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Analysis of a second-tier test panel in dried blood spot samples using liquid chromatography–tandem mass spectrometry in Catalonia's newborn screening programme

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Plain language summary: Expanded neonatal screening is performed using tandem mass spectrometry; similarly to other methodologies, this method may yield false positive results. To address this issue, more specific assays, namely second-tier tests (2TTs), have been developed. To date, several independent 2TT methods have been used depending on the primary altered marker. We present a panel of several 2TT metabolites analysed simultaneously without derivatisation in a single process, thereby optimising resources such as materials, instruments, and personnel. The 2TT metabolites evaluated in this study, namely organic acids, acylcarnitine and acylglycine isomers, homocysteine, and orotic acid, showed 100 % sensitivity and allowed differentiation among all organic acidurias included in our newborn screening programme (except for glutaric aciduria type 1). In addition, this panel helped detect defects in mitochondrial fatty acid β-oxidation and the urea cycle. By implementing this method in our newborn screening programme, we could reduce the rate of false positive results and the need for collecting new samples.

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Abstract

Objectives: Acylcarnitine and amino acid analyses of dried blood spot (DBS) samples using tandem mass spectrometry in newborn screening (NBS) programmes can generate false positive (FP) results. Therefore, implementation of second-tier tests (2TTs) using DBS samples has become increasingly important to avoid FPs. The most widely used 2TT metabolites include methylmalonic acid, 3-hydroxypropionic acid, methylcitric acid, and homocysteine.

Methods: We simultaneously measured 44 underivatised metabolites, including organic acids, acylglycine and acylcarnitine isomers, homocysteine, and orotic acid, in DBS samples using tandem mass spectrometry. To validate this method, we analysed samples from 147 healthy newborns, 160 patients with genetic disorders diagnosed via NBS, 20 patients with acquired vitamin B12 deficiency, 10 newborns receiving antibiotic treatment, and nine external quality control samples.

Results: The validation study revealed that 31 metabolites showed good analytical performance. Furthermore, this method detected key metabolites for all diseases associated with increased levels of the following acylcarnitines: C3, C4, C5, C4DC\C5OH, and C5DC. The sensitivity of this method to detect all diseases was 100 %, and the specificity was 74–99 %, except for glutaric aciduria type 1. This method can also be used to diagnose mitochondrial fatty acid β-oxidation disorders (FAODs) and urea cycle defects (UCDs).

Conclusions: We have described a 2TT panel of 31 metabolites in DBS samples based on an easy and rapid method without derivatisation. Its implementation allowed us to distinguish between different organic acidurias, some FAODs, and UCDs. This new strategy has increased the efficiency of our NBS programme by reducing FP and false negative results, second sample requests, and the time required for diagnosis.

Keywords: second-tier test; newborn screening; organic acids; acylcarnitines; acylglycines; mass spectrometry

Introduction

In the 1990s, with the introduction of tandem mass spectrometry (MS/MS), there was a marked expansion of newborn screening (NBS) programmes. MS/MS enables simultaneous measurement of several metabolites and consequent detection of several diseases in the same sample through a single analysis [1, 2]. Organic acidurias, amino acid disorders, urea cycle defects (UCDs), and mitochondrial fatty acid β-oxidation disorders (FAODs) detected based on acylcarnitine and amino acid (primary markers) analyses of dried blood spot (DBS) samples using MS/MS have been included in several NBS programmes. Similarly to other analytical laboratory techniques, MS/MS analyses of DBS samples may yield false positive (FP) results [3, 4]. In addition, some metabolites share the same mass-to-charge ratio, which makes it difficult to differentiate among them. Although acylcarnitine isomers can be distinguished using derivatisation methods, other metabolites cannot be distinguished using derivatisation or underivatised methods. To address these issues, second-tier tests (2TTs) have been developed; they are conducted using the initial DBS samples. In these tests, the same primary marker is analysed in greater detail by using a more specific methodology or by considering more specific metabolites. This strategy enables the adjustment of the cut-off values for primary markers, thereby reducing the possibility of false negative (FN) results. Furthermore, this approach could increase the diagnostic sensitivity and specificity by reducing the number of FPs and, consequently, increasing the positive predictive value (PPV) of the assay. This approach decreases the need for second sample requests, which improves the response time and reduces anxiety in the patient's family.

Methylmalonic acid (MMA), 3-hydoxypropionic acid, methylcitric acid (MCA), and homocysteine (Hcys) are the most common 2TT metabolites analysed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) in DBS samples. These metabolites are associated with a change in the primary markers propionylcarnitine (C3) and/or methionine (Met) in disorders such as methylmalonic acidurias (MMAs), propionic aciduria (PA), and homocystinurias (HCYs) [3–19].

