995 |
| Female                    | 70427                           | 36                     |       |
| Unknown                   | 17                              | 0                      |       |
| Gestational age (weeks)   |                                 |                        |
| <32                       | 1054                            | 0                      | <0.001 |
| 32–36                     | 10479                           | 11                     |       |
| ≥37                       | 134523                          | 63                     |       |
| Unknown                   | 21                              | 1                      |       |
| Birth weight (g)          |                                 |                        |
| <2500                     | 9677                            | 6                      | 0.049 |
| 2500–2999                 | 24106                           | 21                     |       |
| ≥3000                     | 112266                          | 48                     |       |
| Unknown                   | 28                              | 0                      |       |
| Number of fetus           |                                 |                        |
| Singleton                 | 145872                          | 70                     | <0.001 |
| Twins                     | 205                             | 5                      |       |
| Multiplets                | 0                               | 0                      |       |

Table 1. Baseline characteristics of newborns enrolled by expanded newborn screening program.
Other IEMs were very rare, with only 1 or 2 cases found in 146152 newborns. The disease spectrum and distribution of IEMs are shown in Table 3. In addition, we found two cases of maternal carnitine deficiency with free carnitine concentration of 2.2 μmol/L and 2.08 μmol/L, respectively. With the exception of PCD and ornithine aminotransferase deficiency (OTCD), the average value of the corresponding biomarkers with MSMS of all confirmed infants is higher than the upper limit of the reference value range, abnormal biomarkers were seen for details in Table 3.

Genetic analysis in the confirmed cases. Except for PKU, all other confirmed infants accepted genetic testing by NGS, while only 14 (50.0%) of PKU infants received NGS testing because they could be diagnosed clearly and dietary therapy by specific biochemical markers. Their genetic testing results were shown in Table 4. Eighteen different mutations in the PAH gene were detected in fourteen patients with PKU, of which 77.8% (14/18) occurred in exon 7, and the most common mutation was c.728G>A (p. Arg243Gln) (6/28, 21.4%). Three patients with PKU were homozygous mutations in the PAH gene, none of them are inbreeding, and the others were compound heterozygous mutations.

Genetic analysis of the 47 infants with non-PKU IEMs showed that 91.5% (43/47) had two variants in specific disease-causing genes. In total, sixty-four different mutations were detected in fourteen specific genes related to IEMs, including twenty-seven missense mutations, sixteen nonsense mutations, nine frameshift mutations, seven in-frame mutations, and five splice site mutations. Based on the American College of Medical Genetics and Genomics (ACMG) classification, 75.0% (48/64) of variants were pathogenic, 15.6% (10/64) were likely pathogenic and 9.4% (6/64) were of uncertain significance. In addition, 76.6% (49/64) of variants were found in only one case. Compound heterozygous mutations were detected in 76.6% (36/47) of the cases, homozygous mutations were detected in 10.6% (5/47), none of them are inbreeding, mono-heterozygous mutations were detected in 10.6% (5/47), and hemizygous mutations were detected in 2.1% (1/47).

Among 21 patients diagnosed with MMA, 10 cases with combined MMA and homocysteinemia carried thirteen different mutations in the MMACHC gene mutations, the common mutations were c.609G>A (p. Trp203Ter) and c.567dupT (p. Ile190fs), accounting for 40% of MMACHC mutation alleles. While 11 cases with isolated MMA carried fifteen different mutations in the MMUT gene, the most common mutant allele was c.323G>A (p. Arg108His) (3/22, 13.6%), which involves the alteration of a conserved nucleotide located at the substrate binding (beta alpha) 8 barrel domain. This variant has been reported in multiple affected individuals mostly as compound heterozygotes and the patients' fibroblasts presented with a marked decrease in methylmalonyl-CoA mutase enzyme activity. Heterozygous mutation of the SLC25A13 gene (c.852_855del) was detected in 4 patients with CD, with a mutation frequency of 50%. In addition, some hotspot mutations were observed for other IEMs, including MCCCG2 gene c.592G>T, SLC22A5 gene c.1400C>G, ACADS gene c.1031A>G, ACADM gene c.985A>G, and ACADVL gene c.1843C>T.

Among the confirmed patients, there were five cases of double pregnancy. Two cases, identical twins, carried compound heterozygous c.1630_1631delGGinsTA/c.323G>A mutations in the MMUT gene, were diagnosed with isolated MMA. Two other cases were assisted reproductive dizygotic twins, both of which were confirmed with MCADD, and a homozygous c.985A>G mutation in the ACADM gene was detected. Besides, one of the naturally conceived dizygotic twins had complex heterozygous c.136C>T/c.310_312delGAG mutation in the ACADG gene, was diagnosed as SCADD.

