Introduction

The aim of newborn screening is to detect newborns with serious treatable disorders to facilitate appropriate interventions and avoid or ameliorate adverse outcomes. Mass biochemical testing of newborns was pioneered in the 1960s with the introduction of screening for phenylketonuria, a rare inborn error of metabolism, tested by using a dried blood spot sample. Most of these disorders are autosomal recessive. Inborn errors of metabolism (IEM) is a permanent and inherited biochemical disorder generally caused by...
lack of a functional enzyme, transmembrane transporter, or similar protein resulting in the blockage of the corresponding metabolic pathway. There may be an accumulation of metabolites before the metabolic block and/or deficiency in the ultimate products of the pathway. In metabolic diseases, an increase in one analyte in the metabolic pathway may result in an increase or a decrease in another analyte or production of secondary metabolic byproducts. Relevant ratios can be calculated and used as secondary markers.

Detection of IEM through screening is the key element in the early treatment of these disorders. Undiagnosed and untreated disorders can cause irreversible mental retardation, physical disability, neurological damage, and even fatal outcomes.

Tandem mass spectrometry (MS/MS) is a powerful technology that can be used for the rapid detection of many IEMs, including some diseases that could not be previously detected by other methods. MS/MS has some advantages over other currently available analytical methods and is widely used for newborn screening. MS/MS is mainly aimed at the detection of the urea cycle, amino acid, organic acid, and fatty acids abnormalities. The specificity and sensitivity of this method could be up to 99% for the mentioned disorders. This technique has been applied to measure over 50 metabolites; thus, it can screen more than 40 IEMs using dry blood samples.

Each newborn screening laboratory must determine its own cut-off values, normal values, and analyte ratios by measuring 1000–2000 normal dried blood spot (DBS) samples. The cut-off value is set above 95% of the normal range, 10% lower than the SD cut-off, and then, the analysis is repeated. In a screening test, the positive predictive value (PPV) and negative predictive value (NPV) are informative parameters. Sensitivity and specificity should be provided for NPV and PPV calculation. PPV is the percentage of newborns with an abnormal result that have the disease. On the other hand, NPV is the percentage of cases with normal results that do not have this disease.

Second-tier tests were developed to decrease false-positive results and consequently increase PPV. Thus, it is essential to provide a separate testing protocol for second-tier tests. These tests are available for some neonatal screening programs of IEMs, such as tyrosinemia type I, propionic acidemia, maple syrup urine disease. Detection of IEM through screening is the key element in the early treatment of these disorders. Undiagnosed and untreated disorders can cause irreversible mental retardation, physical disability, neurological damage, and even fatal outcomes.

In the present retrospective study, we collected the results of 39987 DBS samples sent for analysis to Nilou Medical Laboratory, Tehran, Iran. These samples were unique cases. The newborn screening test was performed on DBS samples between March 2017 and February 2020.

Most samples were taken on the 3rd-7th days of birth. Each participant completed a questionnaire at the time of referral to the laboratory, containing clinical data, such as the birth date and weight, familial history, the presence of any metabolic disorders in siblings or other relatives, and type of feeding.

After taking heel stick blood of every participant, the relevant test was implemented to assess the 50 metabolic analytes, including amino acids and acyl carnitines using the instrument of Sciex Tandem Mass and Recipe ClinSpot® LC-MS/MS Complete Kit. In the cases of blood transfusion or after parenteral feeding, a second specimen was collected after seven days of birth. A second specimen was also collected after 15 days of birth in the cases of preterm or low birth weight babies (<2400 g) in cases suspicious for congenital hypothyroidism. Finally, collection of a third specimen was required after 30 days of birth for extremely low birth weight babies (<1800 g).

The cut-off values used were initially set according to the literature. Subsequently, the data of 3000 healthy newborns were reviewed and the analyte concentrations corresponding to the 95th percentile were calculated and considered as the reference levels. The cut-off values were updated following every 6000 analytes assessments. We also applied age-dependent criterion levels.

In this study, based on the determined cut-offs, as the primary markers, we determined the secondary markers. For instance, in methylmalonic academia, an increase in propionyl carnitine (C3) level was considered as a primary biomarker and its ratio with acetylcarnitine (C2) was regarded as a secondary biomarker. Therefore, if the abnormal level was observed in this ratio, the probability of having the metabolic disease was increased. In these cases, a high level of secondary biomarkers without any recall was introduced for second-tier or confirmatory tests.

