Quantitative Determination of Branched-Chain Amino Acids in Dried Blood Spot Samples by LC-MSMS and its Application in Diagnosis and Follow-Up of Chilean Patients with Maple Syrup Urine Disease

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Abstract

Elevation of branched-chain amino acids (BCAAs) in biological fluids indicates a deficiency in the branched-chain ketoacid dehydrogenase complex, which causes maple syrup urine disease (MSUD). Detection of increased levels of alloisoleucine confirms the diagnosis, while routine monitoring of leucine concentration is crucial for preventing metabolic decompensation and neurological dysfunction. In the metabolic center at Universidad de Chile, we have confirmed and monitored more than fifty MSUD patients in the last 20 years. Most diagnoses were made by clinical and sibling diagnosis, as MSUD is not included in the Chilean national newborn screening program. Shortening diagnosis time has a fundamental impact on the outcome of patients, therefore we focused on implementing detection of BCAAs in dried blood spot by liquid chromatography mass spectrometry (LC-MSMS) for disease confirmation as well as for biochemical monitoring. Retrospective analysis of samples from 9 diagnosed MSUD patients were performed; BCAAs values were determined via MSMS and LC-MSMS conducted in parallel. Leucine and alloisoleucine levels were positively correlated with patient's diagnosis age. Alloisoleucine was significatively elevated as early as 24 hr after birth. A predictable variation in BCAAs levels after nutritional intervention among diagnosed MSUD patients was found.

Keywords: Maple syrup urine disease, second tier test, alloisoleucine, newborn screening.

Introduction

Maple syrup urine disease (MSUD) is a rare autosomal disorder characterized by the accumulation of branched-chain amino acids (BCAAs) leucine (Leu), isoleucine (Ile), alloisoleucine (Allo), valine (Val) in urine, blood and spinal fluids, as a consequence of a deficiency in the branched-chain keto-acid dehydrogenase complex.[1] The elevated concentrations of Leu and its keto-acid are known to be neurotoxic leading to clinical manifestations such as, feeding intolerance, vomiting, metabolic acidosis, encephalopathy, lethargy and seizures that are presented in the first days of life in severe forms of disease.[2,3]

Early diagnosis and treatment are crucial to prevent brain damage and achieve normal development for MSUD patients.[4] Due to favorable outcomes when disease is detected between 48 and 72 hours of birth and positive cost-effectiveness of nutritional treatment, MSUD has been included in several newborn screening (NBS) programs around the world, predominantly in developed countries.[5–7] MSUD screening was initially conducted by bacterial inhibition assay (BIA), which measures increased Leu concentration in dried blood spot (DBS) samples.[8] Currently, NBS by tandem mass spectrometry (MSMS) analyze total Leu levels (XLeu, sum of Leu, Ile, Allo and Hydroxyproline [OHPro]) and Val in DBS samples.[9] Consensus about the cut-off value (COV) for Xleu and Val is not fully defined, thus values are independently established in each center according to local NBS program experience.[6] Elevation leucine/phenylalanine (Leu/Phe)

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ratio has been shown to be useful to avoid false positive results as well as a complementary parameter for the confirmation of MSUD.[5,10,11] Despite that, misdiagnosis may occur if infants are under parenteral nutrition or present the benign condition of hydroxyprolinemia, where isobaric OHPro is increased leading to an elevation of XLeu.[9,12] To reduce false positive results in MSUD screening, a second tier test has emerged as a reliable approach to enhance positive predictive value. The method consists in separating each isobaric form of XLeu and quantifying them independently using liquid chromatography-tandem mass spectrometry (LC-MSMS) in the original DBS sample used for first test.[7,13–16] As early as in the first 12 to 24 hours of life, MSUD newborns exhibit elevated plasma concentration of BCAAs Leu, Ile and Val together with Allo, undetected in normal individuals, making this the pathognomonic marker of disease[17] and required for confirmatory diagnosis.[18] Several protocols for BCAAs and Allo quantification in DBS have been published and have demonstrated subtle differences that make implementation feasible for specialized metabolic laboratories.[13–16] Outside of its initial application as a second-tier test for MSUD, the LC-MSMS method for BCAAs quantification in DBS samples may be useful for rapid confirmatory diagnosis when MSUD patients are diagnosed by clinical findings or by family history due to sibling diagnosis, as generally happens in nations like Chile and most of South American countries where the local NBS program does not include MSUD. Detection and quantification of Allo in DBS samples of potential MSUD cases, in conjunction with determination of Leu/Phe ratio would be a useful approach for shortening the time during the confirmatory diagnosis process.