Follow-up in the confirmed cases. Prior to diagnosis, most infants did not show typical clinical manifestations, while 14 patients developed suspicious symptoms, including poor response, milk refusal, infection, metabolic acidosis, hyperammonemia, hypoglycemia, etc. 11 patients died within 1–2 months of age due to

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**Table 2. Parameters of the expanded newborn screening program.**

| Parameters                          | Value                          |
|-------------------------------------|-------------------------------|
| Total screening number              | 146152                        |
| Positive cases of initial screening | 3387                          |
| Positive rate of initial screening  | 2.32                          |
| Positive recall cases of initial screening | 2729          |
| Positive recall rate of initial screening | 80.57                       |
| Positive cases after recall         | 305                           |
| Positive rate of recall             | 11.18                         |
| Confirmed cases                     | 77                            |
| Positive predictive value (%)       | 25.25                         |
| Sensitivity (%)                     | 100.00                        |
| Specificity (%)                     | 99.84                         |
| Incidence                           | 1:1898 (1:1949 without mother) |
| Total amino acid disorders          | 1:4176                        |
| Total organic acid disorders        | 1:5220                        |
| Total fatty acid oxidation disorders| 1:10439 (1:12179 without mother) |
infection, feed difficulties, acute metabolic crisis or multiple organ failure, including five patients with isolated MMA, three patients with VLCADD, two patients with GA-1 and one patient with OTCD. The details of these 14 patients are shown in Table 5. Exception for one child with argininosuccinic aciduria (ASA) whose parents refused to follow up, the remaining 63 children received regular follow-up, and all are developing normally at present. The longest follow-up period is approximately 6 years.

**Discussion**

There are no national detailed expanded NBS standards in China due to regional differences in the application of MSMS and lack of sufficient and valid studies. Some work has been done on the epidemiology, technical aspects and clinical validity of MS/MS screening in several provinces and cities in China, indicated that NGS combined with MS/MS is an enhanced plan for IEMs as well as disease spectrum and genetic characteristic of IEMs vary

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**Table 3.** Abnormal MSMS markers and results statistics of all confirmed infants.