Other 2TT methodologies using DBS samples have also been reported, including C5-acylcarnitine isomers (isovalerylcarnitine [IVC]\2-methylbutyrylcarnitine [2MBC]\pivaloylcarnitine [PVC]) to differentiate isovaleric acidaemia (IVA) and 2-methylbutyryl-CoA dehydrogenase deficiency (2-MBDD) from interference by pivalic acid [15, 17, 18, 20–25], and isovalerylglycine (IVG) to detect IVA [5]. Similarly, 2TT analysis of the isomeric forms of C4-acylcarnitine (butyrylcarnitine [BC]\isobutyrylcarnitine [IBC]) [20], ethylmalonic acid (EMA) [17, 26], isobutyrylglycine (IBG), and butyrylglycine (BG) [18] has been implemented for the differential diagnosis of short-chain acyl-CoA dehydrogenase deficiency (SCADD) and isobutyryl-CoA dehydrogenase deficiency (IBCDD). Recently, 2TT strategies to detect alterations in C5OH-isomers (3-hydroxyisovalerylcarnitine\2-methyl-3-hydroxy-butirylcarnitine) and glutarylcarnitine (C5DC) have been described [15, 27]. Depending on the primary marker that is altered, distinct 2TT methods have been employed through separate analyses.

In 2015, we implemented an LC–MS/MS method in Catalonia's NBS programme for 2TT analysis of MMA, Hcys, and MCA in initial DBS samples. We conducted this analysis for samples showing elevated levels of C3, C3/C2, C3/Met, and C17, and for samples showing increased or decreased Met levels [12]. When we noted alterations in the concentrations of primary markers associated with other organic acidurias, we used dried urine spot samples to analyse organic acids via gas chromatography–MS. This strategy facilitated the differential diagnosis of several organic acidurias; however, because this approach requires a second sample, it delays the detection time and increases the anxiety of the patient's family. To overcome these issues, we established a methodology that enables simultaneous analysis of a 2TT panel in initial DBS samples, including organic acids, acylcarnitine and acylglycine isomers, Hcys, and orotic acid. There have only been two studies on the use of a 2TT panel for DBS samples, both published in 2023 [18, 19]. Compared with those studies, our DBS panel includes additional metabolites. Our panel is similar to the one included in the NBS programme of Galicia (a region of Spain), although that panel was developed exclusively for dried urine spot samples [28].

Materials and methods

The details on the chemicals, reagents, patients, and controls are described in the Supplementary Material.

Sample preparation

A 4.7-mm spot was punched from each DBS sample and transferred to an Ostro 96-well plate (Waters, Milford, MA, USA). Then, 250 µL of a solution containing a mixture of isotopically labelled standards as an internal standard (IS) (Table 1) and DL-dithiothreitol (42 mmol/L) in Milli-Q water/methanol (90:10) with 0.4 % formic acid was added to each well. The plate was covered, placed on a shaker for 1 h at 1,350 rpm and room temperature, and filtered using Otto SPEcialist-Positive Pressure

Manifold (Waters, Milford, MA, USA) for 5 min. The eluate was transferred to a clean 96-well filter plate, centrifuged for 40 min at 4,000 rpm and room temperature, and then transferred to a clean 96-well round-bottom plate for injection. An 11-point calibration curve prepared with spiked concentrations of each metabolite in DBS samples was used for quantification (Table 1). Spiked DBS samples at three concentrations for each metabolite were used as internal quality control (QC) sample in each batch (Table 1).

Ultra-high pressure LC–MS/MS (UPLC–MS/MS) conditions

Organic acids, acylglycine and acylcarnitine isomers, Hcys, and orotic acid were analysed using UPLC–MS/MS (Acquity UPLC I-Class-Xevo TQS, Waters). Chromatographic separation was performed on an Acquity BEH C18 column (2.1 × 100 mm, 1.7 µm, Waters). The mobile phase for isocratic elution at 60 °C consisted of 95 % mobile phase A (0.4 % formic acid in Milli-Q water) and 5 % mobile phase B (0.4 % formic acid in methanol) at a flow rate of 0.5 mL/min for 8 min. The injection volume was 5 µL.

Detection was performed in the multiple reaction monitoring (MRM) mode; the positive ion mode was used for Hcys, Hcys-$_{d8}$, and acylcarnitines, whereas the negative ion mode was used for organic acids and acylglycines. The MRM transitions for each metabolite are presented in Table 2. Nitrogen and argon were used as nebulising and collision gases, respectively. The dwell time for each transition was 60 ms, and the inter-channel delay time was 20 ms. The following instrumental settings were used: source temperature, 450 °C; desolvation temperature, 1,200 °C; and capillary voltage, 0.4 kV. Data acquisition and analysis were performed using the MassLynxTM (V4.1) software. Compounds were quantified relative to their corresponding IS using an external calibration curve.

Method validation

The method was validated for linearity, the limit of detection, the limit of quantification, imprecision, and recovery. Within-day imprecision, between-day imprecision, and recovery were determined by analysing the internal QC samples at three concentrations.

Table 1: Calibrators, internal quality controls, and, internal standard used for each metabolite.