Currently, thirty-eight MSUD patients are being followed-up in the National Reference Center for Inborn Errors of Metabolism at INTA, University of Chile. Treatment management guidelines include: maintaining plasma Leu levels below 200 µmol/L for children under five years of age and below 300 µmol/L thereafter, together with Val and Ile in the range of 200-400 µmol/L. These thresholds have been shown to promote normal growth, avoid decompensation and prevent neurological manifestations of disease.[4,19]

The aim of the present study was to evaluate the use of BCAAs quantification in DBS samples using the LC-MSMS method for the confirmatory diagnosis of MSUD and follow-up of patients. By retrospective analysis of DBS samples from MSUD patients at time of diagnosis, we compared the concentration of Xleu and each isobaric amino acid determined by the two spectrometric methods MSMS and LC-MSMS. We evaluate the potential use of the Leu/Phe ratio for confirmatory diagnosis correlating this ratio, Leu and Allo values with age at diagnosis. Also, we described changes in BCAAs concentration of MSUD patients after diagnosis and under nutritional treatment in order to explore the potential use of the LC-MSMS method for routine biochemical monitoring of patients.

Methodology

Materials and Reagents

Amino acids standard mix (including acidic, neutral and basic amino acids (L-Leu, L-Ile, L-Allo, L-Val and L-Phe), Butanol/HCl 3M, Formic Acid, Hydrazine hydrate and 4-Aza-DL-leucine dihydrochloride were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Acetonitrile (ACN), formic acid (FA), water and methanol, from Merck-Sigma (Saint Louis, Missouri, USA). Isotopically labeled internal standards for BCAAs and Phe were obtained from Cambridge Isotopes Laboratories. Filter paper used for blood spot collection was Whatman # 903. Quality control samples were prepared for each BCAAs collecting whole blood samples from MSUD patients in follow-up. Fifty µL of the whole blood pool from one patient was added to filter paper until complete dryness. Linear calibration curves for each amino acid ranged from 1 to 400 µmol/L and were constructed presenting a regression coefficient of 0.99795 for Allo; 0.99778 for Ile; 0.99905 for Leu and 0.99550 for Val. We established limits of detection and quantification of 0.5 and 2 µmol/L for Leu, Val, Allo and Ile, respectively considering signal/noise ratio >15. Intra- and inter-assay imprecision were determined at concentration of 50 µmol/L. Intra-assay variation determined in 6 replicate analysis conducted on the same day was 4.3% for Leu, 6.5% for Ile, 4.6 % for Allo and 3.2 % for Val. Inter-assay imprecision was determined in six independent experiments conducted on consecutive days. The value obtained for Leu was 6.3%, for Ile 8.3 %, for Allo 13% and for Val 10.4%. Accuracy values were determined by analyzing external proficiency testing samples from ERNDIM (European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism) achieving values >90%.

Clinical Samples

DBS samples of MSUD patients diagnosed between 2012 to 2020 (n=9) were elected for MSMS re-analysis and LC-MSMS comparison studies. Thirty-one DBS samples were taken within 6 months of diagnosis from patients under active treatment and 23 DBS samples from healthy newborns (control group) were eligible for inclusion in this retrospective study, collected from our laboratory and reanalyzed by MSMS and LC-MSMS method. Thirty-two DBS samples obtained from MSUD patients in follow-up were analyzed in parallel through BIA and LC-MSMS method for BCAAs. Ethical approval and patient informed consent were approved by the ethical committee of INTA, Universidad de Chile. To address the degree of XLeu (Ile+Leu+Allo+OHPro) and Val degradation during long-term storage of DBS samples (from one to eight years), 24 samples from MSUD patients were reanalyzed by MSMS. Samples that were stored for ≤ 12 months suffered a variation no greater than 12% in XLeu and

Val concentrations. In contrast, samples stored from two to eight years presented unpredictable variations that ranged from 1% to 43% without a time-dependent correlation for XLeu nor Val.