| Disorders                        | n  | Incidence | Abnormal MSMS markers | Concentration of initial screening (μmol/L) (mean, range) | Concentration of the second testing (μmol/L) (mean, range) | Reference range (μmol/L) |
|----------------------------------|----|-----------|-----------------------|----------------------------------------------------------|-----------------------------------------------------------|--------------------------|
| Amino acid disorders             | 35 | 1:4176    | Phe                   | 1085.14 (347.93–2766.42)                                  | 1840.29 (581.34–2369.21)                                  | 20.00–120.00             |
|                                  |    |           | Phe/Tyr               | 19.61 (4.74–50.53)                                         | 34.23 (5.75–41.70)                                         | 0.20–1.50                |
| tyrosinemia type III             | 1  | 1:146152  | Tyr                   | 1177.00                                                  | 904.71                                                    | 25.00–280.00             |
|                                  |    |           | Tyr/Phe               | 23.62                                                   | 26.09                                                     | 0.60–5.00                |
| Citin deficiency (CD)            | 4  | 1:36538   | Cit                   | 139.70 (88.26–208.18)                                     | 315.55 (119.60–489.59)                                     | 6.50–37.00               |
|                                  |    |           | Cit/Phe               | 2.55 (1.19–4.02)                                          | 3.37 (2.73–4.16)                                          | 0.10–0.75                |
|                                  |    |           | Cit/Arg               | 4.87 (2.24–6.55)                                          | 5.16 (1.75–9.12)                                          | 0.25–4.50                |
| Ornithine transcarbamylase       | 1  | 1:146152  | Cit                   | 3.3                                                      | 6.14                                                      | 6.50–37.00               |
| deficiency (OTCD)                |    |           | Cit/Phe               | 0.03                                                     | 0.06                                                      | 0.10–0.75                |
|                                  |    |           | Cit/Arg               | 0.10                                                     | 0.06                                                      | 0.25–4.50                |
| Argininosuccinic aciduria (ASA)  | 1  | 1:146152  | Cit                   | 223.86                                                   | 375.82                                                    | 6.50–37.00               |
|                                  |    |           | Cit/Phe               | 3.73                                                     | 11.37                                                     | 0.10–0.75                |
|                                  |    |           | Cit/Arg               | 18.47                                                    | 50.09                                                     | 0.25–4.50                |
| Organic acid disorders           | 28 | 1:5220    | C3                    | 8.73 (1.69–29.28)                                         | 11.59 (2.34–67.68)                                         | 0.35–4.00                |
| Methylmalonic acidemia (MMA)     | 21 | 1:6960    | C3/C2                 | 0.85 (0.28–2.43)                                          | 1.28 (0.34–4.26)                                          | 0.03–0.18                |
|                                  |    |           | C3/Met                | 0.79 (0.14–2.84)                                          | 1.23 (0.18–4.04)                                          | 0.02–0.25                |
|                                  |    |           | C3/C0                 | 0.78 (0.14–2.38)                                          | 0.65 (0.13–1.22)                                          | 0.02–0.22                |
| 3-Methylcrotonyl-CoA carboxylase  | 3  | 1:48717   | C4DC + CSOH            | 4.92 (3.05–7.23)                                          | 5.57 (4.28–7.30)                                          | 0.06–0.38                |
| deficiency (3MCCD)              |    |           | (C4DC + CSOH)/C8      | 0.88 (0.15–1.27)                                          | 1.92 (0.51–3.29)                                          | 0–0.02                   |
| Glutaric acidemia I (GA-1)       | 2  | 1:73076   | CSDC + C6OH           | 2.47 (1.95–2.99)                                          | 2.25 (1.66–2.84)                                          | 0.03–0.26                |
| Holocarboxylase synthetase       | 1  | 1:146152  | C4DC + CSOH            | 0.90                                                     | 1.35                                                      | 0.06–0.38                |
| deficiency (HCS)                |    |           | (C4DC + CSOH)/C0      | 0.03                                                     | 0.04                                                      | 0–0.02                   |
|                                  |    |           | (C4DC + CSOH)/C8      | 22.50                                                    | 45.00                                                     | 1.20–12.00               |
| Isobutyryl-CoA dehydrogenase     | 1  | 1:146152  | C4                    | 2.50                                                     | 1.36                                                      | 0.09–0.45                |
| deficiency (IBD)                |    |           | C4/C2                 | 0.57                                                     | 0.29                                                      | 0–0.03                   |
|                                  |    |           | C4/C3                 | 1.51                                                     | 1.72                                                      | 0.06–0.35                |
| Fatty acid oxidation disorders   | 12 | 1:12179   | C0                    | 4.82 (2.31–8.07)                                          | 3.07 (1.94–4.28)                                          | 8.50–50.00               |
| Primary carnitine deficiency     | 3  | 1:48717   | C4                    | 1.62 (1.39–1.89)                                          | 1.51 (0.95–2.23)                                          | 0.09–0.45                |
| (PCD)                            |    |           | C4/C2                 | 0.10 (0.09–0.12)                                          | 0.17 (0.10–0.24)                                          | 0–0.03                   |
| Short-chain acyl-CoA dehydro-    | 3  | 1:48717   | C4                    | 1.17 (0.97–1.46)                                          | 2.38 (2.16–2.56)                                          | 0.06–0.35                |
| genase deficiency (SCADD)        |    |           | C8                    | 2.01 (1.60–2.26)                                          | 1.94 (0.67–3.70)                                          | 0.01–0.17                |
| Medium chain acyl-CoA dehy-      | 3  | 1:48717   | C8                    | 0.21 (0.16–0.26)                                          | 0.29 (0.09–0.40)                                          | 0–0.10                   |
| drogenase deficiency (MCADD)     |    |           | C8/C10                | 12.57 (8.69–14.55)                                        | 15.33 (5.58–24.33)                                        | 0.40–1.30                |
| Very long chain acyl-CoA dehy-   | 3  | 1:48717   | C14:1                 | 3.97 (2.77–4.76)                                          | 2.68 (1.28–3.09)                                          | 0.02–0.19                |
| drogenase deficiency (VLCADD)    |    |           | C14:1/C2              | 0.93 (0.69–1.34)                                          | 0.76 (0.60–1.03)                                          | 0–0.01                   |
|                                  |    |           | C14:1/C16             | 0.95 (0.83–1.05)                                          | 0.79 (0.54–1.20)                                          | 0.01–0.11                |
| Disorders | Gene (n) | Nucleotide variant | Amino acid variant | Mutation type | Region | Pathogenic | Allele frequency % (n) |
|-----------|----------|--------------------|-------------------|---------------|--------|------------|-----------------------|
| **Amino acid disorders** | | | | | | | |
| **Phenylketonuria (PKU)** | PAH (14) | c.728G>A | p.Arg243Gln | Missense | E7 | P | 21.4 (6) |
| | | c.721C>T | p.Arg241Cys | Missense | E7 | P | 10.7 (3) |
| | | c.208_210del | p.Ser70del | In-frame | E3 | P | 7.1 (2) |
| | | c.331C>T | p.Arg111Ter | Nonsense | E3 | P | 7.1 (2) |
| | | c.611A>G | p.Tyr204Cys | Missense | E6 | P | 7.1 (2) |
| | | c.137G>A | p.Glu46Asp | Missense | E2 | LP | 3.6 (1) |
| | | c.442-1G>A | Splice site | I4 | P | 3.6 (1) |
| | | c.482C>T | p.Phe161Ser | Missense | E5 | P | 3.6 (1) |
| | | c.498C>G | p.Tyr166Ter | Nonsense | E5 | P | 3.6 (1) |
| | | c.526C>T | p.Arg176Ter | Nonsense | E6 | P | 3.6 (1) |
| | | c.754C>T | p.Arg252Trp | Missense | E7 | P | 3.6 (1) |
| | | c.781C>T | p.Arg261Ter | Nonsense | E7 | P | 3.6 (1) |
| | | c.782G>A | p.Arg261Gln | Missense | E7 | P | 3.6 (1) |
| | | c.833C>T | p.Thr278Ile | Missense | E7 | P | 3.6 (1) |
| | | c.842+2T>A | Splice site | I7 | P | 3.6 (1) |
| | | c.842C>T | p.Pro281Leu | Missense | E7 | P | 3.6 (1) |
| | | c.889C>T | p.Arg297Cys | Missense | E8 | P | 3.6 (1) |
| | | c.1238G>C | p.Arg413Pro | Missense | E12 | P | 3.6 (1) |
| **Tyrosinemia type III** | HPD (1) | c.460G>A | p.Gly154Ser | Missense | E8 | VUS | 100.0 (1) |
| **Citrin deficiency (CD)** | SLC25A13(4) | c.852_855del | p.Met285fs | Frameshift | E9 | P | 50.0 (4) |
| | | Not detected | | | | | 50.0 (4) |
| **Ornithine transcarbamylase deficiency (OTCD)** | OTC (1) | c.958C>T | p.Arg320Ter | Nonsense | E9 | P | 100.0 (1) |
| **Argininosuccinic aciduria (ASA)** | ASL (1) | c.260A>G | p.Lys69Arg | Missense | E3 | VUS | 50.0 (1) |
| | | c.637C>T | p.Arg217Ter | Nonsense | E9 | P | 50.0 (1) |
| **Organic acid disorders** | | | | | | | |
| **Methylmalonic acidemia (MMA)** | MMACHC (10) | c.696G>A | p.Trp203Ter | Nonsense | E4 | P | 20.0 (4) |
| | | c.567dupT | p.Ile190fs | Frameshift | E4 | P | 20.0 (4) |
| | | c.80A>G | p.Gln27Arg | Missense | E1 | P | 10.0 (2) |
| | | c.217C>T | p.Arg73Ter | Nonsense | E2 | P | 5.0 (1) |
| | | c.315C>G | p.Tyr105Ter | Nonsense | E3 | P | 5.0 (1) |
| | | c.331C>T | p.Arg111Ter | Nonsense | E3 | P | 5.0 (1) |
| | | c.394C>T | p.Arg132Ter | Nonsense | E3 | P | 5.0 (1) |
| | | c.430-2A>C | Splice site | I4 | P | 5.0 (1) |
| | | c.445_446del | p.Cys149fs | Frameshift | E4 | P | 5.0 (1) |
| | | c.482G>A | p.Arg161Gln | Missense | E4 | P | 5.0 (1) |
| | | c.648_650del | p.216_217del | Frameshift | E4 | P | 5.0 (1) |
| | | c.656_660del | p.Lys220del | In-frame | E4 | LP | 5.0 (1) |
| | | c.658_660del | p.Lys220del | In-frame | E4 | LP | 5.0 (1) |
| **Methylenetetrahydrofolate dehydrogenase (MMUT)** | MMUT (11) | c.323G>A | p.Arg108His | Missense | E2 | P | 13.6 (3) |
| | | c.729_730insTT | p.Glu244del | Frameshift | E3 | P | 9.1 (2) |
| | | c.1106G>A | p.Arg369His | Missense | E6 | P | 9.1 (2) |
| | | c.1280G>A | p.Glu427Asp | Missense | E6 | P | 9.1 (2) |
| | | c.1630_1631delGGinsTA | p.Glu544Ter | Nonsense | E9 | P | 9.1 (2) |
| | | c.1630G>T | p.Glu544Ter | Nonsense | E9 | P | 9.1 (2) |
| | | c.679G>T | p.Glu544Ter | Nonsense | E9 | P | 9.1 (2) |
| | | c.599T>C | p.Lle200Thr | Missense | E3 | LP | 4.5 (1) |
| | | c.682C>T | p.Arg228Ter | Nonsense | E3 | P | 4.5 (1) |
| | | c.861C>G | p.Tyr287Ter | Nonsense | E4 | P | 4.5 (1) |
| | | c.914T>C | p.Leu305Ser | Missense | E5 | LP | 4.5 (1) |
| | | c.1038_1040del | p.346_347del | In-frame | E5 | LP | 4.5 (1) |
| | | c.1676_1678insT | p.357_358insT | Splice site | I10 | VUS | 4.5 (1) |
| | | c.1677_1679insT | p.358_360insT | Splice site | I10 | P | 4.5 (1) |
| | | c.1741C>T | p.Arg581Ter | Nonsense | E10 | P | 4.5 (1) |