	Calibrators range	Low QC	Medium QC	High QC	Internal standard (IS)
		(µmol/L)			
Homocysteine	0.2–100	3.9	31.2	100	Homocysteine-3,3,3′,4,4,4′,4′-$_{d8}$
Methylmalonic acid	0.6–318	18.45	61.52	153.8	Methylmalonic-$_{d3}$ acid
Methylcitric acid	0.2–100	5.76	19.2	48	Methylcitric-$_{d3}$ acid
Propionylglycine	0.03–15.9	0.92	3.07	7.69	Propionylglycine-[3,3,3-$_{d3}$]
Glutaric acid	0.3–79.5	4.61	15.38	38.45	Glutaric-$_{d4}$ acid
3-Hydroxyglutaric acid	0.3–79.5	4.61	15.38	38.45	3-Hydroxy-1,5-pentanedioic-2,2,3,4,4-$_{d5}$ acid
2-Hydroxyglutaric acid	0.3–79.5	4.61	15.38	38.45	3-Hydroxy-1,5-pentanedioic-2,2,3,4,4-$_{d5}$ acid
Ethylmalonic acid	0.3–79.5	4.61	15.38	38.45	Glutaric-$_{d4}$ acid
Isobutyrylglycine\butyrylglycine	0.03–15.9	0.92	3.07	7.69	Isobutyrylglycine-$_{d7}$
Isobutyryl-L-carnitine	0.01–5.2	0.3	1	2.5	Butyrylcarnitine-$^{2}H_3$
Butyrylcarnitine	0.01–5.2	0.3	1	2.5	Butyrylcarnitine-$^{2}H_3$
Isovalerylcarnitine	0.01–5.2	0.3	1	2.5	Isovalerylcarnitine-$^{2}H_9$
2-Methylbutyrylcarnitine	0.01–5.2	0.3	1	2.5	Isovalerylcarnitine-$^{2}H_9$
Pivaloylcarnitine	0.01–5.2	0.3	1	2.5	Isovalerylcarnitine-$^{2}H_9$
Isovalerylglycine	0.3–79.5	4.61	15.38	38.45	Isovalerylglycine-$_{d9}$
2-Methylbutyrylglycine	0.03–15.9	0.92	3.07	7.69	2-Methylbutyrylglycine-$_{d9}$
3-Methylcrotonylglycine\tiglylglycine	0.03–15.9	0.92	3.07	7.69	3-Methylcrotonylglycine-$^{13}C_2$
3-Methylcrotonylcarnitine	0.01–5.2	0.3	1	2.5	Isovalerylcarnitine-$^{2}H_9$
Tiglylcarnitine	0.01–5.2	0.3	1	2.5	Isovalerylcarnitine-$^{2}H_9$
3-Hydroxyisovaleric acid	0.3–79.5	4.61	15.38	38.45	3-Hydroxyisovaleric-$_{d6}$ acid
3-Hydroxy-2-methylbutyric acid	0.2–100	5.76	19.2	48	3-Hydroxyisovaleric-$_{d6}$ acid
3-Hydroxy-3-methylglutaric acid	0.2–100	5.76	19.2	48	3-Hydroxy-1,5-pentanedioic-2,2,3,4,4-$_{d5}$ acid
3-Methylglutaric acid	0.3–79.5	4.61	15.38	38.45	3-Hydroxyisovaleric-$_{d6}$ acid
Adipic acid	0.3–79.5	4.61	15.38	38.45	3-Hydroxyisovaleric-$_{d6}$ acid
Suberylglycine	0.03–15.9	0.92	3.07	7.69	Isovalerylglycine-$_{d9}$
Orotic acid	0.3–79.5	4.61	15.38	38.45	3-Hydroxy-1,5-pentanedioic-2,2,3,4,4-$_{d5}$ acid
4-Hydroxyphenyllactic acid	0.3–79.5	4.61	15.38	38.45	3-Hydroxyisovaleric-$_{d6}$ acid
Mevalonic acid	0.3–79.5	4.61	31.2	100	3-Hydroxy-1,5-pentanedioic-2,2,3,4,4-$_{d5}$ acid
N-acetyl-L-aspartic acid	0.3–79.5	4.61	61.52	153.8	3-Hydroxy-1,5-pentanedioic-2,2,3,4,4-$_{d5}$ acid

The concentration of the IS used were 0.48 µmol/L for methylmalonic-$_{d3}$ acid; 0.24 µmol/L for homocysteine-3,3,3′,4,4,4′,4′-$_{d8}$, methylcitric-$_{d3}$ acid, glutaric-$_{d4}$ acid, 3-hydroxy-1,5-pentanedioic-2,2,3,4,4-$_{d5}$ acid, and 3-hydroxyisovaleric-$_{d6}$ acid; 0.18 µmol/L for butyrylcarnitine-$^{2}H_3$, isovalerylcarnitine-$^{2}H_9$ and isovalerylglycine-$_{d9}$; 0.06 µmol/L for propionylglycine-[3,3,3-$_{d3}$], isobutyrylglycine-$_{d7}$, 3-methylcrotonylglycine-$^{13}C_2$ and 2-methylbutyrylglycine-$_{d9}$. QC, quality control.

Table 2: Optimized MS parameters in the MRM mode and retention times.