Sample Preparation

For BCAAs analysis by LC-MSMS, a 3.2mm disc was placed in microfuge tubes with 200 µL methanol containing labeled internal standard. After shaking for 30 min at room temperature on an orbital shaker, eluents were collected in 1.5 ml glass vials and evaporated under a stream of nitrogen at 40°C. Dried analytes were derivatized by adding 50µL Butanol/HCl 3M. Amino acids butylation was conducted at 65°C for 7.5 minutes, after which solution was evaporated by a stream of nitrogen until the sample dried. Resuspension was conducted by adding 150 µL of aqueous mobile phase (ACN 20% in water, with FA 0,1%). For MSMS analysis, a single 3.2 mm disc (3.2 mm in diameter) was punched out from the DBS into a microplate well containing 100 µL of a working solution (isotopically labeled metabolites in Methanol 80%, Oxalic Acid 0.5 M and Hydrazine 0.06% v/v). The plate was shaken in the incubator/shaker (Wallac NCS incubator, Perkin-Elmer) at 45 °C for 45 min at a speed of 750 rpm. Then, 75 µL of well content was transferred to another microplate. Analytical measurements were performed in the multiple reaction monitoring mode. In order to monitor the performances of our assays, quality control was conducted together with samples in the same plate. Leu determination by BIA was performed as described: 4 mm disc of DBS sample was placed on the surface of an agar culture medium containing 4-azaleucine and *Bacillus subtilis (*ATCC 6051). Growth inhibition of *B. subtilis* by 4-azaleucine in a minimal culture medium was prevented in the presence of Leu. Leu concentration was calculated by measuring the diameter of bacterial growth on patient's disc sample and comparing it to the diameter obtained by using different Leu standard disc. Values are reported in µmol/L after conversion from mg/dL, considering 1mg/dL equal to 76.23 µmol/L, and as range: <152.4 µmol/L, 152.4 – 305 µmol/L; 305 - 457.4 µmol/L and 457.4 -610 µmol/L.

Instrumentation and Analysis

BCAAs analysis was performed using the Shimadzu UFLC system coupled to a SCIEX 3500 ESI-MSMS detector (Applied Biosystems). Chromatographic system was equipped with a Symmetry C8 (150mm x 4.6 mm, 5µm) column to separate isobaric amino acid Leu, Ile, Allo by isocratic flow of 0.8 ml/min with a mobile phase comprising 20% ACN and 0.1% FA maintained at 40°C. Total run was of 10.5 minutes and the sample volume injection was 10 µL. MSMS detector was adjusted in positive electrospray ionization through multiple reaction monitoring mode at a temperature of 550 °C and a source voltage of 3500 V. ESI-MSMS parameter was optimized for each butylated amino acid and its corresponding labeled standard. Analyte concentration was determined measuring the ratio of BCAA peak areas to the respective internal standard peak area with known concentration using MultiQuant^{tm} 3.0.2 software. For Allo, labeled Leu was used as internal standard.

Underivatized Xleu and Valine analysis for NBS of DBS were performed on a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters Corporation, Milford MA) operating in positive electrospray ionization mode. The capillary voltage was 3.3 kV. A 40-V cone voltage was used for all analytes. The source temperature was 120°C and the desolvation temperature was 400°C. Each sample was injected by an autosampler (Waters 2777 C, Waters Corporations, Manchester, UK) and eluted by an HPLC pump (Waters 1525µ) at a flow rate of 0.07 mL/min, for 2 minutes. For quantification, the instrument was operated in multiple reaction monitoring mode at unit resolution. A 50-ms dwell time was used between transitions. A 10-eV collision energy was used for collision-induced dissociation.

