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SECOND-TIER TESTS DO NOT ALWAYS REQUIRE CHROMATOGRAPHIC SEPARATION: THE CASE OF HYDROXYLATED AND DICARBOXYLIC ACYL-CARNITINE COUPLES

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***ABSTRACT* – Objective:** Newborn screening is used to diagnose inherited metabolic diseases (IEMs), many of which are diagnosed via flow injection analysis coupled with tandem mass spectrometry (FIA-MS/MS). Some metabolites are isobaric and thus are not distinguished. Second-tier tests have been developed to facilitate this separation, often involving liquid chromatography. Some isobaric acyl-carnitines are associated with IEMs characterized by the suppression of ketogenesis. These are acquired as a sum of signals at the first screening, as in the case of dicarboxylic and hydroxylated acyl-carnitines (C3DC\C4OH, C4DC\C5OH, C5DC\C6OH). In this regard, a second-tier test based on FIA-MS/MS has been developed.

Materials and methods: Internal standards (ISs) of the NeoBase™ 2 non-derivatized MSMS kit (Revvity, ex PerkinElmer) were employed; for the isobaric couples, the corresponding ISs were $^2H_3$-C4, $^2H_9$-C5, and $^2H_6$-C5DC. A derivatization step using 3N HCl in n-butanol was added to the normal sample preparation of the kit. Samples were analyzed by FIA-MS/MS through the RenataDX™ screening system coupled to a Xevo™ TQD IVD mass spectrometer.

Results: This approach allowed rapid differentiation between isobaric couples without a chromatographic separation. The method is accurate and precise for routine diagnostics. In this context, a clinical suspect was identified between 3-methylcrotonylglycinuria, multiple carboxylase deficiencies, 3-methylglutaconic aciduria type I, and 3-hydroxy-3-methylglutaric aciduria in a newborn showing elevated methylmalonyl-carnitine (C4DC)/3-hydroxyisovaleryl-carnitine (C5OH) levels at the first screening. The second-tier test highlighted alterations in the levels of C5OH rather than C4DC.

Conclusions: For the clinical approach, the availability of this simple second-tier test supports a rapid differential diagnosis among IEMs that differ in the degree of metabolic decompensation through suppression of ketogenesis.

Keywords: FIA-MS/MS, Fischer esterification, Inborn errors of metabolism, Isobaric couples of acyl-carnitines, Newborn screening, Second-tier test.

INTRODUCTION

Ketone bodies represent an alternative energy source, mostly in the brain, when hypoglycemia occurs. As a matter of fact, they are produced to face acetyl-CoA overloads as glucose levels are low for prolonged periods and fatty acids still undergo oxidation. Under these conditions, β-hydroxybutyrate and acetate can be converted to acetyl-CoA and entered into the tricarboxylic acid cycle[1]. Some inherited metabolic diseases (IEMs) display suppressed ketogenesis due to the impaired catabolism of ketogenic amino acids (i.e., lysine, leucine) or the altered fatty acid β-oxidation (FAO).

The importance of ketogenic amino acids

Lysine (Lys) is an essential amino acid used for both protein synthesis and ketogenesis[2]. It is known to be mostly catabolized within mitochondria and, in part, peroxisomally[3]. As depicted in Figure 1, any alterations in the pool of enzymes involved in Lys breakdown can result in hyperlysinemia, which is associated with neurological symptoms, probably driven by either the accumulation of pipecolate, a recently established pro-apoptotic stressor in neurons[4], or the reduced ketogenesis[5]. Notably, glutaric aciduria type 1 (GA-1) is an autosomal recessive disorder arising from deficient glutaryl-CoA dehydrogenase[6] that impairs the physiological catabolism of not only Lys but also hydroxylysine (Hyl) and tryptophan (Trp), whose metabolites (i.e., 2-aminoadipic-semialdehyde and 2-oxoadipate, respectively) enter the same pathways for Lys degradation. Therefore, GA-1 is characterized by accumulating levels of glutaryl-CoA and its corresponding dicarboxylic species, such as glutaric acid (GA), 3-hydroxyglutaric acid (3-OH-GA), glutaconic acid, and glutaryl-carnitine (C5DC)[3]. Affected patients can suffer from dystonic-dyskinetic movement disorders that might occur after bilateral striatal injury and white matter lesions possibly caused by encephalopathic crisis[3]. Beyond administering neuroprotective agents and antiepileptic drugs[7], GA-1 management requires fine control of the energy intake with a low-protein/high-glucose diet and carnitine supplementation to prevent hypoglycemia and limit GA and 3-OH-GA production.