Each sample with an abnormal result was repeated from the original DBS. If it was still high, the baby was recalled for confirming the diagnosis with follow-up testing. We reduced the false-positive rate and improved PPV using second-tier tests (Table 1).

Since we did not detect any case of adenosylcobalamin synthesis defects (CblC, D), we did not include the relevant tests for this kind of disorder.

Because we used the RECIPE newborn screening, we could not detect succinylacetone levels. Thus, when we detected a high level
of tyrosine in newborn screening, we evaluated succinylacetone levels using a PerkinElmer kit. Succinylacetone is considered a second-tier test.

Finally, by entering the related information in the laboratory software (self-developed) for reported disorders, the risk of IEMs was assessed and written answers, which included interpretive comments, were delivered to the families. Statistical data, such as baby status and metabolic symptoms, were recorded via telephone interviews 6–8 months after their screening tests.

4 RESULTS

After determining newborn screening cut-off values in our laboratory, we observed that normal values of some acylcarnitines levels were lower than reference values from the literature. It can be due to different nutrition regimens. The newly established normal range caused a decrease in the false-positive or false-negative results. Detailed demographic and clinical data of cases are summarized in Table 2.

The mean age of neonates was 3.9 ± 1.1 days; 5.1% of neonates were over 13 days and 7.7% were preterm or underweight. A total of 11384 cases (29.4%) came from a consanguineous marriage and the type of delivery in 8332 (51.3%) of valid cases was a cesarean section.

Detailed clinical problems of neonates in 3472 neonates from 14967 valid data are summarized in Table 3. A total of 3008 of cases had jaundice. Convulsion was reported in 344 (2.3%) cases. In addition, 164 (1.1%) of babies had no weight gain as the major problem. Finally, 254 (1.7%) of cases and 239 (1.6%) had hospitalization history and lethargy, respectively. At least 32.0% of patients had more than one problem in their histories.

Prevalence, FPR, DR, and PPV of newborn screening by MS/MS are summarized in Table 4. PPV and FPR values for this disorder and its prevalence were 40%, 0.007%, and 1:19993, respectively. In addition, a total of 102 cases had abnormal results and finally, 41 of these cases were confirmed by second-tier tests. Therefore, the overall PPV and FPR values and prevalence of these disorders were 40.2%, 0.15%, and 1:975, respectively.

After initial screening, 175 cases had positive results. We checked these screen-positive results in the original DBS. If the repeated test on the first sample revealed positive results, available second-tier tests were performed on the original samples. Positive second-tier results were considered as a positive screen result and reported for clinicians. Using this strategy, we reached the final results for 83 newborns with confidence. For other 92 newborns, no second-tier

| TABLE 1 Second-tier tests and their evaluation methods |
| --- |
| **Analytes** | **Methods** | **Disorders** | **Reference** |
| Allo-isoleucine | HPLC | MSUD | 13 |
| Homocystein | LC-MS/MS | Homocystinuria Maternal Vit B12 deficiency | 14 |
| Succinylacetone | LC-MS/MS | Tyrosinemia type1 | 15 |

| TABLE 2 Demographic and clinical data of patients (valid number column indicates the available data for each parameter) |
| --- |
| **Parameter** | **Valid No.** | **Median** | **Mean** | **SD** | **Minimum** | **Maximum** |
| Maternal Age (Yrs.) | 39987 | 31.7 | 32.3 | 4.7 | 18 | 51 |
| In person/referral | 39984 | 35188 (88.0%): In person 4796 (12.0%): Referral |
| Gestational age at delivery | 35473 | 38W+0D | 1W+5D | 26W+0D | 41W+4D |
| Consanguinity | 38721 | 11384 (29.4%): Related 27377 (70.6%): Non-Related |
| Type of delivery | 16243 | 8332 (51.3%): Cesarean Section 7911 (48.7%): Normal Delivery |
| Neonate age (Day) | 39721 | 3.7 | 3.9 | 1.1 | 2 Days | 13 Days |
| Weight of neonates (Kg) | 38546 | 2.97 | 3.17 | 0.44 | 0.92 | 4.5 |
| Maternal problem at delivery | 10837 | 116 (1.07%): had problems |
| Neonatal problems | 14967 | 3472 (23.2%): had problem |
| Preterm Delivery | 35473 | 1277 (3.6%): <37W+0D |
| Under weight (<2.5 Kg) | 38546 | 1580 (4.1%) |
| Previous history of problems in sibling | 9875 | 107 (1.09%): had problem |
test was available, thus they were recalled for diagnostic tests. In 92 newborns with a first positive result, a new specimen was collected (Recall Rate = 0.26), showing a 0.18% reduction in recall rate. For the second sample collected from the newborn, in the cases of amino acid disorders, plasma amino acids evaluation by the HPLC method was performed. For acylcarnitines abnormalities, plasma acylcarnitines, and urine organic acids were evaluated by LC-MS/MS method. A positive second result was determined to be positive and was referred for further genetic testing. Finally, 41 infants were diagnosed with one of the IEMs.