Statistical Analysis

For all statistical analysis, first the distribution for each variable was determined using the Shapiro-Wilk test. Depending on its normality, the mean or median was calculated, and, depending on the statistic used, the SD or the 1stand 99th percentile ranging reported. Spearman's correlation was performed to verify the association of variables. A p-value <0.05 was considered statistically significant. Graph Pad Prism 8.4.0, LLC. were used for analyses.

Results

Retrospective MSUD Samples Analysis

In MSUD diagnosed DBS samples (n=9), we quantified the levels of Leu, Ile, Allo and Val by the implemented LC-MSMS method in parallel with the reprocessing of the samples by MSMS. Allo, the pathognomonic biomarker of MSUD[18], was detected in all samples and presented a mean value of 85 µmol/L with the 1st and 99th percentile ranging from 8 to 154 µmol/L. For Leu the mean was 971 µmol/L and for Ile and Val the average level in the diagnosis samples were 204 µmol/L and 305 µmol/L, respectively (Table 1). Twenty seven percent of variation was found in the mean value of Val when two different spectrometric methods were used for quantification.

Since each MSUD patient was diagnosed (clinically or by sibling diagnosis) at a different age, we evaluated whether a correlation between each BCAA and age existed. We analyzed Leu, Ile, Allo, Val and Leu/Phe ratio. As shown in Figure 1A and 1B, Leu and Allo had a positive correlation with diagnosis age (rho= 0.8108, p value <0.05 for Leu; rho= 0.8469, p value <0.05 for Allo). Leu/Phe ratio, mostly used in the first MSUD screening[5,6,10], did not show a significant positive correlation, nevertheless a trend is outlined (Figure 1C). No significant correlation was found with Ile and Val concentrations and

diagnosis age. To further explore the significance of Leu/Phe ratio in MSUD confirmation, we compared the average Leu/Phe ratio of MSUD diagnosis samples with a group of control newborn samples (n=30). Mean of Leu/Phe ratio in healthy control newborn group was 1.87 with 1st and 99th percentile ranging from 1.2 to 4.3. The analysis of samples of diagnosed MSUD patients, showed an average of 27.7 with values in the range of 10.3 and 51.2 for the 1st-99th percentile, respectively. To be considered, the DBS sample taken earlier (at 24 h of life) in a MSUD patient was the one that presented the lowest Leu/Phe ratio (10) as well as for Allo (8 µmol/L) and Leu (258 µmol/L) (Figure 1).

BCAA Levels Among MSUD Patients Being Followed-Up

After diagnosis, MSUD patients undergoing a metabolic crisis and those who were pre-symptomatic began nutritional treatment at our center. We quantified the BCAA levels in samples from MSUD patients within the next six months after diagnosis, with the purpose of evaluating the impact of nutritional management on blood concentration of Leu, Ile, Allo and Val. MSUD patients presented a reduction in Leu mean levels from 971 µmol/L to 99 µmol/L; an elevation in average Allo from 85 µmol/L (at time of diagnosis) to 170 µmol/L; and a moderate increase in Val and Ile averages were found after nutritional intervention and metabolic compensation (Table 2). In the control group of healthy newborns, the highest Leu levels were 283 µmol/L with a mean of 106 µmol/L. No quantifiable levels of Allo were found in the newborn healthy control group analyzed.

We have been employing the BIA weekly during routine monitoring of Leu levels in MSUD patients in follow-up. BIA results are reported as a range of Leu levels as given by the semi-quantitative scope of the technique. Therefore, we were interested in determining Leu value quantified by LCMSMS in a set of DBS MSUD samples (n=32) previously analyzed by BIA, as first approach to establish the mean of Leu concentration for each range determined semi-quantitative method and if there is linearity in this comparison. Quantitative mean Leu value for each range of BIA determination is shown in Figure 2.

Table 1. BCAAs levels in first dried blood spot samples from MSUD patients. Retrospective analysis by two methods MSMS and LC-MSMS.