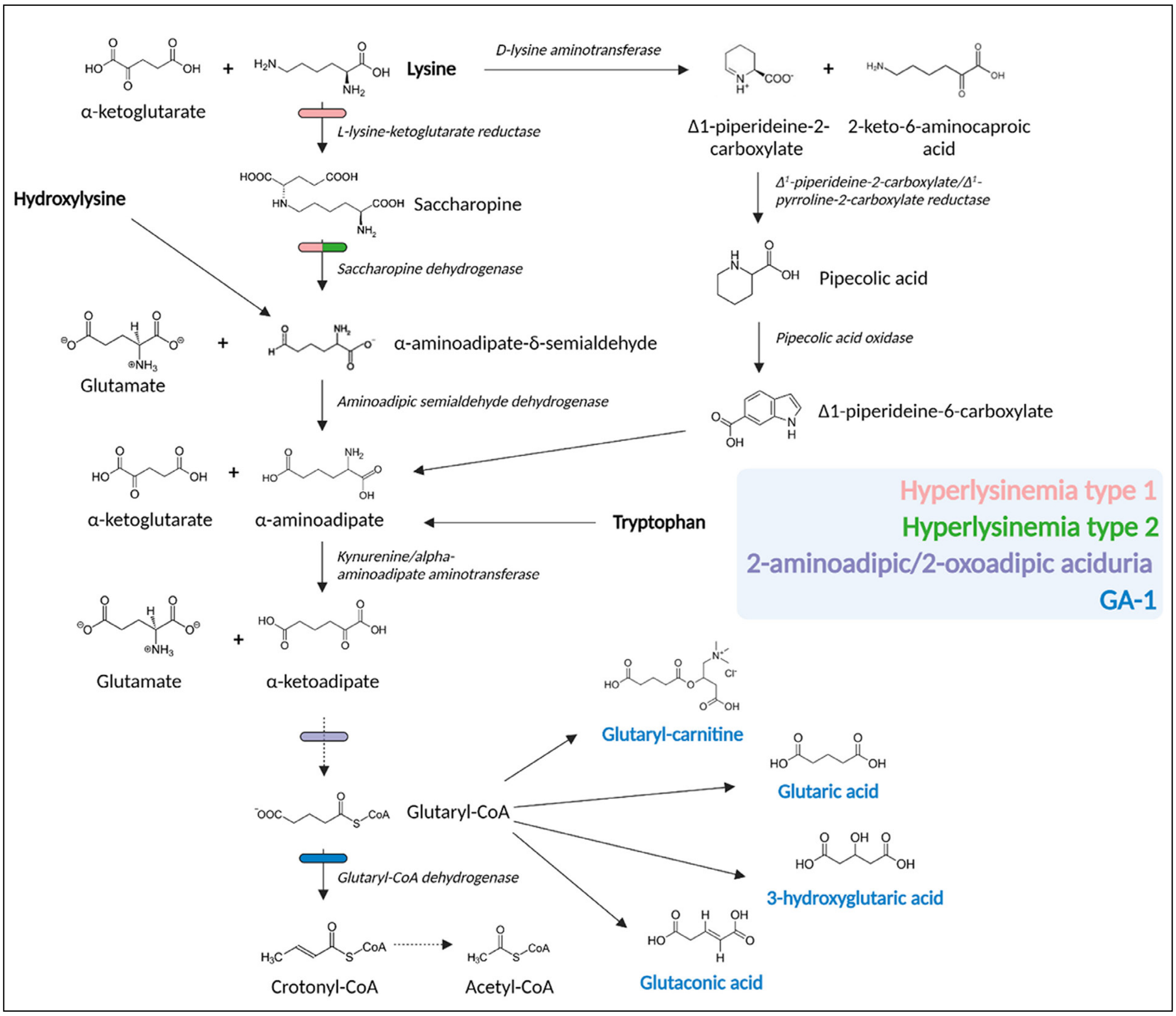

Figure 1. Biochemical reactions around Lys, Hyl, and Trp metabolism. The possible enzymatic deficiencies are highlighted by colored bars (pink for hyperlysinemia type 1, green for hyperlysinemia type 2, grey for 2-aminoadipic/2-oxoadipic aciduria, blue for GA1). Image created with BioRender.com.