	Retention time	Monitored transition	CV, V	CE, V
Homocysteine	0.52	136>90	30	10
Homocysteine-3,3,3′,4,4,4′,4′-$_{d8}$	0.52	140>94	30	10
Orotic acid	0.54	155>111	−13	−13
3-Hydroxyglutaric acid	0.61	147>85	−20	−8
2-Hydroxyglutaric acid	0.61	147>129	−20	−8
3-Hydroxy-1,5-pentanedioic-2,2,3,4,4-$_{d5}$ acid	0.61	152>89	−20	−8
N-acetyl-L-aspartic acid	0.6	174>88	−17	−15
Propionylglycine	0.8	130>74	−30	−13
Propionylglycine-[3,3,3-$_{d3}$]	0.8	133>74	−30	−13
Mevalonic acid	0.82	147>59	−25	−10
3-Hydroxy-3-methylglutaric acid	0.84	161>99	−13	−14
Methylmalonic acid	0.88	117>73	−30	−10
Methylmalonic-$_{d3}$ acid	0.88	120>76	−30	−10
Methylcitric acid	0.85	205>125	−30	−10
Methylcitric-$_{d3}$ acid	0.85	208>128	−30	−10
Glutaric acid	1	131>87	−20	−8
Glutaric-$_{d4}$ acid	1	135>91	−20	−8
Isobutyrylglycine\butyrylglycine	1.3	144>74	−45	−15
Isobutyrylglycine-$_{d7}$	1.3	151>75	−20	−25
3-Hydroxyisovaleric acid	1.39	117>59	−21	−14
3-Hydroxyisovaleric-$_{d6}$ acid	1.39	123>59	−30	−11
Butyrylcarnitine-$^{2}H_3$	1.68	235.2>85	24	20
Isobutyryl-L-carnitine	1.5	232.3>85	24	20
Ethylmalonic acid	1.73	131>87	−20	−8
Butyrylcarnitine	1.68	232.3>85	24	20
Adipic acid	2.17	145>83	−10	−15
3-Hydroxy-2-methylbutyric acid	1.63	117>73	−15	−15
4-Hydroxyphenyllactic acid	2.12	181>163	−30	−16
3-Methylglutaric acid	2.17	145>101	−22	−10
Pivaloylcarnitine	3.53	246.3>85	24	22
3-Methylcrotonylglycine\tiglylglycine	2.77	156>74	−45	−15
3-Methylcrotonylglycine-$^{13}C_2$	2.77	158.15>76	−20	−15
2-Methylbutyrylglycine	2.8	158>74	−50	−15
2-Methylbutyrylglycine-$_{d9}$	2.8	167>75	−30	−25
Tiglylcarnitine	3.2	244.2>85	28	22
Isovalerylcarnitine	4.26	246.3>85	24	22
Isovalerylglycine	3.09	158>74	−50	−15
Isovalerylcarnitine-$^{2}H_9$	4.26	255.2>85	24	22
Isovalerylglycine-$_{d9}$	3.09	167>75	−20	−15
Suberylglycine	6.69	230>74	−40	−25
2-Methylbutyrylcarnitine	3.99	246.3>85	24	22
3-Methylcrotonylcarnitine	3.58	244.2>85	28	22

CE, collision energy; CV, conus voltage; V, voltage.

Statistical analysis

Sensitivity, specificity, and the range of baseline and pathological values were analysed with Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). Sensitivity and specificity were calculated by analysing 160 DBS samples from patients with distinct diagnoses as well as external QC samples. Positive samples for other diseases that did not share the specific 2TT as the key metabolite were included to calculate specificity. The cut-off values were set at the <97.5th percentile.

Results

2TT method validation

Among the initial set of 44 tested metabolites, only 31 showed good analytical performance after the validation study. Hence, we excluded the remaining metabolites (suberic, glycolic, malic, malonic, ketoglutaric, pyroglutamic, 4-hydroxybutyric, fumaric, 3-hydroxybutyric, lactic,

and 3-methylglutaconic [3MGA] acids; thymine; and hexanoylglycine) from the panel because they showed poor performance in the validation study. The results of the method validation are shown in Table 3. Most metabolites exhibited within- and between-day imprecision values that met the acceptability criteria, with imprecision values of <15 % at the high internal QC concentration and <20 % at the low internal QC concentration. Furthermore, the method showed excellent recovery (>90 %) and good linearity (r^2>0.98).

We obtained sufficient resolution for the isomeric forms of C4- and C5-acylcarnitines. In contrast, the isomeric forms of two acylglycines (3-methylcrotononylglycine [3-MCG]\tiglylglycine [TGG]) and IBG\BG could not be completely separated chromatographically.

Simultaneous 2TT method

2TTs related to alterations of the primary markers C3 and Met

When an alteration of the primary markers C3, Met, C3/C2, C3/Met and C17, was noted, we focused on MMA, MCA, Hcys, and PG in the simultaneous 2TT panel (Table 4). The 2TTs for the key metabolites of MMAs, PA, HCYs, and acquired vitamin-B12 deficiency (AVitB12D) exhibited 100 % sensitivity and 92–98 % specificity, including MMA for all types of MMAs, MCA and PG for PA, and Hcys for all types of HCYs. MCA and PG levels can increase in MMAs because they belong to the same metabolic pathway. Furthermore, the Hcys level may be increased in MMAs with homocystinuria; however, the presence of MMA in combination with these metabolites facilitates the differentiation of MMAs from other disorders.

Although the detection of AVitB12D was not a specific target of our NBS panel, we observed an overlap in the levels of the primary markers and specific 2TT metabolites between the acquired and genetic conditions (Table 4). Therefore, a differential diagnosis must be performed after referral to the clinic. Hcys and MMA are the most sensitive biomarkers for the detection of AVitB12D.