Patient #	Year of Diagnosis	BCAA Level in reprocessed diagnosis samples by MSMS - (µmol /L)		BCAA Levels in diagnosis samples by LC-MSMS (µmol/L)			
		X-LEU	VAL	LEU	ILE	ALLO	VAL
1	Nov-2012	1762	254	1308	249	95	238
2	Mar-2013	315	213	258	110	7	258
3	May-2013	478	260	302	161	18	251
4	May-2016	1469	143	1254	97	84	154
5	May-2016	2068	374	1349	305	154	405
6	Jun-2016	1311	171	936	174	95	176
7	Aug-2018	2779	507	1201	307	138	434
8	Sept-2019	2547	458	1607	227	150	435
9	Oct-2020	967	592	529	207	26	394
	1st percentile	328	145	261	98	8	156
	99th percentile	2760	585	1586	307	154	435
	Mean	1522	330	971	204	85	305

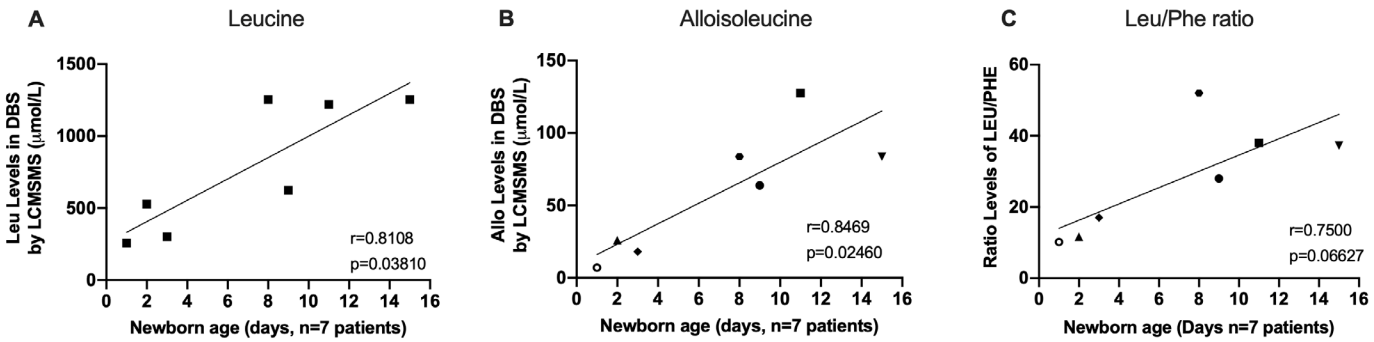

Figure 1. Correlation of Leu and Allo blood levels and Leu/Phe ratio with age. Analysis of Leu, Allo and Leu/Phe ratio of each dried blood spot sample of diagnosed MSUD patients was correlated with newborn age. Each point represents one patient; in total, data from seven patients are displayed (r: Spearman rho, p value <0.05, for Leu and Allo).

Table 2. Comparison of branched-chain amino acids (BCCAs) levels quantified by liquid-chromatography mass spectrometry (LC-MSMS) in dried blood spot samples of MSUD patients at diagnosis and follow-up with control newborn samples.

		LEU (mmol/L)		ILE (mmol/L)		ALLO (mmol/L)		VAL (mmol/L)	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range
MSUD	Diagnosis samples (n=9)	971	358 - 1607	204	97- 307	85	7 - 155	305	154 - 435
	Samples within six months of diagnosis (n=31)	99	9 - 540	356	45 - 1018	170	9 - 615	426	31 - 986
Control	(n=23)	106	38 - 283	47	18 - 145	0	0	133	21 - 411