Continued
dramatically in different regions. As far as we know this is the first single center report of the incidence, disease spectrum, and genetic characteristics of IEMs in a northwestern Chinese population.

A total of 146,152 newborns underwent expanded NBS for IEMs until December 2019, and seventy-five infants and two mothers were diagnosed with IEMs in our 6-year study, and the average incidence of IEMs detected by MS/MS in Xi'an was 1:1898, which is higher than the average prevalence in previous reports from mainland China (1:3795) and the incidence in most Asian countries. Moreover, compared with the incidence of IEMs in different regions in China, the local incidence was similar to that in Jining city (1:1941) in northern China and slightly higher than that in Quanzhou city (1:2804), Jiangsu province (1:2763), Zhejiang province (1:5626), Taiwan (1:6219), Hong Kong (1:4122) in south China. This indicates that IEMs are not rare in northwest China, however, facing the current situation that large-scale expanded NBS has not been carried out in this region, it is necessary to consider the incorporation of MS/MS into the neonatal screening system.

Although there are significant differences in the disease spectrum of IEMs in different regions, it has been widely reported that PKU is the most common amino acid disorder. Moreover, numerous studies have shown that PKU is more common in northern China than in southern China, thus, our study further confirmed these conclusions. Patients with BH4 deficiency were not identified during the study period, which may further confirm the regional differences in PKU typing. Genetic analysis showed that the most common mutation was c.728G>A in the PAH gene, which is consistent with previous reports.