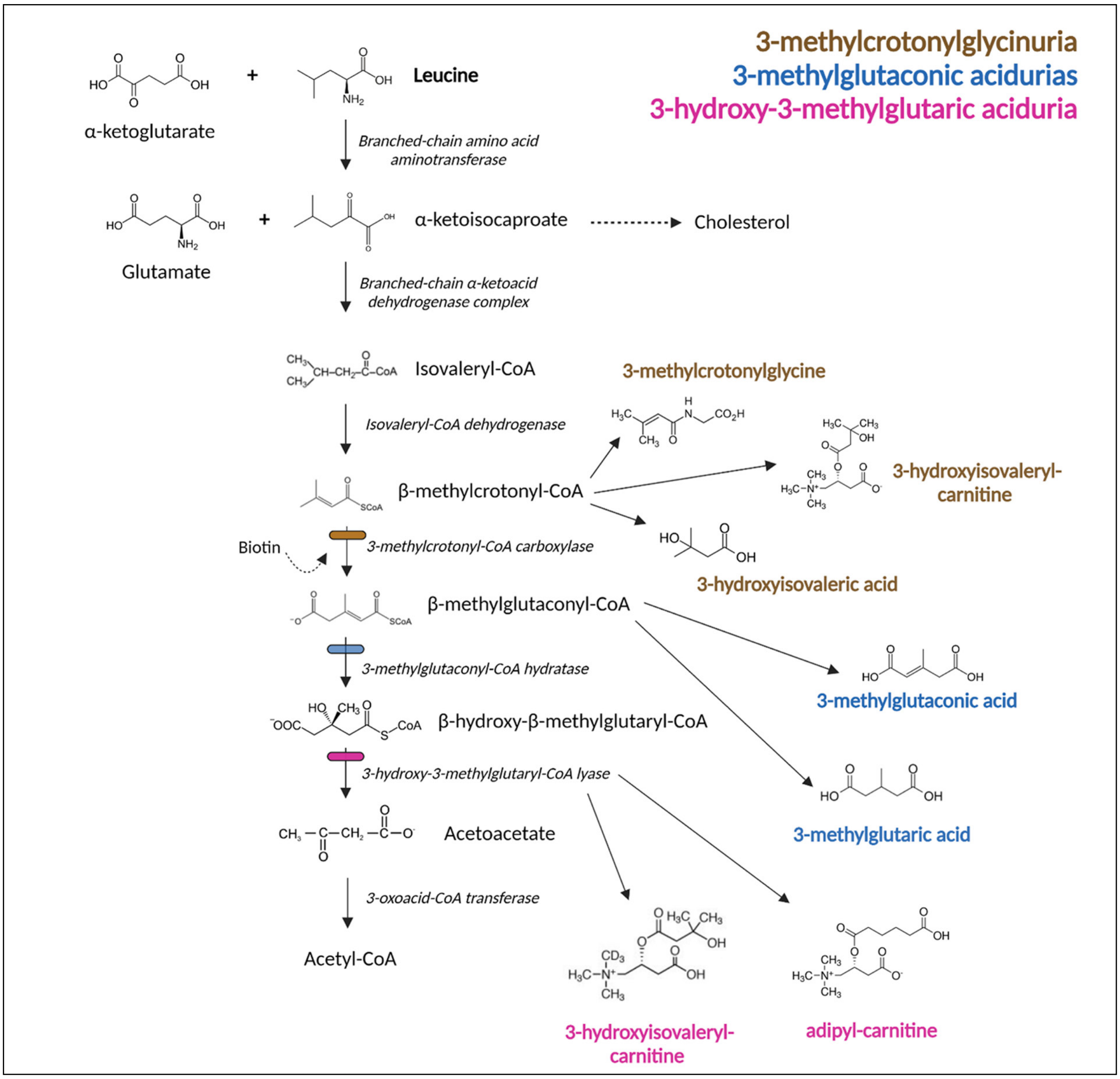

Figure 2. Biochemical reactions around Leu metabolism. The possible enzymatic deficiencies are highlighted by colored bars (brown for 3-methylcrotonylglycinuria, blue for 3-methylglutaconic acidurias, pink for 3-hydroxy-3-methylglutaric acidemia). Image created with BioRender.com.

As Lys, leucine (Leu) is another essential ketogenic amino acid. According to the metabolic reactions leading to Leu breakdown (Figure 2), 3-methylcrotonylglycinuria results from the deficiency of the mitochondrial 3-methylcrotonyl-CoA carboxylase (3-MCC), which converts 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA in a biotin-dependent manner. Diagnosis is based on elevated levels of 3-methylcrotonylglycine, 3-hydroxyisovaleric acid, and 3-hydroxyisovaleryl-carnitine (C5OH)[8]. Since MCC is a biotin-dependent carboxylase, 3-methylcrotonylglycinuria is part of a heterogeneous group of known multiple carboxylase deficiencies that must be considered for differential diagnosis[9]. A second group of Leu-related diseases refers to 3-methylglutaconic acidurias, which are characterized by increased levels of 3-methylglutaconic acid and 3-methylglutaric acid due to 3-methylglutaconyl-CoA hydratase deficiency. Another Leu-related disease, the so-called 3-hydroxy-3-methylglutaric aciduria, is caused by the deficiency of HMG-CoA lyase, which catalyzes the conversion of HMG-CoA to acetoacetate and acetyl-CoA. This disorder is biochemically associated with elevated levels of adipoyl-carnitine and C5OH. All these Leu-related diseases can be characterized by harmful hypoglycemia with neurological abnormalities, lethargy, and hypotonia. Therefore, treatments are aimed at preventing low glucose levels and metabolic acidosis and are accompanied by restricted protein intake.

Other paths for impaired ketogenesis

The acute decompensation driven by the impaired ketogenic amino acids described above may alternatively arise from aberrant FAO. As a matter of fact, malonic aciduria results from malonyl-CoA decarboxylase deficiency, an enzyme involved in malonyl-CoA conversion to acetyl-CoA: such alteration completely suppresses the activity of carnitine palmitoyltransferase I, and consequently, mitochondrial oxidative degradation of fatty acids[10]. Diagnosis is based on increased levels of malonyl-carnitine (C3DC) and must be made promptly because of the severe clinical manifestations due to suppression of ketogenesis. Conversely, a partial FAO blockade is given by short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (SCHADD), which is characterized by high levels of 3-hydroxybutyryl-carnitine (C4OH). In both cases, hypoglycemia must be prevented to avoid acute metabolic crisis, although patients with SCHADD are often asymptomatic because fair compensation by other acyl-CoA dehydrogenases can occur[11].