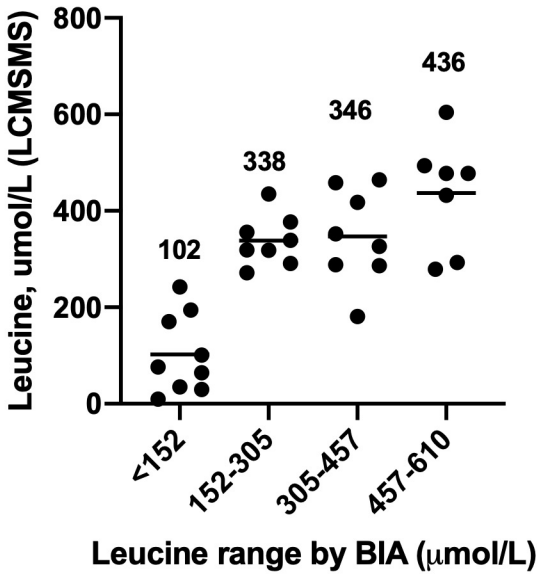

Figure 2. Comparison of Leu levels measured by BIA versus LC-MSMS. Thirty-two dried blood spot samples of MSUD patients in follow-up were analyzed by bacteria inhibition assay (BIA) and LCMSMS method for BCAAs. Average Leu values are shown for each range of values analyzed by BIA.

The LC-MSMS quantitative mean Leu value for BIA level of <152.4 μmol/L was 102 μmol/L; 338 μmol/L for the range of 152.4 - 305 μmol/L; 346 μmol/L for 305 - 457.4 μmol/L and 436 μmol/L for 457.4 - 610 μmol/L.

Discussion

In the last decade, second-tier testing methods have emerged as a reliable and necessary approach to improve the positive predictive value in the screening of several inborn errors of metabolism, moreover, they are important for nations that are progressively expanding the number of pathologies included in their NBS programs.[20–22] In Chile, a national NBS program was initiated in 1992 with the screening of two pathologies, phenylketonuria and congenital hypothyroidism. An expanded NBS, including a total of 26 disorders, is done under particular (parent and clinician) requirements in specialized metabolic laboratories, such as our reference center at INTA, University of Chile and as part of a national pilot program carried out in 2017.

In our clinical center, there are thirty-eight MSUD patients under active follow-up, most of them diagnosed clinically, by siblings and one by NBS. Disease manifestation occurs early in classical MSUD form, within 2-3 days of life and fatal or irreversible consequences may occur if diagnosis and management are delayed. We implemented a traditional method for MSUD second-tier testing to be applied to DBS samples that after MSMS analysis present XLeu levels above the COV of 250 μmol/L and 253 μmol/L for Val with the purpose of: i) shortening the confirmatory process of MSUD and, ii) to count with quantitative Leu, Ile, Allo and Val levels in blood for the biochemical follow-up of patients, oriented for an early nutritional intervention in cases of catabolic crisis or patient's decompensation, where fast modification in Leu intake is essential to prevent greater neurological damage and death.

Retrospective analysis by LC-MSMS of samples of MSUD patients revealed that all of them presented quantifiable levels of Allo with values above the upper COV reported in literature for Allo (2-5 μmol/L).[13,14,23] Allo seems to be stable over time since it was detected even in samples that were stored for more than five-years in a non-controlled environment. Thus, it appears to be a highly sensitive biomarker for samples taken as early as 24h of life (7.1 μmol/L), as was the case for the MSUD patient diagnosed by family history. Our analysis of control NBS samples,

taken between 48h and 72h of birth, presented, in most cases, undetectable levels of Allo below the limits of quantification (2 μmol/L). Therefore, no overlap between the 99th percentile of control samples and the 1st percentile in MSUD samples (7.9 μmol/L) existed in the analyzed samples. A retrospective study in Australia, indicated Allo levels ranging from 6-73 μmol/L in NBS samples from classical MSUD patients and an average value of 16 μmol/L was found in mild/intermediate MSUD patients.[15] Our data are consistent with this report; however, increasing the sample size would strengthen our observation. The introduction of MSUD second-tier testing in the NBS program in British Columbia, Canada showed that all false-positive samples (n=7) presented normal values of Allo-Ile (<2 μmol/L) whereas all true positives (n=3) had markedly higher levels of Allo-Ile (>22 μmol/L).[14] Although Allo-Ile is a well-recognized biomarker for MSUD, in an intermittent MSUD phenotype, Allo elevation may not be present in NBS samples, but its formation and detection can be observed under catabolic state.[24] Also, it has been reported that elevated levels of Allo and Leu may be found in PKU patient's (6 of 11 patients studied) due to liver damage which could lead to false positive result.[11]