2TTs related to alterations of the primary C4DC\C5OH-acylcarnitine isomer markers

Table 5 presents the 2TT results when we evaluated positive samples with known alterations in the C4DC\C5OH-acylcarnitine isomers, which we used as primary markers. Our 2TT panel enabled us to distinguish among all C5OH-related disorders. The combination of 3-hydroxyisovaleric acid (3-IVA), 3-hydroxy-3-methylglutaric acid (3OH3MEGLUT), and 3-methylglutaric acid allowed us to detect 3-hydroxy-3-methylglutaryl-CoA lyase deficiency (HMGCLD) with 100 % sensitivity. The most specific and exclusive metabolite for this disease was 3OH3MEGLUT. There were increased 2-methyl-3-hydroxy-butyric acid and tiglyl-L-carnitine levels in a patient with ketothiolase deficiency (BKTD), with a specificity of 92 and 99 %, respectively. We were unable to include a patient with 17β-hydroxysteroid dehydrogenase type 10 deficiency (HSD10D). However, because 2-methylacetatoacetate acid is not included in our panel, it would not be possible to differentiate between BKTD and HSD10D, as both entities present increased 2-methyl-3-hydroxybutyric and tiglyl-L-carnitine levels. In addition, we observed increased 3-MCG\TGG levels in patients with 3-methylcrotonyl-CoA carboxylase deficiency (3-MCCD). 3-MCG\TGG showed higher sensitivity than 3-methylcrotonylcarnitine for this disease, and an increase in both metabolites alongside an increase in the 3IVA level led to 100 % sensitivity. We were unable to analyse patients with holocarboxylase synthetase or biotinidase deficiency (BTD). However, we evaluated an external QC sample of BTD and found diagnostic concordance (Table 6).

2TTs related to alterations of the primary C5-acylcarnitine isomer markers

Table 7 presents the 2TT results when we evaluated positive samples with the C5-acylcarnitine isomer markers. It is necessary to differentiate between C5-acylcarnitine isomers to accurately discriminate between the related disorders and secondary interference from pivalic acid, which results from antibiotic administration or body cream application. IVC and IVG exhibited 100 % sensitivity and 99 % specificity to detect IVA. Although multiple acyl-CoA dehydrogenase deficiency (MADD) is associated with increased IVC and IVG levels, the levels observed in the patient with IVA were clearly higher than those observed in the patient with MADD. Furthermore, our panel includes additional key markers to detect MADD; they were also elevated in the analysed MADD case.

We noted an isolated increase in the 2MBC and 2-methylbutyrylglycine (2MBG) levels for the 2-MBDD case. We only analysed one patient from our NBS programme who showed a FN result: the level of the primary marker C5 was lower than our cut-off value: 0.37 µmol/L, control values (C.V.)<0.9. The 2TT analysis revealed a slight increase in the 2MBC level.

In addition, to identify FP results related to pivalic acid interference, we included PVC as a 2TT metabolite. We investigated 20 newborns who had slightly elevated C5 levels

Table 3: Validation results of 2TT on DBS by LC-MS/MS.

	LD, µmol/L	LQ, µmol/L	Intra-day assay CV, %			Inter-day assay CV, %			Linearity (r^2)	Recovery, %		
			Low QC	Medium QC	High QC	Low QC	Medium QC	High QC		Low QC	Medium QC	High QC
Homocysteine	0.19	50	8	19	13	33	25	23	0.97	104	93	87
Methylmalonic acid	0.62	2.48	20	13	22	22	15	10	0.99	98	99	108
Methylcitric acid	0.05	0.19	2	6	8	15	16	9	0.99	98	100	104
Propionylglycine	0.008	0.015	19	20	11	22	23	13	0.98	98	99	100
Glutaric acid	4.9	9.93	20	12	10	23	16	11	0.99	106	93	97
3-Hydroxyglutaric acid	2.48	9.93	15	12	11	18	18	11	0.99	119	98	102
2-Hydroxyglutaric acid	2.48	9.93	13	14	13	22	18	13	0.98	147	98	102
Ethylmalonic acid	0.62	4.9	25	9	14	18	18	15	0.99	116	97	104
Isobutyrylglycine\butyrylglycine	0.008	0.06	33	19	22	26	20	20	0.98	88	89	84
Isobutyryl-L-carnitine	0.015	0.12	0	13	9	18	26	12	0.99	100	91	95
Butyrylcarnitine	0.06	0.24	0	9	9	20	15	12	0.99	106	96	99
Isovalerylcarnitine	0.06	0.24	15	9	8	16	15	8	0.99	106	98	101
2-Methylbutyrylcarnitine	0.03	0.12	13	10	8	16	15	8	0.99	111	105	106
Pivaloylcarnitine	0.06	0.24	15	8	8	11	14	8	0.99	100	98	105
Isovalerylglycine	0.31	0.62	13	7	7	15	18	11	0.99	110	102	108
2-Methylbutyrylglycine	0.03	0.06	43	58	43	19	23	17	0.97	123	102	82
3-Methylcrotonylglycine\tiglylglycine	0.015	0.03	26	18	9	17	19	21	0.99	118	105	109
3-Methylcrotonylcarnitine	0.06	0.24	13	9	8	14	17	10	0.99	100	102	108
Tiglylcarnitine	0.03	0.12	13	9	8	15	20	11	0.99	100	108	99
3-Hydroxyisovaleric acid	0.019	0.075	24	12	17	21	22	14	0.95	71	84	99
3-Hydroxy-2-methylbutyric acid	6.25	12.5	22	24	15	24	15	27	0.97	105	81	118
3-Hydroxy-3-methylglutaric acid	0.39	0.78	18	18	11	17	18	16	0.99	154	94	103
3-Methylglutaric acid	4.9	9.93	16	15	16	18	21	19	0.99	99	82	108
Adipic acid	4.9	9.93	31	23	16	26	19	20	0.96	91	80	105
Suberylglycine	0.12	0.24	14	6	11	14	17	12	0.98	113	103	113
Orotic acid	1.24	4.9	8	12	7	15	14	12	0.99	88	79	100
4-Hydroxyphenyllactic acid	0.019	0.0375	79	14	13	18	19	18	0.98	101	85	108
Mevalonic acid	0.62	2.48	17	17	13	23	24	15	0.99	114	91	110
N-acetyl-L-aspartic acid	0.04	1.24	11	13	11	15	18	10	0.99	121	98	104