IEMs with impaired ketogenesis in the context of newborn screening

Newborn screening (NBS) plays a crucial role in early diagnosis and management of IEMs. Despite the lack of homogeneity among NBS programs throughout the countries, most of the diseases are easily identified by flow injection analysis coupled with tandem mass spectrometry (FIA-MS/MS), which enables the detection of charged species (i.e., amino acids, acyl-carnitines, organic compounds) on dried blood spot (DBS) without any prior chromatographic separation[12]. However, it is known that some metabolites quantified through the NBS platform are isobars and share the same mass-to-charge ratios (m/z). Therefore, they cannot be distinguished as they are, unless a fair derivatization takes place. The early screening programs used to imply derivatizing steps during sample preparation; however, these strategies were associated with many drawbacks, including the handling of hazardous reagents and long procedures, that imposed the shift towards non-derivatization techniques[13]. Second-tier tests have been massively implemented to support the need to separate analytes, thus facilitating further separation of ions by MS, often involving liquid chromatography[14,15]. Precisely, such need arises for some isobaric acyl-carnitines metabolically linked to those IEMs associated with suppression of ketogenesis: the m/z sharing is applied for C3DC and C4OH – biomarkers for malonic aciduria and SCHADD, respectively; methylmalonyl-carnitine (C4DC) and C5OH – the former possibly related to methylmalonyl-CoA epimerase deficiency[16], the latter to 3-methylcrotonylglycinuria, multiple carboxylase deficiencies, 3-methylglutaconic aciduria type I or 3-hydroxy-3-methylglutaric aciduria, respectively; C5DC and 3-hydroxyhexanoyl-carnitine (C6OH) – biomarkers for GA-1 and possible ketosis, respectively (Figure 3).

Considering that each couple of the isobaric acyl-carnitines listed above is acquired as a sum of signals at the first screening (C3DC\C4OH, C4DC\C5OH, C5DC\C6OH), we have developed a second-tier test unexpectedly based on FIA-MS/MS, thanks to a simple and fast derivatization step of the different number of carboxylic groups available on each acyl-carnitine moiety. Figure 4 shows the decisional workflow that our NBS lab is currently using to better understand the alterations of such isobaric acyl-carnitines.

MATERIAL AND METHODS

Standards and QCs preparation

Internal standards (ISs) from the NeoBase™ 2 Non-derivatized MSMS kit (Revvity, ex PerkinElmer, Turku, Finland) were reconstituted with 1.4 mL of the NeoBase 2 extraction solution, thoroughly mixed for 60 min, and sonicated in an ultrasonic bath for 3 min. Low and high QCs were provided by the kit itself and contained only C5DC among the analytes of our interest. For the isobaric couples C3DC/C4OH, C4DC/C5OH and C5DC/C6OH, the corresponding ISs were $^{2}H_{3}$-C4, $^{2}H_{9}$-C5 and $^{2}H_{6}$-C5DC, respectively. The mean concentrations of the ISs over the used kits were 1.46, 1.53, and 1.47 µM for $^{2}H_{3}$-C4, $^{2}H_{9}$-C5, and $^{2}H_{6}$-C5DC, respectively.

Sample extraction

The same protocol used daily in our NBS laboratory for the sample preparation of DBS specimens was slightly modified to perform the derivatization of the isobaric species[12]. After the extraction of the metabolites from a 3.2 mm diameter disk, clear supernatants were dried using a Savant SpeedVac® system from Thermo Scientific (Waltham, MA, USA).