To prevent misdiagnosis of false positive results and improve diagnosis, Leu/Phe and Val/Phe ratios have been proposed both for first-tier test algorithms and for MSUD diagnosis.[5,9,11,13,25] We analyzed those ratios in samples of MSUD and control newborns revealing a difference of 14 times in the means of Leu/Phe ratio and 3.8 times for Val/Phe between the two groups (ratios from MSUD vs ratios from control). Meanwhile the value of the percentile 99th for Leu/Phe ratio in control samples was 4.3, first percentile in MSUD patient samples was 10.3, exhibiting a difference of 2.4 times. It is important to remark that the lowest Leu/Phe ratio in MSUD was the sample taken at 24h of life in the case of MSUD sibling diagnosis. Conversely, for the Val/Phe ratio we observed that the highest percentile value in control samples (6.2) was above the 1st percentile in MSUD samples (2.9), being unsuitable to be considered for MSUD diagnosis. Thus, taking in account our results and the published data we suggest that Leu/Phe ratio would be an appropriate complementary parameter to the Allo value if a confirmatory approach is intended.

In our Centre, monitoring of biochemical parameters in MSUD patients undergoing follow-up is carried out weekly in children under one-year, then biweekly or monthly in plasma or DBS samples depending on the frequency, age of patient and patient adherence to nutritional treatment. Leu levels should be nutritional adjusted according to consensus-based guideline recommendations (200 μmol/L in patients younger than 5 years old or 300 μmol/L in older patients).[19] BIA is a low-cost test that allows semi-quantitative Leu levels in blood (dried blood spot samples) and it has been the alternative of choice in developing countries to provide accessibility to the majority of MSUD patients. Despite this social-economic advantage, this semi-quantitative analysis presents some difficulties for dieticians when adjustment of Leu intake is needed, especially when blood levels are below 200 μmol/L. In this particular case, it is necessary to gradually introduce Leu intake, to prevent this amino acid from remaining at values below those recommended, as this could induce catabolism due to Leu deficiency. LC-MSMS-based determination of each BCAAs allowed us to compare Leu levels from the semi-quantitative method currently used in MSUD patient monitoring with a reliable quantitative method, besides counting with other BCAAs values (e.g., Allo, Ile and Val). Application of this methodology as part of our routine monitoring program is under evaluation given that its cost is slightly higher than BIA, but much lower than the quantification of amino acids in plasma sample that might not be affordable for patients. The Chilean government subsidizes the medical formula provided to MSUD patients, but not the costs associated with the quantification of amino acids, which, according to our protocol, must be performed at least once a month. These costs are also not covered by health insurance, which means they must be paid by families, illustrating the importance of providing quality, low-cost exams, in order to maintain an adequate metabolic state and allow growth and development within normal ranges.

Conclusion

We determined values of Leu, Val, Ile and Allo in DBS samples using LC-MSMS. Although this technique has been used successfully as a second-tier testing method in some NBS programs, in the Chilean context, it would allow a rapid diagnosis in patients with selective screening (e.g. clinical suspicion due to family diagnosis), as well as for the follow-up of patients with a MSUD diagnosis.

DBS analysis of each BCAAs by LC-MSMS is simple, fast and no major pre-analytical processing is required. The fact that diagnosis can be made in DBS helps to receive critical samples from places located more than 1000 kilometers from the NBS center without any special handling requirement. Shortening MSUD diagnosis results is incredibly important for the prevention of metabolic decompensation and the onset of severe complications. Thus, improving our time-response has been one of our objectives. In relation to the BCAA method for routine biochemical monitoring of MSUD patients, further studies are required to demonstrate the cost effectiveness of this technique that should consider the socio-economic situation of the patients served.

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Declaration of Conflicting Interests

The authors declare no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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