LD, limit of detection; LQ, limit of quantification; CV, coefficient of variation; QC, quality control.

Table 4: Sensitivity, specificity and pathological values of 2TT related with C3 and methionine-disorders.

			Primary marker	C3				Met	
			Disease	Propionic aciduria (n=9)	Isolated methylmalonic aciduria (n=9)	Combined methylmalonic aciduria with homocystinuria (n=6)	Acquired vitamin B12 deficiency (n=20)	CBS deficiency (n=5)	MTHFR deficiency (n=1)
2TT	MMA	Cut-off (3.1 µmol/L)[a]	SE, %	–	100	100	50	–	–
			SP, %	–			98	–	–
			PV, µmol/L	–	111.7 (4.7–796.2)	84.8 (4.2–335.5)	3.3 (0.6–53.3)	–	–
	HCYS	Cut-off (7 µmol/L)[a]	SE, %	–	–	83	65	100	100
			SP, %	–	–				92
			PV, µmol/L	–	–	61.2 (6.9–288.9)	8.7 (4.9–48.1)	19.2 (8.04–39.5)	106.29
	MCA	Cut-off (0.9 µmol/L)	SE, %	100	89	50	65	–	–
			SP, %				75	–	–
			PV, µmol/L	42.6 (8.4–88.4)	3.3 (0.9–24.6)	9.2 (5.8–16.5)	0.4 (0.1–5.4)	–	–
	PG	Cut-off (0.04 µmol/L)	SE, %	100	56	67	5	–	–
			SP, %				90	–	–
			PV, µmol/L	11.6 (3.9–29.2)	0.1 (0–9.2)	0.35 (0–0.6)	0 (0–0.1)	–	–

Diseases that not shared common 2TT were considered as true negative and false positive for the estimation of specificity. PV are expressed as median (minimum value-maximum value). [a]MMA and HCYS had two levels of cut-off, the first cut-off (MMA: 3.1 µmol/L, HCYS: 7 µmol/L) and second cut-off (MMA: 5 µmol/L, HCYS: 14 µmol/L). A second request sample is performed if result is between first and second cut-off, while case is referred to clinical unit if result is above second cut off. C3, propionyl-carnitine; CBS, cystathionine-beta-synthase; HCYS, homocysteine; MCA, methylcitric acid; Met, methionine; MMA, methylmalonic acid; MTHFR, methylenetetrahydrofolate reductase; PG, propionylglycine; PV, pathological values; SE, sensitivity; SP, specificity; 2TT, second tier-test.

due to antibiotic treatment. We found an increase in PVC levels, showing 80 % sensitivity and 93 % specificity, without any alteration in the other C5-related metabolites.

2TTs related to alterations of the primary C4-acylcarnitine isomer, C5DC, and other markers

Table 7 includes the 2TT results when we evaluated positive samples containing the C4-acylcarnitine isomer, C5DC, and other markers for the detection of IBCDD and SCADD and differential diagnosis of MADD. The key metabolites IBC and IBG\BG, used as 2TT metabolites to detect IBCDD, exhibited 100 and 75 % sensitivity, respectively, with 99 % specificity for IBC. Twenty patients with SCADD showed an increase in EMA, IBG\BG, and BC levels. EMA was the most sensitive marker (90 %); the specificity was 98 %. Although we did not include IBCDD and SCADD in our panel-screening conditions, this methodology allowed us to detect and differentiate these conditions.

An increase in the C5DC level is suggestive of glutaric aciduria type I (GA1). We evaluated glutaric acid (GLUT) levels in the seven patients with GA1, revealing 100 % sensitivity but low specificity (36 %). Moreover, 3-hydroxyglutaric acid (3OHGLUT) was not a sensitive biomarker as only two of seven patients exhibited slightly higher 3OHGLUT levels in DBS samples. Notably, six of the seven patients presented a low excretory biochemical phenotype and mild-to-moderate 3OHGLUT urine levels in the confirmatory analysis after referral to the clinic. We also included a FN case of GA1: the C5DC concentration in the DBS sample was within the reference range. However, in the same sample, the GLUT level was elevated, whereas the 3OHGLUT level was normal.