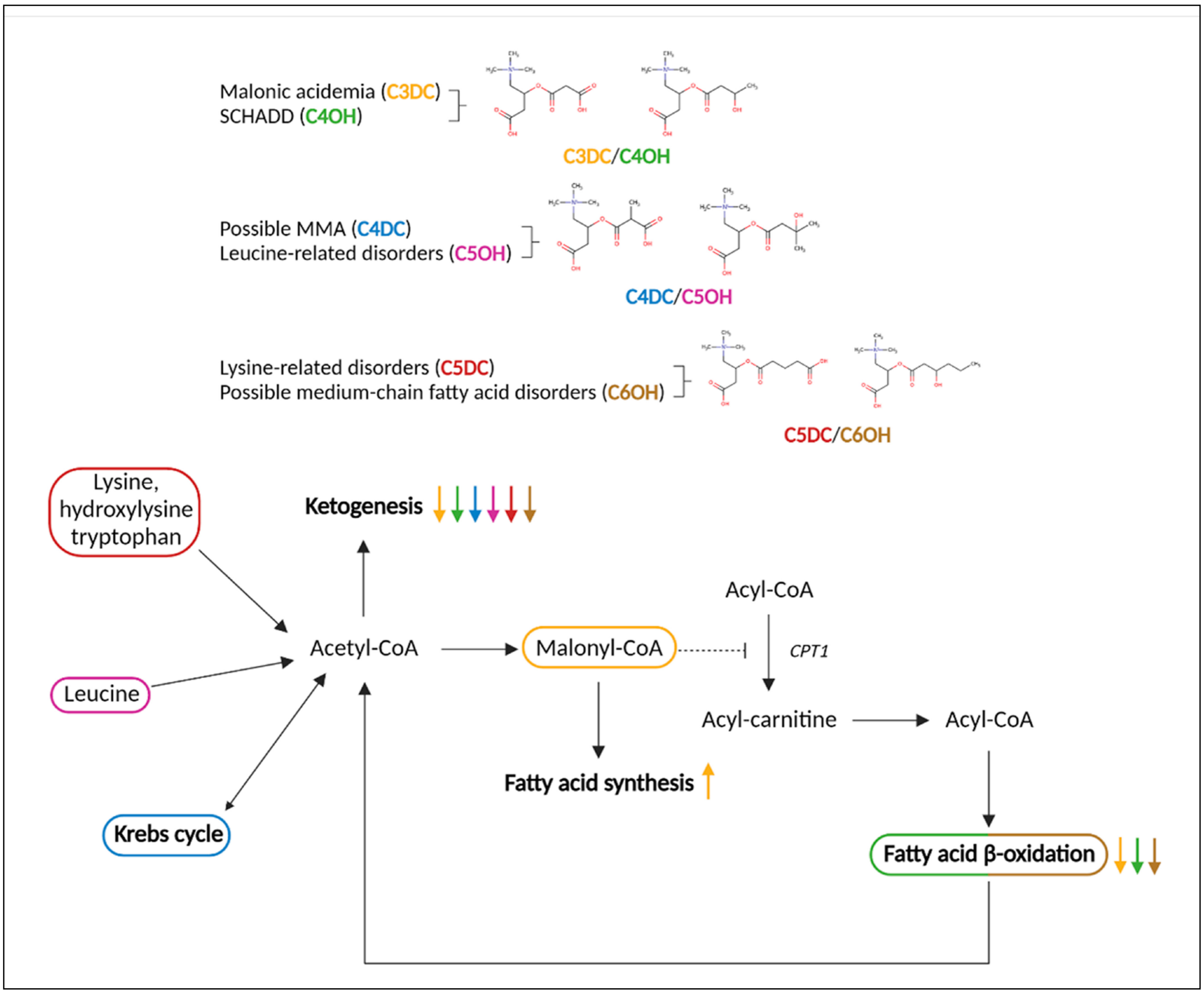

Figure 3. Isobaric couples of acyl-carnitines associated with their disorders. For each disease, the affected metabolic character has been highlighted with colors, as well as the downstream effect (yellow for malonic acidemia, green for SCHADD, blue for organic acidemia, violet for Leu-related disorders, red for Lys/Hyl/Trp-related disorders, brown for fatty acid disorders). Ketogenesis is the most impaired function in such disorders. Image created with BioRender.com.

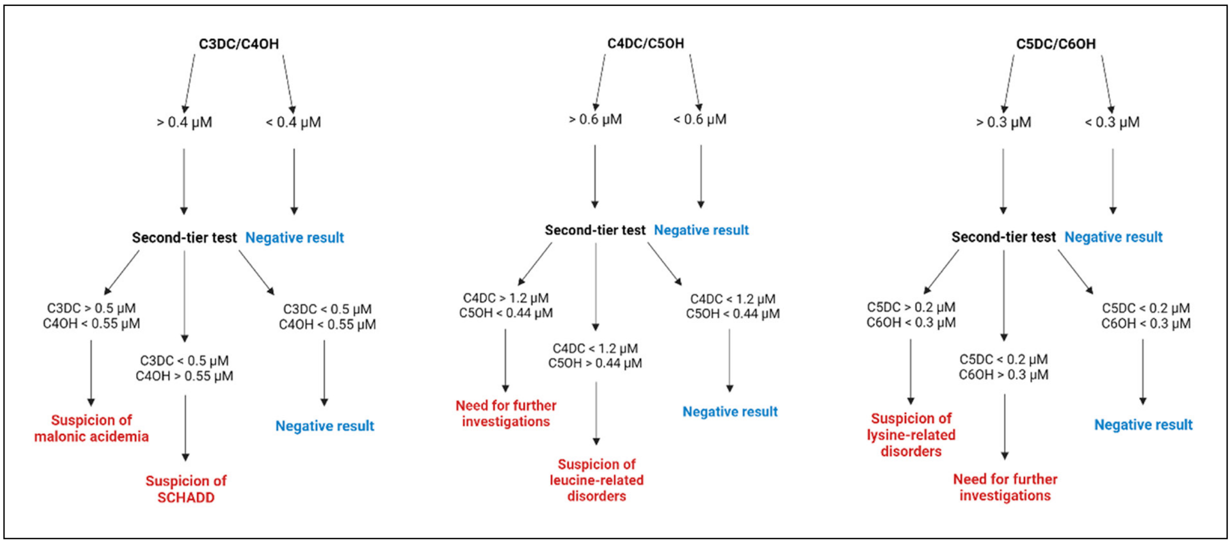

Figure 4. Decisional workflow to investigate the alterations of the isobaric couples of acyl-carnitines. Image created with BioRender.com.

Then, the residues were reconstituted with 100 µL of 3N HCl in n-butanol (Sigma-Aldrich, St. Louis, MO, USA) to derivatize the analytes into their respective butyl esters, following an incubation at 60°C for 20 min. The derivatized specimens were dried over again and reconstituted with 100 µL of Flow-Solvent solution provided by the NeoBase™ 2 kit.