As an example, propionyl carnitine level was shown to be elevated (5.9 mM) and its methionine level was 7 µM in the DBS sample of a 12 days newborn. We determined homocysteine (16 µM) level as a second-tier test for vitamin B12 deficiency. This newborn was referred to another center for evaluation of folate, and Holo-Tc levels. These tests confirmed the diagnosis of maternal vitamin B12 deficiency.

According to this study data, phenylketonuria (classic) had more prevalence than other amino acid disorders. The highest FPR belonged to tyrosinemia (Type 1). In our laboratory, we tried to reduce FPR through performing second-tier tests, such as succinylacetone evaluation and isovaleryl-carnitine (C5) in DBS. C5 in MSUD patients was decreased dramatically.

Second-tier tests were usually conducted on the first samples. However, when it was necessary, these tests were conducted on second samples obtained from newborns. In congenital hypothyroidism, in the cases of birth weight<2400 g or gestational age<36 weeks, when tyrosine levels were high, we recommended repeated test on the second samples even in the presence of normal succinylacetone. This has led to increase in the rate of re-sampling in cases suspicious to tyrosinemia.

We additionally calculated the ratios of Xle/alanine/C5, and Val/alanine/C5. High Xle/Ala/C5 and Val/alanine/C5 ratios were the best indicators for MSUD.

In fatty acid disorders and organic acid disorder groups, medium-chain acyl CoA dehydrogenase deficiency (MCAD) and glutaric acidemia type 1 (GA) had the highest frequencies.

In this experience, we did not report any false-negative results. Positive screen test results were confirmed by confirmatory and genetic tests. Figure 1 shows newborn screening protocol to increase PPV and decrease recall.

### 5 | DISCUSSION

Newborn screening aims to diagnose inherited metabolic disorders before they cause irreversible sequelae. Every newborn screening laboratory must determine its normal ranges and cut-off values as well as secondary biomarker ratio levels for enhancing the diagnostic value of related tests. The data of healthy newborns can be determined and the analyte concentrations corresponding to the 95th percentile can be calculated and considered as reference levels for this purpose.

McHugh et al. in 2011 determined the cumulative percentiles of amino acids and acylcarnitines in DBS samples of approximately 25–30 million normal newborns from 130 sites in 45 countries. The obtained cumulative evidence has been used for the generation of 91 high and 23 low cut-off target ranges. Our study is concordant with the study conducted by Wilcken et al. in 2008, Australia. They confirmed that adapting the cut-off value is essential for newborn screening. The cut-off values obtained from these studies were applied in the current study.

Our study showed that the overall PPV and FPR values and the prevalence of IEMs were 40.2%, 0.15%, and 1:975, respectively. These results are not concordant with those of Rinaldo et al. In the mentioned study on a total of 176,185 cases, the overall performance parameters were as follow: DR of 1:1,816, PPV of 37% (54% and FPR in our study may be due to the high rate of consanguineous marriages in Iran. Moreover, since our laboratory is a referral laboratory, we collected samples from lots of high-risk infants, for instance, those having positive screening results from other laboratories.