Methylmalonic acidemia was the second most common IEM in this region and also had the highest incidence of organic acidemias (1:6960), which was slightly lower than reported in Jining city (1:5590) and Henan province (1:6032), but significantly higher than in Zhejiang province (1:46500), Jiangsu province (1:35734), Quanzhou city (1:121515), and Taiwan (1:101625) in southern China. Thus, our research provides further evidence that the incidence of MMA in northern China is higher than that in southern China. However, 21 patients were diagnosed with methylmalonic acidemia in this study, 52.4% of patients with isolated MMA and 47.6% with combined MMA with homocysteinemia, which were different from most reported conclusions that combined MMA with homocysteinemia caused by the MMACHC gene mutation is the common type of MMA in the mainland of China. It is worth noting that our study was performed at a single center, may result in

| Disorders | Gene (n) | Nucleotide variant | Amino acid variant | Mutation type | Region | Pathogenic Allele frequency % (n) |
|-----------|----------|-------------------|-------------------|--------------|--------|----------------------------------|
| 3-Methylcrotonyl-CoA carboxylase deficiency (3MCCD) | MCCC2 (3) | c.592C>T | p.Gln198Ter | Nonsense | E6 | P | 33.3 (2) |
| | | c.659G>A | p.Gly220Glu | Missense | E7 | P | 16.7 (1) |
| | | c.690_692del | p.230_231del | In-frame | E7 | P | 16.7 (1) |
| Glutaric acidemia I (GA-1) | GCDH (2) | c.416G>C | p.Ser139Trp | Missense | E6 | P | 25.0 (1) |
| | | c.533G>A | p.Gly178Glu | Missense | E7 | P | 25.0 (1) |
| Holocarboxylase synthetase deficiency (HCS) | HLCS (1) | c.782delG | p.Glu261fs | Frameshift | E5 | P | 50.0 (1) |
| Isobutyryl-CoA dehydrogenase deficiency (IBD) | ACAD8 (1) | c.705+1G>A | Splice site | I6 | P | 50.0 (1) |
| | | c.1129G>A | p.Gly377Ser | Missense | E10 | P | 50.0 (1) |
| Fatty acid oxidation disorders |  |  |  |  |  |  |
| Primary carnitine deficiency (PCD) | SLC22A5 (3) | c.1400C>G | p.Ser467Cys | Missense | E8 | P | 33.3 (2) |
| | | c.51G>C | p.Phe17Leu | Missense | E1 | P | 16.7 (1) |
| | | c.322_331del | p.108_111del | In-frame | E1 | LP | 16.7 (1) |
| | | c.824+5G>C | p.Leu280Pro | Missense | E1 | VUS | 16.7 (1) |
| Short-chain acyl-CoA dehydrogenase deficiency (SCADD) | ACADS (3) | c.1031A>G | p.Glu344Gly | Missense | E9 | P | 33.3 (2) |
| | | c.136C>T | p.Arg46Trp | Missense | E2 | P | 16.7 (1) |
| | | c.164C>T | p.Pro55Leu | Missense | E2 | P | 16.7 (1) |
| | | c.310_312del | p.Glu104del | In-frame | E3 | LP | 16.7 (1) |
| | | c.950T>C | p.Met317Thr | Missense | E8 | LP | 16.7 (1) |
| Medium chain acyl-CoA dehydrogenase deficiency (MCADD) | ACADM (3) | c.985A>G | p.Lys329Glu | Missense | E11 | P | 66.7 (4) |
| | | c.449_452del | p.Thr150fs | Frameshift | E6 | P | 16.7 (1) |
| | | c.1238G>A | p.Arg413His | Missense | E12 | VUS | 16.7 (1) |
| Very long chain acyl-CoA dehydrogenase deficiency (VLCAADD) | ACADVL (3) | c.1843C>T | p.Arg615Ter | Nonsense | E20 | P | 33.3 (2) |
| | | c.298_299del | p.Gln100fs | Frameshift | E5 | P | 16.7 (1) |
| | | c.996dupT | p.Ala333fs | Frameshift | E10 | P | 16.7 (1) |
| | | c.1396G>T | p.Asp466Tyr | Missense | E14 | P | 16.7 (1) |

Table 4. Gene mutation spectrum of 61 infants with IEMs.
selection bias. Therefore, it is necessary to continuously increase the samples from different cities in this region in order to obtain more objective and reliable information about the disease spectrum of MMA. c.609G>A and c.567dupT in the \textit{MMACHC} gene were two common mutations, which is consistent with previous reports\textsuperscript{26}. Similar to the report by Hu et al.\textsuperscript{28}, in our population, c.323G>A was the most prevalent mutation in the \textit{MMUT} gene. However, previous other studies have suggested that c.729_730insTT is the most common \textit{MMUT} mutation in China\textsuperscript{29,30}. This difference should be taken into account the small number of cases in this study and the impact of geographical differences.