As mentioned previously, detection of MADD involves alterations in 2TT markers that are shared with other conditions, such as increased C4, C5, and C5DC levels. Nonetheless, MADD can be distinguished because most cases exhibit an acylcarnitine profile that differs from that observed in other diseases. The included patient with MADD showed increased levels of GLUT, 2-hydroxyglutaric acid (2OHGLUT), EMA, acylglycines (IBG\BG, IVG, and 2MBG), and the C4- and C5-acylcarnitine isomers (IBC, BC, IVC, and 2MBC).

2TTs for other conditions

We evaluated adipic acid (AD) and suberylglycine (SG) as potential 2TT metabolites for other FAODs (Table 7,

Table 5: Sensitivity, specificity and pathological values of 2TT related with C5OH-disorders.

			Primary marker	C5OH		
			Disease	HMGCLD (n=2)	BKTD (n=1)	3-MCCD (n=20)
2TT	3-IVA	Cut-off (7.8 µmol/L)	SE, %	100	–	85
			SP, %	53	–	53
			PV, µmol/L	641.9 (82.9–1,200.8)	–	128.8 (34.3–993.9)
	3-MCC	Cut-off (0 µmol/L)	SE, %	–	–	10
			SP, %	–	–	98
			PV, µmol/L	–	–	0 (0–0.3)
	3-MCG\TGG	Cut-off (0 µmol/L)	SE, %	–	0	55
			SP, %	–		93
			PV, µmol/L	–	0	0.15 (0–38.4)
	TGC	Cut-off (0 µmol/L)	SE, %	–	100	–
			SP, %	–	99	–
			PV, µmol/L	–	0.3	–
	3OH2MEBUT	Cut-off (8.6 µmol/L)	SE, %	–	100	–
			SP, %	–	92	–
			PV, µmol/L	–	117.3	–
	3OH3MEGLUT	Cut-off (36 µmol/L)	SE, %	100	–	–
			SP, %	100	–	–
			PV, µmol/L	429.9 (424.2–435.7)	–	–
	3MEGLUT	Cut-off (0.64 µmol/L)	SE, %	100	–	–
			SP, %	74	–	–
			PV, µmol/L	3.8 (2.9–4.7)	–	–

Diseases that not shared common 2TT were considered as true negative and false positive for the estimation of specificity. PV are expressed as median (minimum value-maximum value). BKTD, ketothiolase deficiency; C5OH, 3-hydroxy-isovaleryl-carnitine\2-methyl-3-hydroxy-butiryl-carnitine; HMGCLD, 3-hydroxy-3-methylglutaryl-CoA lyase deficiency; 3-IVA, 3-hydroxyisovaleric acid; 3-MCCD, 3-methylcrotonyl-CoA carboxylase deficiency; 3-MCC, 3-methylcrotonyl-carnitine; 3-MCG\TGG, 3-methylcrotononylglycine\tiglylglycine; 3MEGLUT, 3-methyl-glutaric acid; 3OH2MEBUT, 3-hydroxy-2-methyl-butyric acid; 3OH3MEGLUT, 3-hydroxy-3-methyl-glutaric acid; PV, pathological values; SE, sensitivity; SP, specificity; TGC, tiglyl-carnitine; 2TT, second tier-test.

Supplementary Material). Only SG enabled the detection of medium chain acyl-CoA dehydrogenase deficiency (MCADD); the sensitivity was 74 % and the specificity was 99 %. However, as the initial markers used for screening this condition already exhibit good sensitivity and specificity, the utility of this 2TT marker may be limited. Given the low sensitivity (3 %) and specificity (64 %) of AD, we decided to exclude it from our routine workflow.

Unfortunately, we did not have access to samples from patients with ornithine transcarbamylase deficiency to evaluate the performance of orotic acid. However, we evaluated patients with citrullinemia type I and argininosuccinate lyase deficiency (ASLD). The orotic acid levels were normal, although one external control sample from a patient with ASLD showed an increased orotic acid level (Table 6).

There was an increased level of p-OH-phenyllactic in four of five patients with tyrosinemia type 1 and in one patient with tyrosinemia type II. However, this metabolite is not specific; thus, we removed it from our 2TT panel. Other 2TT targets that exhibited good performance were n-acetylaspartic acid and mevalonic acid, but they have not yet been associated with routine primary markers in NBS programmes.

External QC performance of the 2TT methodology

The nine external QC samples (Supplementary Material) showed good performance (Table 6). In particular, analysis of QC samples based on external quality assessment conducted by the Italian Society for the Study of Inherited Metabolic Diseases and Newborn Screening was useful to validate the 2TT profiles for ethylmalonic encephalopathy, ASLD, and biotinidase deficiency, as we did not have access to samples from patients with those specific conditions. The results revealed an increase in the levels of key metabolites, confirming a strong diagnostic agreement.

Discussion

Only a few methods have been reported for the detection of organic acids using DBS samples, and even fewer methods have been reported without derivatisation because multiple carboxyl groups present in their structures make the analysis challenging [29]. Thus, analytical methods for derivatising samples have mostly been adopted in laboratories

Table 6: External quality control results.