The prevalence of IMDs was as follows: 12 for PKU, 8 for MSUD, 5 for GA1, 4 for MMA, 3 for TYR, 2 for IVA, 2 for NKH, 1 for CUD, 1 for MCAD, 1 for VLCAD, and 2 for UCD. The prevalence of metabolic disease in our study was more than Tornio’s study in Turkey, as described by Ozben. However, our results are consistent with the reports of the annual MENA (Middle East North Africa) region meeting, as the results of the conference showed a high rate of metabolic disease in infants in the MENA region due to the high endogamy.
| Disorders                        | Primary | Informative ratios | 2TT | First sample | second sample | Confirmed by genetic tests | PPV | FN | Detection Rate | FPR | Prevalence | Others |
|---------------------------------|---------|--------------------|-----|--------------|---------------|-----------------------------|-----|----|----------------|-----|------------|--------|
| Urea Cycle Disorders            |         |                    |     |              |               |                             |     |    |                |     |            |        |
| Arginemia                       | Arg     | Arg/Orn Cit/Arg    | -   | 3            | 3             | 1                           | -   |    |                |     |            |        |
| OTC<sup>a</sup>                 | Orn     | -                  | -   | 2            | 2             | 1                           | -   |    |                |     |            |        |
| Total Urea Cycle Disorders      |         |                    |     | 5            | 5             | 2                           | 40.0 % | 100% | 0.007 %       | 1:19993 | 0.23       |        |
| Amino acid Disorders            |         |                    |     |              |               |                             |     |    |                |     |            |        |
| PKU                             | Phe     | Phe/Tyr            | -   | 32           | 24            | 12                          | -   |    |                |     |            |        |
| MSUD                            | Val-Xle | Xle/Phe Xle/Ala Val/Phe | 23 | 9            | 8             | -                           |     |    |                |     |            |        |
| NKH<sup>b</sup>                 | Gly     | Gly/Ala            | -   | 7            | 7             | 2                           | -   |    |                |     |            |        |
| Tyrosinemia                     | Tyr     | Tyr/Cit Succylacetone | 72 | 14           | 3             | -                           |     |    |                |     |            |        |
| Total Amino acid Disorders      |         |                    |     | 134          | 54            | 25                          | 40.29 % | 100% | 0.07 %        | 1:1599 | 0.23       |        |
| Fatty acid Disorders            |         |                    |     |              |               |                             |     |    |                |     |            |        |
| MCAD<sup>c</sup>                | C6 C8 C10 | C8/C2 C8/C10      | -   | 3            | 2             | 1                           | -   |    |                |     |            |        |
| CUD<sup>d</sup>                 | C0      | Ac3/Cit            | -   | 8            | 6             | 1                           | -   |    |                |     |            |        |
| VLCAD<sup>e</sup>               | C14 C14:1 C14:2 C16 C18 C18:1 | C14:1/C12 C14:1/C2 C14:1/C4 C14:1/C16 C14:1/C5 C14:1/C8 | - | 4 | 4 | 1 | - |
| Total Fatty acid Disorders      |         |                    |     | 15           | 12            | 3                           | 25.0 % | 100% | 0.023 %       | 1:13329 | 0.23       |        |
| Organic acid Disorders          |         |                    |     |              |               |                             |     |    |                |     |            |        |
| MMA<sup>f</sup>                 | C3      | C3/C2 C3/C16       | HCY | 8            | 5             | 4                           | -   |    |                |     |            |        |
| GA<sup>f</sup>                  | C5DC    | C5DC/C5OH C5DC/C8 C5DC/C16 | 9 | 6 | 5 | - |
| IVA<sup>h</sup>                 | C5      | C5/C0 C5/C2 C5/C3 | 4  | 3            | 2             | -                           |     |    |                |     |            |        |
| Total Organic acid Disorders     |         |                    |     | 21           | 14            | 11                          | 52.4 % | 100% | 0.025 %       | 1:3635 | 0.23       |        |
| 39987 DBS samples               |         |                    |     | 175          | 92            | 41                          | 44.60 % | 100% | 0.13 %        | 1:975  | Recall = 0.23 |      |