FIA-MS/MS analysis

Specimens underwent FIA-MS/MS analysis through the RenataDX™ Screening system coupled to a Xevo™ TQD IVD mass spectrometer (Waters™ Corporation, Milford, MA, USA), operating in ESI+ by MRM acquisition. Ten µL of specimens were directly injected in a run time of 1.1 min, injection-to-injection. The mobile phase consisted of the Neo MSMS Flow Solvent provided by the kit. The flow rate followed the manufacturer's instructions, as in Table 1. The parameters referring to MRM functions for detecting the derivatized compounds are shown in detail in Table 2. Data were processed using MassLynx™ V4.2 and NeoLynx software (Waters™ Corporation, Milford, MA, USA).

Table 1. Flow rate used to inject the derivatized compounds.

Time (min)	Flow rate (mL/min)	Curve
0	0.150	Initial
0.17	0.010	11
0.98	0.600	11
1.08	0.150	11

Table 2. MRM parameters for C3DC di-butyl ester, C4OH mono-butyl ester and their correspondent ISs ($^2H_3$-C4 mono-butyl ester); C4DC di-butyl ester, C5OH mono-butyl ester a nd their correspondent ISs ($^2H_9$-C5 mono-butyl ester); C5DC di-butyl ester, C6OH mono-butyl ester and their correspondent ISs ($^2H_6$-C5DC di-butyl ester).

MRM Function	Transitions *(m/z)*	Compounds	Dwell (s)	Cone (V)	Collision energy (eV)
	360.0>85.0	C3DC 2but			
	304.0>85.0	C4OH but		36	18
	291.0>85.0	$^2H_3$-C4 but			
	374.0>85.0	C4DC 2but			
1	318.0>85.0	C5OH but	0.025	38	20
	311.0>85.0	$^2H_9$-C5 but			
	388.0>85.0	C5DC 2but			
	332.0>85.0	C6OH but		40	24
	394.0>85.0	$^2H_6$-C5DC 2but			

RESULTS

Development, setup, and validation of the method

The separation of the isobaric species was successfully achieved through a Fischer esterification mechanism that enabled the derivatization of the carboxylic groups in the acyl-carnitine moiety. Since each couple includes an acyl-carnitine with two carboxylic groups and another acyl-carnitine containing a single carboxylic group, we exploited the different availability of these reactive groups to be differentially

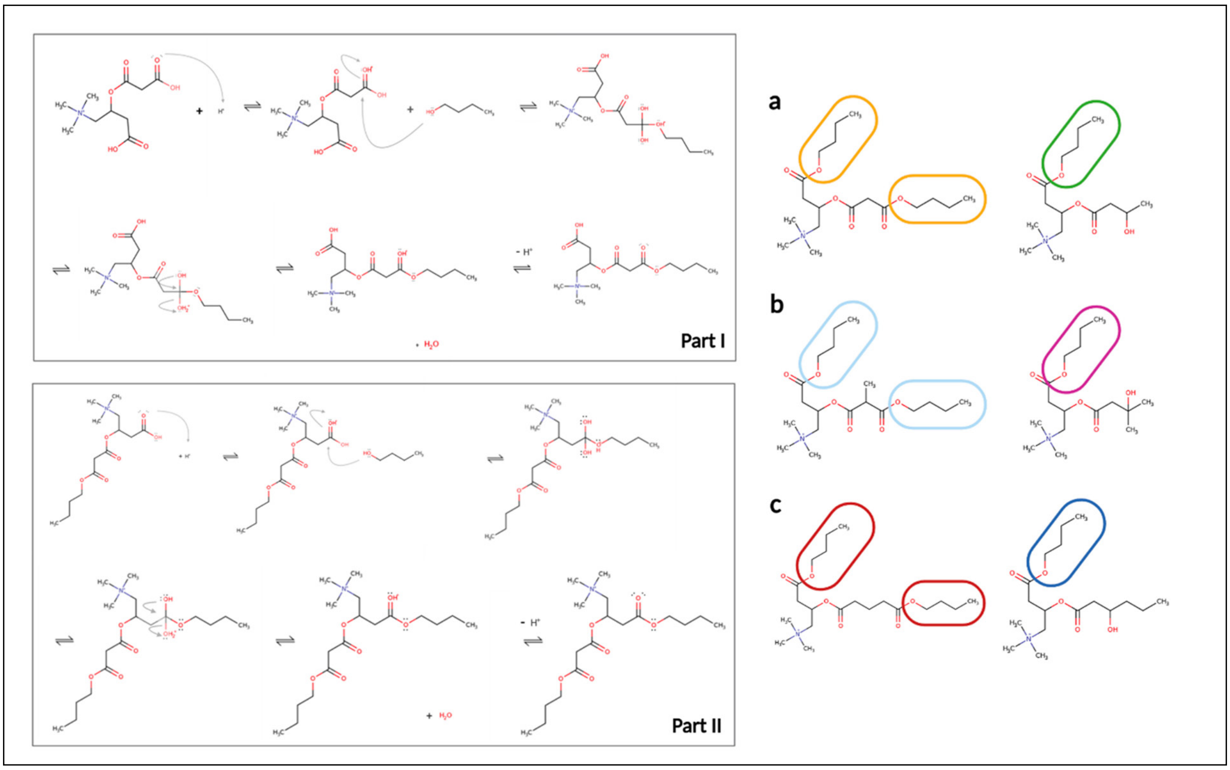

Figure 5. The molecular mechanism of Fischer esterification is illustrated on C3DC as exhaustive example, being one of the acyl-carnitines displaying two carboxylic groups: the reaction requires protonation of the carboxylic group to favor the nucleophilic addition of the alcohol, followed by the collapse of the tetrahedral intermediate to form a single butyl ester (part I). If present, the environmental reaction with low pH drives the derivatization of the second carboxylic group (part II). In panels a, b, and c, the resulting ester groups for each acyl-carnitine are highlighted to underline the structural difference between the isobaric species from the same couple after derivatization (C3DC/C4OH, C4DC/C5OH, C5DC/C6OH, respectively). Image created with BioRender.com.