Citrin deficiency was another common amino acid disorder detected in this study with an incidence of 1:36538, comparable with those reported in Jining city (1:39556)\textsuperscript{10} and Quanzhou city (1:36455)\textsuperscript{11}. The incidence in our study was significantly higher than the calculated incidence based on the carrier rate of 1:940 in

| No. | Diagnosis | Age of onset (days) | Symptom | Age at initial screening (days) | Age at confirmed diagnosis (days) | Age at start of treatment (days) | Prognosis |
|-----|-----------|---------------------|---------|-------------------------------|---------------------------------|---------------------------------|-----------|
| 1   | VLCADD    | 1                   | Poor response; milk refusal; infection; hypoglycemia | 3                              | 10                             | 10                              | Die       |
| 2   | GA-1      | 3                   | Poor response; milk refusal; encephalopathy; metabolic acidosis | 8                              | 14                             | 14                              | Die       |
| 3   | VLCADD    | 2                   | Poor response; hypoglycemia | 7                              | 10                             | 10                              | Die       |
| 4   | Isolated MMA | 0.5              | Poor response; milk refusal; encephalopathy; sepsis; metabolic acidosis; anemia | 2                              | 4                              | 4                                | Die       |
| 5   | GA-1      | 4                   | Poor response; milk refusal; encephalopathy; sepsis | 4                              | 6                              | 6                                | Die       |
| 6   | Isolated MMA | 9                 | Poor response; milk refusal; metabolic acidosis; jaundice; congenital heart disease | 9                              | 11                             | 11                              | Die       |
| 7   | Isolated MMA | 10               | Poor response; milk refusal; metabolic acidosis; jaundice | 11                             | 11                             | 11                              | Die       |
| 8   | VLCADD    | 1                   | Hypoglycemia | 4                              | 9                              | 10                              | Die       |
| 9   | Isolated MMA | 11               | Poor response; milk refusal; metabolic acidosis; encephalopathy; infection; hypoglycemia; hyperammonemia | 5                              | 12                             | 12                              | Under treatment |
| 10  | Isolated MMA | 10               | Poor response; milk refusal; anemia; encephalopathy; sepsis; seizures; hyperammonemia; congenital heart disease; coagulant function abnormality | 10                             | 11                             | 11                              | Die       |
| 11  | OTCD      | 2                   | Poor response; milk refusal; anemia; encephalopathy; sepsis; hyperammonemia; Congenital heart disease; coagulant function abnormality | 3                              | 4                              | 4                                | Die       |
| 12  | Isolated MMA | 3                 | Poor response; milk refusal; congenital heart disease; pneumonia; metabolic acidosis; hypoglycemia; electrolyte disturbances | 4                              | 5                              | 5                                | Under treatment |
| 13  | Isolated MMA | 2                 | Poor response; milk refusal; sepsis; encephalopathy; metabolic acidosis; electrolyte disturbances; respiratory failure; hyperammonemia; coagulant function abnormality | 3                              | 6                              | 6                                | Die       |
| 14  | Isolated MMA | 6                 | Poor response; milk refusal; anemia; encephalopathy; pneumonia; metabolic acidosis; hyperammonemia | 5                              | 8                              | 8                                | Under treatment |

Table 5. Details of patients who became symptomatic before establishing the diagnosis.
the SLC25A13 gene in northern China\(^3\). However, a recent study conducted by Lin et al.\(^3\) on genetic screening for 29364 newborns at six NBS centers in southern, northern, and border regions of China, showed that the carrying rate of SLC25A13 gene mutations were basically the same in these three regions (1/42, 1/43, and 1/56, respectively). Therefore, more data should be available to determine the true incidence and regional differences of CD. Nevertheless, a large number of studies have shown that c.852_855del in the SLC25A13 gene is a hotspot mutation in Chinese patients with CD, with no regional differences in its incidence. The four patients diagnosed with CD in this study carried c.852_855del heterozygous mutations in the SLC25A13 gene. But unfortunately, CD as an autosomal recessive disorder, we did not detect other mutations in these four patients, which may be related to the limited detection ability of target NGS technology to detect gene intron regions, regulatory regions, large fragment deletions / duplications and complicated structural rearrangements. In recent years, with the reduction in cost and widely application of NGS, newborn genetic screening has received more and more attention\(^3\). In terms of the above cases, biochemical screening is still irreplaceable. Of course, considering the previous reported cases of false negative results with MSMS screening, combining newborn metabolic and genetic screening may be a better choice.

In our study, fatty acid oxidation disorders accounted for only 18.18% of all confirmed cases, including PCD, SCADD, MCADD, and VLCADD, with 3 infants of each identified. However, if maternal carnitine deficiency was included in the analysis, it could be concluded that carnitine deficiency was more common in the region. c.1400C>G in the SLC22A5 gene for PCD and c.1031A>G in the ACADS gene for SCADD were common mutations, consistent with previous reports\(^10\)\^-\(^13\). However, of the 3 cases diagnosed with MCADD, 2 cases with dizygotic twins carried a homozygous c.985A>G (66.7%) mutation in the ACADM gene, so we think that the mutation frequency of c.985A>G seems to be overestimated in this study. c.985A>G is a known high-frequency mutation of MCADD in Caucasian populations, involved in approximately 90% of cases\(^35\), but it has not been found in Japan\(^36\), South Korea\(^37\) and Taiwan\(^38\).