<sup>a</sup>Ornithine transcarbamylase deficiency (orotic aciduria).
<sup>b</sup>Non Ketotic Hyperglycinemia.
<sup>c</sup>Medium-Chain Acyl-CoA Dehydrogenase Deficiency.
<sup>d</sup>Carnitine Uptake (Transport) Defect.
<sup>e</sup>Very Long-Chain Acyl-CoA Dehydrogenase Deficiency.
<sup>f</sup>Methylmalonic Acidemias; Glutaric Acidemia Type I.
<sup>g</sup>Glutaric Acidemia Type I.
<sup>h</sup>Isovaleric Acidemia (Isovaleryl CoA Dehydrogenase Deficiency, Xle (Leu, Ile, Allo-Ile and Hydroxyproline), Acs (C0+C2+C3+C16+C18+C18:1)).
and the high percentage of first-cousin marriages. Our results are not concordant with the results of Kobarfard’s study in Iran. In this study, the screening program was launched as a pilot study in six provinces of Iran in 2017 and a total of 145,169 babies were screened for the expanded panel of IMDs in two years. Positive samples were 3379 samples (2.3%) and 82 cases were confirmed as metabolic diseases (1 in 1770). The prevalence of IMDs was as follows: 1 for IVA, 22 for HPA/PKU, 7 for GA1, 2 for CUD, 2 for NKH, 12 for MSUD, 1 for GA2, 3 for PA, 6 for MMA, 4 for TYR, 4 for MCAD, 6 for UCD. Our results are comparable to those of Khneisser in Lebanon. Among the 276000 babies screened for the expanded MSMS panel, 19 cases with MSUD, six with CIT, three to five cases for each disease (MCAD, VLCAD, IVA, MMA, PA, GA1, and HMG), two with BKT, one with CPT-I, and one with LCHAD were detected. The incidence of MCAD was lesser than what was anticipated, and relatively high consanguinity was detected in a cluster of 18 of 19 MSUD cases that had been detected in a geographically isolated region of 40,000 residents. The prevalence of metabolic disorders in our study was more than in European countries and the United States. It might be a consequence of the high rate of consanguinity and endogamy and the relatively large family size in Iran. Therefore, we observed a high prevalence of autosomal recessive conditions and other monogenic disorders in this population.

Easy second-tier tests are now available to decrease the FPR, improve the PPV, and reduce false tests. Recall rates in various programs in different parts of the world range from 0.01% to 13.3%; the difference being mainly due to various screening methodologies, including screening protocols, different laboratory techniques, and kits, site of sample collection, and different recall criteria (cut-offs) and second-tier tests. This study showed that through establishing our own cut-off values, using secondary biomarkers, and application of second-tier tests, we can reduce recall and false-positive results (about 0.18%). The rate of recall in this study was 0.26%. Therefore, it causes early diagnosis and lifesaving treatment to infants with hereditary metabolic diseases. As a result, the FPR was decreased and the PPV was increased.

Similar findings were reported by Ombrone et al. in Italy in 2016. They proved that the new challenge for the future will be reducing the FPR by using second-tier tests, avoiding false-negative results by using new specific biomarkers and introducing new treatable disorders in newborn screening programs. Finally, we could identify three cases with MSUD using the secondary biomarker by calculating the ratio of Xle/alanine/C5 on the same primary sample, all of which show an increase in alloisoleucine level.

It is worth mentioning that levels of amino acids and acylcarnitines are different in the first and second weeks. Similarly, levels of secondary biomarkers are different. Thus, in order to avoid false-negative or false-positive results, we used age-dependent cut-off values.

In newborn screening, for improving the PPV and decreasing the false-positive and recall rates, it is important to develop a second-tier test, select conservative cut-off, and secondary biomarkers points. Moreover, applying age-dependent criteria for cut-off levels can be helpful. All these strategies markedly enhance the sensitivity and specificity of the initial test in the newborn screening program.

ACKNOWLEDGMENT
This study was financially supported by Grant from Medical School of Shahid Beheshti University of Medical Sciences.

AUTHOR CONTRIBUTIONS
SGF and SY wrote the draft and revised it. MHM and SS designed and supervised the study. BY and MMTA analyzed the data. PS, SJ, SA and HR performed the experiment and collected the data. All the authors read and approved the submitted version.

CONFLICT OF INTEREST
The authors declare they have no conflict of interest.

DATA AVAILABILITY STATEMENT
The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

ETHICAL APPROVAL
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent forms were obtained from all study participants. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences. All methods were performed in accordance with the relevant guidelines and regulations.

CONSENT OF PUBLICATION
Not applicable.
REFERENCES

1. Lanpher B, Brunetti-Pierri N, Lee B. Inborn errors of metabolism: the flux from Mendelian to complex diseases. Nat Rev Genet. 2006;7(6):449-459.

2. Ombrone D, Giocaliere E, Forni G, Malvagia S, la Marca G. Expanded newborn screening by mass spectrometry: new tests, future perspectives. Mass Spectrom Rev. 2016;35(1):71-84.

3. Dietzen DJ, Rinaldo P, Whitley RJ, et al. National academy of clinical biochemistry laboratory medicine practice guidelines: follow-up testing for metabolic disease identified by expanded newborn screening using tandem mass spectrometry; executive summary. Oxford University Press; 2009.