derivatized. In this way, the esters resulting from the reaction with n-butanol necessarily have unique m/z values that allow the separation of the butyl esters originally acquired as the sum of signals at the first screening. The reaction mechanism and the resulting products with their structural differences are illustrated in Figure 5: precisely, the Fischer esterification is explained using C3DC as exhaustive example with two carboxylic groups to be derivatized (part I and II), but the same molecular mechanism refers to all the dicarboxylic species (C4DC and C5DC), while the hydroxylated acyl-carnitines (C4OH, C5OH and C6OH) undergo Fischer esterification as depicted in the part I. Such an approach enabled a fast differentiation between the isobaric couples without the need for chromatographic separation prior to MS analysis. Therefore, the developed second-tier test stands among those analytical approaches that elegantly meet the requirements of automaticity and simplicity through FIA-MS/MS systems.

The degree of agreement among individual tests was established on DBS by analyzing low and high QC levels using replicates from 6 non-consecutive days. Given that the QC levels provided by the NeoBase™ 2 Non-derivatized MSMS kit contained only C5DC, the precision assessment was carried out solely on this analyte. Specifically, C5DC met low and high QC levels requirements, with coefficient of variation (CV) values lower than 15%. Likewise, accuracy was assessed by comparing the measured values of C5DC from the same batches with the concentrations reported in the kit datasheet, referring to 0.35 and 1.7 µM for low and high QC levels, respectively. All data are summarized in Table 3.

NBS and second-tier clinical application in a neonatal case

The application of the FIA-MS/MS method is based on clinical demand, when alterations of the isobaric acyl-carnitine couples at the first screening occur. We observed altered levels of the isobaric C4DC/C5OH couple in a newborn when first tested at the NBS, our second-tier test was carried on accordingly, reveal-

Table 3. Precision and accuracy for C5DC at low and high QC levels through CV and bias values expressed as percentages.

C5DC	Precision		Accuracy	
	% CV	SD	% bias	SD
Low QC	12.78	0.05	6.46	6.53
High QC	7.68	0.15	12.76	8.66

Abbreviations - CV: coefficient of variation; SD: standard deviation.

ing that the alteration was due to C5OH overload rather than C4DC. These observations led to the recognition of a suspected IEM linked to altered metabolism of Leu, thus driving the differential diagnostic path in the attempt to understand what the underlying disease was, among 3-methylcrotonylglycinuria, multiple carboxylase deficiencies, 3-methylglutaconic aciduria type I, and 3-hydroxy-3-methylglutaric aciduria. As depicted from the comparison with a healthy newborn in Figure 6 and Table 4, the second-tier test provided C5OH values exceeding the cut-off, with a mean concentration of 3.45 µM despite the reference value of 0.44 µM. All the other analytes were within the established cut-offs (0.015 µM for C3DC – cut-off 0.5 µM; 0.054 µM for C4OH – cut-off: 0.55 µM; 0.36 µM for C4DC – cut-off: 1.2 µM; 0.041 µM for C5DC – cut-off: 0.2 µM; 0.031 µM for C6OH – cut-off: 0.3 µM). The identification of such alteration led to the prompt transfer of the newborn to the intensive care unit to prevent the life-threatening sequelae resulting from Leu's impaired metabolism. Nevertheless, the lack of specificity of C5OH needed further confirmatory tests to support the differential diagnosis among the heterogeneous group of disorders associated with high levels of the aforementioned biomarker. Lastly, these in-depth analyses were able to provide a differential diagnosis of 3-MCC deficiency (synonym 3-methylcrotonylglycinuria) due to a heterozygous mutation in the *MCCC2* gene that encodes for MCCase subunit beta. This enzyme catalyzes the fourth step in the Leu catabolic pathway (Figure 2, brown enzymatic deficiency). The definitive diagnosis based on genetic testing provided the exact cause of the observed alteration of C5OH, corroborating the usefulness of such second-tier test as a strategy to support the differential diagnosis in the clinical path.