In this study, there was one patient confirmed with HCS by expanded NBS. This disease is a type of multiple carboxylase deficiency (MCD) caused by abnormal biotin metabolism. The clinical manifestations are non-specific, and usually mainly include nervous system and skin damage. The results of MSMS showed elevated 3-hydroxypropionylcarbinol. In mainland China, except for 3 cases of MCD confirmed in 186126 newborns screened in Zhejiang province\(^39\), there were no patient reported in other regions by expanded NBS\(^3\)\^-\(^11\). In addition, Han et al.\(^30\) screened 18303 patients with suspected IEMs by MSMS, 24 patients were diagnosed with HCS. Likewise, a comparative study of selective screening and expanded NBS in Asian countries suggested that larger number of patients with MCD were identified via selective screening, whereas very few were discovered with expanded NBS and it is easier to detect when dietary biotin insufficiency\(^39\). Therefore, the diagnosis of MCD depends more on the identification of clinicians, and of course, early diagnosis through expanded NBS can lead to effective intervention before the onset of clinical symptoms, thus reducing the risk of a poor prognosis.

This study had several limitations of note. With regard to MSMS screening efficiency, like most newborn screening centers in China, we did not use any second tier testing so that we had a high positive rate of initial screening. The Region4Stork (R4S) collaborative project has provided an optimized tool for laboratory management, clinical validation and interpretation of results of newborn screening by MS/MS, which effectively reduced the false positive rate and promoted the improvement of screening efficiency\(^39\)\^-\(^40\). However, this system has not been widely used in China, and we still use traditional interpretation rules, which may also result in a high positive rate of initial screening. The artificial intelligence interpretation model based on localized big data under this study is expected to further promote the interpretation of newborn screening results by MSMS. In addition, due to the lack of relevant screening policies and the inadequacy of education on expanded NBS, nearly 10% of the infants in this study were not recalled for secondary testing. Although we did not find false negative cases during the study period, which was related to the strict cut-off value we set, it also caused a high initial screening positive rate, which in turn caused a psychological and economic burden on neonatal guardians. The identification of false negative results mainly relies on child care management system. In this way, the current imperfect child care network may also lead to failure to effectively identify false negative cases. Therefore, how to further optimize the MSMS screening system and relevant policy support to improve screening efficiency will become the focus of our following work. Besides, a potential source of bias in sample acquisition may exit due to its single-center design, thus, our findings may not accurately reflect the prevalence of IEMs in Shaanxi province and even northwest China. However, considering the lack of massive information on expanded NBS in this region, so the incidence and disease spectrum of IEMs, including amino acid disorders, organic acidemia and fatty acid oxidation disorders derived from this study still have important reference value for the implementation of expanded NBS by MSMS in Northwest China.

Conclusions

We presented expanded NBS results with MS/MS in a northwestern Chinese population. The prevalence, disease spectrum, and genetic characteristics of confirmed IEMs were initially clarified. Our experience showed that IEMs were not uncommon in northwest China, PKU and MMA were the most common IEMs in this region, which provided effective clinical guidance for the popularization and application of MS/MS with genetic analysis for NBS and diagnosis of IEMs. In addition, the mutational hotspots we found could be potential candidates for gene screening, this will be of value for genetic counseling and genetic diagnosis of IEMs. Of course, we also need to accumulate more clinical data to standardize and optimize expanded NBS with MSMS, and the combination of MS/MS with gene screening is necessary in future.
Materials and methods

Subjects and study protocol. From January 2014 to December 2019, a total of 146152 newborns were referred to expanded NBS by MS/MS at the Neonatal Screening Center of Northwest Women’s and Children’s Hospital in Xi’an, Shaanxi, China. The baseline characteristics of the enrolled infants is shown in Table 1. The study protocol was reviewed and approved by the Ethics Committee of Northwest Women’s and Children’s Hospital. Written informed contents were obtained from all the infants’ parents.

All procedures were in accordance with the technical standards for newborn screening of the Ministry of Health of China (2010). In brief, blood samples were collected from the heel stick at more than 72 h after birth, spotted onto a Whatman 903 filter paper, air-dried at room temperature, and then sent to the laboratory for MSMS analysis. Blood specimens of preterm (< 32 weeks of gestational age) or low birth weight neonates (< 1000 g) were similarly assayed, but second specimens of these infants were collected and assayed again after one month of life. Newborns with positive initial screening results were recalled for repeated test, if the second test was still positive, the infant was transferred to a specialist for further diagnosis, treatment, and follow up. During the study period, the panel of screened disorders via MSMS was not officially regulated in China, and all disorders recommended in the US panel were screened for in our center.

Expanded NBS by MSMS. Dried blood spot (DBS) samples were pre-processed following the instruction of the NeoBase non-derivative MSMS Kit (PerkinElmer, Waltham, United States). Each 3.2-mm diameter blood DBS sample was added to a PerkinElmer 96-well microtiter plate with 100 μl of extraction buffer containing amino acid and acylcarnitine internal standards. After incubation at 45 °C for 45 min with shaking in a PerkinElmer shaking incubator, 80 μl was transferred to a new microtiter plate and placed in an ACQUITY TQD mass spectrometer (Waters, Milford, MA, USA) for quantitative analysis10,12. The analytes included 11 amino acids and 31 acylcarnitine. The 11 amino acids were alanine (Ala), citrulline (Cit), arginine (Arg), glycine (Gly), leucine/isoleucine (Leu/Ile/Pro-OH), methionine (Met), ornithine (Orn), phenylalanine (Phe), Valine (Val), Proline (Pro), and tyrosine (Tyr); the 31 acylcarnitine were C0, C2, C3, C3DC+C4OH, C4, C4DC+C5OH, C5, C5:1, C5DC+C6OH, C6, C6DC, C8, C8:1, C10, C10:1, C10:2, C12, C12:1, C14, C14:1, C14:2, C14OH, C16, C16:1, C16:1OH, C16OH, C18, C18:1, C18:2, and C18OH.