4. Lehotay D, Hall P, Lepage J, Eichhorst J, Etter M, Greenberg A. Use of steroid profiling by UPLC-MS/MS as a second-tier test in newborn screening for congenital adrenal hyperplasia: the Utah experience. Pediatr Res. 2009;66(2):230-235. PubMed PMID: 19390483. Epub 2009/04/25. eng.

5. Yang L, Wang H, Shen Q, Feng L, Jin H. Long non-coding RNAs involved in autophagy regulation. Cell Death Dis. 2017;8(10):e3073.

6. Sarker SK, Islam MT, Biswas A, et al. Age-specific cut-off values of amino acids and acylcarnitines for diagnosis of inborn errors of metabolism using liquid chromatography tandem mass spectrometry. Biomed Res Int. 2019;2019:3460902.

7. Pitt JJ. Newborn screening. The. Clinical Biochemist Reviews. 2010;31(2):57.

8. Burlina AB, Polo G, Rubert L, Gueroldi C, Cazzorla C, Duro G, et al. Development of second-tier liquid chromatography-tandem mass spectrometry analysis for expanded newborn screening in Japan. Int J Neonatal Screen. 2021;7(3):44.

9. Schwarz E, Liu A, Randall H, et al. Use of steroid profiling by UPLC-MS/MS as a second-tier test in newborn screening for congenital adrenal hyperplasia: the Utah experience. Pediatr Res. 2009;66(2):230-235. PubMed PMID: 19390483. Epub 2009/04/25. eng.

10. Ozben T. Expanded newborn screening and confirmatory follow-up testing for inborn errors of metabolism detected by tandem mass spectrometry. Clin Chem Lab Med. 2013;51(1):157-176.

11. Longo N, Sass JO, Jurecka A, Vockley J. Biomarkers for drug development in propionic and methylmalonic acidemias. J Inherit Metab Dis. 2022;45(2):132-143.

12. Oglesbee D, Sanders KA, Lacey JM, et al. Second-tier test for quantification of alloisoleucine and branched-chain amino acids in dried blood spots to improve newborn screening for maple syrup urine disease (MSUD). Clin Chem. 2008;54(3):542-549. PubMed PMID: 18178665. Epub 2008/01/08. eng.

13. Pajares S, Arranz JA, Ormazabal A, et al. Implementation of second-tier tests in newborn screening for the detection of vitamin B12 related acquired and genetic disorders: results on 258,637 newborns. Orphanet J Rare Dis. 2021:16(1):1-12.

14. Chinsky JM, Singh R, Ficicioglu C, et al. Diagnosis and treatment of tyrosinemia type I: a US and Canadian consensus group review and recommendations. Genet Med. 2017;19(12):PubMed PMID: 28771246. Epub 08/03. eng.

15. McHugh DM, Cameron CA, Abdenur JE, et al. Clinical validation of cutoff target ranges in newborn screening of metabolic disorders by tandem mass spectrometry: a worldwide collaborative project. Genet Med. 2011;13(3):230-254.

16. Wilcken B, Wiley V. Newborn screening. Pathology. 2008;40(2):104-115.

17. Rinaldo P, Zafari S, Tortorelli S, Matern D. Making the case for objective performance metrics in newborn screening by tandem mass spectrometry. Ment Retard Devabil Res Rev. 2006;12(4):255-261.

18. Skrinska V, Khneisser I, Schielen P, Loeber G. Introducing and Expanding Newborn Screening in the MENA Region. Multidisciplinary Digital Publishing Institute; 2020.

19. Rozmariç T, Mitulović G, Konstantopoulo V, et al. Elevated homocysteine after elevated propionylcarnitine or low methionine in newborn screening is highly predictive for low vitamin B12 and holotranscobalamin levels in newborns. Diagnostics. 2020;10(9):626.

20. Konstantopoulo V, Zeyda M. 25th Annual Meeting of the German Society of Newborn Screening. Multidisciplinary Digital Publishing Institute; 2018.

21. Sander J, Janzen N, Peter M, et al. Newborn screening for hepato-renal tyrosinemia: tandem mass spectrometric quantification of succinylacetone. Clin Chem. 2006;52(3):482-487.

How to cite this article: Younesi S, Yazdani B, Taheri Amin MM, et al. Incorporation of second-tier tests and secondary biomarkers to improve positive predictive value (PPV) rate in newborn metabolic screening program. J Clin Lab Anal. 2022;36:e24471. doi:10.1002/jcla.24471.