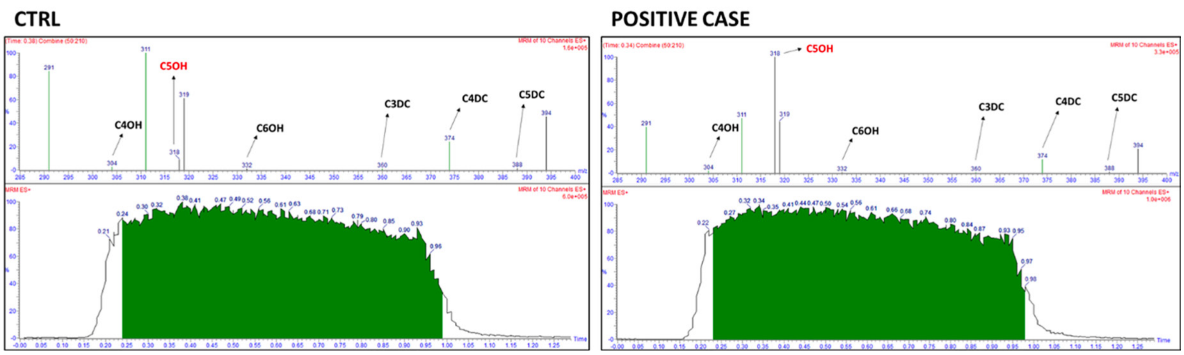

Figure 6. Spectra extrapolated from flow chromatograms after integration in the scanning range 50-210 from the healthy newborn (on the left - CTRL) and the affected newborn with an altered C5OH value.

DISCUSSION

For many years, MS has emerged as a promising tool for clinical analyses, research, and routine laboratory procedures, of which NBS is a noteworthy example[15]. Since NBS programs have greatly increased the number of diagnosed IEMs, the need arises to offer simple and routine second-tier tests that can reduce the number of false positives in case of clinical suspicions and help interpret the first screening results[16]. As previously described, countless metabolic pathways are altered in many heterogenous IEMs, whose unspecific biomarker can be detected at the NBS level, as in the case of isobaric acyl-carni-

Table 4. Measured levels of C3DC, C4OH, C4DC, C5OH, C5DC, C6OH by second-tier testing on DBS from a healthy newborn and the patient diagnosed with 3-methylcrotonylglycinuria (whose C5DC value has been highlighted to underline the excess from the reference limit).

Analyte	Healthy newborn	Positive newborn
C3DC (cutoff: 0.50 μM)	0.018	0.015
C4OH (cutoff: 0.55 μM)	0.042	0.054
C4DC (cutoff: 1.20 μM)	0.370	0.360
C5OH (cutoff: 0.440 μM)	0.137	**3.450**
C5DC (cutoff: 0.20 μM)	0.040	0.041
C6OH (cutoff: 0.30 μM)	0.027	0.031

tines (C3DC/C4OH, C4DC/C5OH, C5DC/C6OH), usually acquired as a sum of signals at the first screening test, unless a derivatizing step occurs. In this complex scenario, exploiting reminiscences of organic chemistry, we have developed a rapid FIA-MS/MS method that allows the simultaneous distinction of the aforementioned isobaric acyl-carnitine couples. It has proved to be associated with good values of reproducibility and accuracy, as well as an uncommon analytical strategy as second-tier testing to be used instead of chromatographic separation. N-butanol is implied to promote differential esterification on the available carboxylic groups exposed by acyl-carnitine moieties in each isobaric couple. Therefore, the different derivatized species will carry unique m/z values that will enable their separation by MS. A further advantage from derivatization is associated with the overall improvement of electrospray ionization (ESI) when acyl-carnitines are in their butyl ester forms[17]. From a clinical point of view, the availability of this second-tier test supports the differential diagnosis among IEMs that differ in the degree of metabolic decompensation via suppressed ketogenesis. Indeed, malonic aciduria and disorders referring to the metabolism of ketogenic amino acids (i.e., Leu, Lys, Hyl and Trp) can be life-threatening from the earliest days of life, while SCHADD is often asymptomatic in affected patients; additionally, other alterations (C4DC and C6OH) may be observed for nonspecific reasons that need to be further investigated. Overall, this second-tier test can justify the origin of the alterations detected at the first screening by indicating the species responsible for the fluctuation in each isobaric couple, thus leading to specific diagnosis and management of the affected newborns. Here, we have presented an application of our FIA-MS/MS method as a second-tier testing strategy to better understand the alterations highlighted by the first screening. The newborn showed high levels of C4DC\C5OH, which imposed the need for diagnostic interpreting because of the possible involvement of impaired Leu metabolism. Data from second-tier testing revealed the increase in C5OH, driving the differential diagnosis among IEMs, possibly revolving around Leu. Subsequent confirmatory biochemical and genetic tests provided the definitive outcome of the diagnostic process in the form of 3-MCC (or 3-methylcrotonylglycinuria) with a heterozygous mutation in the *MCCC2* gene, leading definitively to the exclusion of all the other conditions considered. Altogether, the case described here certifies the clinical relevance of strategies like the one proposed here, which relies on a fast, robust, and reproducible assay providing invaluable aid in the diagnostic process while respecting the same timing as NBS. Although this manuscript is a methodological work, it is important to underline that the described neonatal case supports the clinical relevance of our strategy, as confirmed by the definitive diagnosis that was made possible thanks to the coupling of second-tier and genetic testing. The use of FIA allows automaticity and easy handling without requiring the use of demanding chromatographic techniques. Accordingly, the overall NBS system can benefit from it, especially since our strategy does not impose any additional costs on the analyses other than purchasing the derivatizing agent.

CONCLUSIONS

In conclusion, we strongly recommend adopting our second-tier test for separating isobaric acyl-carnitine couples, as it has been proven analytically and clinically useful.

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