The reference values were initially set by determining 3000 normal-term infants (0.5th–99.5th percentile), and then modified over time as more samples were analyzed and more clinical data were available to minimize both false positives and negatives. Each IEM had one or more indicators including metabolites and ratios, and the positive rules of IEMs are set by three experienced physicians (including two metabolic disease specialists and a laboratory technician). When MSMS results met the positive rules of IEMs, they were considered as positive. All the positive rules and disorder lists of IEMs were shown in Supplementary material.

Genetic analysis. Genetic analysis was performed by amplicon target capture and next generation sequencing (NGS) technology (Uni-Medica, Shenzhen, China). Peripheral blood or DBS were collected from patients diagnosed with one kind of IEM by clinical and biochemical tests. Genomic DNA was extracted using Peripheral Blood or Dry Blood Spot Nucleic Acid Extraction Kits (Uni-Medica, Shenzhen, China) according to the manufacturer’s protocol. The Qubit dsDNA HS assay (Invitrogen, Carlsbad, CA, USA) was used to quantify DNA concentrations. Then, the sequencing library was established using multiplex polymerase chain reaction (PCR) (Uni-Medica, Shenzhen, China) and quantified by Qubit and quantitative PCR. Library dilution and denaturation was performed following the kit instructions and sequenced on an Illumina HiSeq X10 (Illumina, San Diego, CA) platform. The data were analyzed using the Uni-Medical GTS Analysis System. Pathogenicity analysis of each variant was performed to comply with the ACMG guidelines13. Next, variants identified by NGS were further validated by Sanger sequencing of the parents.

The panel contained 95 genes related to IEMs (PAH, PTS, QDPR, FAH, TAT, HPD, BCKDHA, BCKDHB, DBT, CPS1, OTC, ASS1, NAGS, SLC25A13, ASL, ARG1, OAT, SLC25A15, CBS, MAT1A, PRODH, GLDC, MMACHC, MUT, MMAA, MMAB, PCCA, PCB1, IVD, GCDH, MCCCI, MCCE2, AUH, HMGC1, HLC5, BTD, ACAT1, ACADSB, ACAD8, HSD17B10, L2HGDH, SLC22A5, CPT1A, CPT2, SLC25A20, ACADS, ACADM, ACADVL, HADHA, HADHB, ETFa, ETFb, ETFDH, ETHE1, MYLCD, SMPD1, NPC1, NPC2, GBA, GLA, IDUA, IDS, GANNS, GLB1, ARSB, G6PC, SLC37A4, GAA, AGL, CYP11B1, GALC, PSAP, ARSA, ABCD1, ATP7B, GALT, ALDOB, DNACJ12, GCH1, PBRD1, MTHFR, DLL3, PC, HADH, NADK2, SUCLA2, SUCLG1, LMBRD1, HCFC1, TAZ, GNMT, AHHC4, MMADHC, MCEE, and, ABCD4).

Diagnosis, treatment and follow-up. In consideration of early diagnosis and intervention for positive cases, we adopted a strategy of diagnosis and treatment at the same time. Diagnosis was made by combining clinical manifestations, individualized assistant examinations and genetic testing, of which genetic testing was a very important basis. For the positive cases after recall, individualized auxiliary examinations were conducted according to disease type, which included routine blood and urine tests, liver and kidney function assessment, myocardial zymogram, blood glucose, blood ammonia and blood gas analysis. For organic acid disorders and some cases of amino acid disorders, urine gas chromatography was used for differential diagnoses.

We conducted standard treatment and follow-up for all confirmed children according to the type of disease and clinical manifestation, which mainly included: (1) combination of diet and drug therapy, such as isolated MMA, glutaric aciduria type 1 (GA-1), etc.; (2) only diet treatment, such as PKU and CD, etc.; (3) only drug treatment, such as combined MMA with homocysteinemia, PCD and holocarboxylase synthetases deficiency (HCS), etc.; and (4) only regular follow-up, such as 3MCCD and SCADD, etc. These patients were followed up every 3 ~ 6 months, including blood MSMS, urine gas chromatography, blood biochemical analysis, imaging,
physical examination and development assessment, etc. All infants with negative screening results were included in the children's health care management system for follow-up.

**Statistical analysis.** Statistical analysis was performed using SPSS19.0. The difference of categorical data was compared using Chi-square test. The difference of measurement data was compared by t test between two groups. P<0.05 was considered statistically significant.

**Ethical approval.** The study protocol was reviewed and approved by the Ethics Committee of Northwest Women's and Children's Hospital.

**Informed consent.** Informed consent was obtained from all families enrolled in the study.

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Author contributions
R.Z. conceived and designed the research, performed the statistical analysis, and wrote the manuscript. R.Q. participated in its design and coordination and helped draft the manuscript. C.S., Y.Z., R.W., W.Y., L.Y., B.W. and N.C. conducted experiments and collected data. X.M. carried out genetic consultations. F.L. and M.F. took part in diagnosis and treatment of infants with IEMs. All authors reviewed the manuscript.

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Competing interests
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