Qualitative external control quality[a]	
Disease	**Alterations detected**
Glutaric aciduria type 1	Elevated GLUT but normal 3-OH-GLUT
Ethylmalonic encephalopathy	Increase of EMA, IBC, BC and IVC
Argininosuccinate lyase deficiency	A considerable increment of orotic acid
Biotinidase deficiency	Elevation of 3-IVA, MCA and PG
Quantitative external control quality[b]	
2TT analyzed	**Results**
MMA Hcys MCA EMA	Quantitative results very similar with the theoretical mean from laboratories.

[a]Samples from External Quality Assessment (EQA) from the Italian Society for the study of inherited metabolic diseases and newborn screening.
[b]Samples from Quality Control Program provided by the CDC, Centers for Disease Control and Prevention, Atlanta, GA, USA. BC, butyryl-carnitine; EMA, ethylmalonic acid; GLUT, glutaric acid; Hcys, homocysteine; IBC, isobutyryl-carnitine; 3-IVA, 3-hydroxyisovaleric acid; IVC, isovaleryl-carnitine; MCA, methylcitric acid; MMA, methylmalonic acid; 3OHGLUT, 3-hydroxyglutaric acid; PG, propionylglycine.

[5, 20, 22, 24, 28, 30]. However, researchers have described 2TTs based on the measurement of underivatised organic acids using DBS samples over the past 2 years [15, 18, 19, 27, 31]. The measurement of underivatised metabolites takes less time and requires fewer resources for sample preparation. However, a major limitation of this approach is the lack of a highly sensitive instrument that enables a rapid switch between the positive and negative ion modes to detect all metabolites simultaneously. Nonetheless, this limitation has largely been overcome.

In 2015, we implemented simultaneous 2TT analysis of MMA, MCA, and Hcys without derivatisation using UPLC–MS/MS in our NBS programme. Using this strategy, we avoided a second sample request in 98 % of the cases, thereby reducing anxiety and stress in the patients' families. In addition, this change reduced the FP rate from 4.1 to 0.005 % and increased the PPV from 1.4 to 92 % [32]. MMA, 3-hydroxypropionic acid, MCA, and Hcys are employed most often for 2TTs using LC–MS/MS in DBS samples because an elevated C3 level leads to the most FPs in expanded NBS programmes [3, 4]. This strategy has improved the PPV across all programmes [3–5, 8, 16–18, 33].

Recently, there has been a trend to unify analytical processes and to develop metabolite panels that allow differentiation of conditions [18, 19, 34, 35]. The NBS programme in Galicia, Spain, applies a 2TT panel to dried urine samples obtained simultaneously with DBS samples at birth [28]. However, in our programme it is not feasible to collect dried urine samples together with DBS samples from each newborn owing to the substantial complexity of handling a large number of newborns (approximately 60,000) at several maternity centres (>80).

In 2013, our group first implemented a 2TT panel of 48 amino acids in DBS samples to detect aminoacidopathies in our NBS programme (including maple syrup urine disease, tyrosinemia, conditions related to low or high Met, and UCDs). This strategy improved the performance of our programme [32]. In contrast, other programmes implement the 2TTs for aminoacidopathies as independent assays [4, 15, 36, 37].

Based on our previous experience with the amino acid 2TT method, we established another 2TT panel mainly to detect organic acidurias, including organic acids, acylglycine and acylcarnitine isomers, Hcys, and orotic acid. The method is performed without derivatisation and with simultaneous detection via UPLC–MS/MS. In October 2022, we switched from our previous 2TT method we had used to measure MMA, MCA, and Hcys in DBS samples to the new simultaneous 2TT method in our routine NBS protocol. We observed a good correlation between both methodologies (data not shown).

Another parameter that generates a high FP rate in NBS programmes is an increase in C5 levels due to interference from pivalic acid. The 2TT methods used to measure C5 isomer levels have also demonstrated good performance by increasing the PPV [5, 16, 17, 20–25, 38, 39]. Our method could accurately distinguish among all acylcarnitine forms, similarly to the previously described independent methods [5, 16, 17, 20–25, 38, 39], and thus identify true positives and FPs as a result of increased PVC levels.

2TT methods based on increased C4 levels have been described to differentiate between SCADD, IBCDD, and MADD [17–20, 26]. Our new 2TT panel also allows us to differentiate between these conditions. Other studies have also demonstrated that the combined analysis of EMA, GLUT, 2OHGLUT, 3OGLUT, and methylsuccinic acid as 2TTs improves the PPV to detect SCADD compared with the *ACADS* sequencing strategy. It can also distinguish between GA1, other C4-acylcarnitine-related conditions, and MADD [27]. In addition, a previous study described a molecular 2TT for MADD using Matrix Assisted Laser Desorption/Ionization-time-of-flight (MALDI–TOF) mass spectrometry mass spectrometry [40].

To detect other FAODs, such as very long chain acyl-CoA dehydrogenase deficiency, researchers have used C14--acylcarnitine derivatives as 2TT metabolites to reduce the FP rate [18]. Our 2TT strategy can also be used to detect SCADD, MADD, and MCADD. We are currently developing a

Table 7: Sensitivity, specificity and pathological values of 2TT related with C5DC and other acylcarnitine.

		Primary marker	C5DC	Several ACN	Medium chain ACN	C4		C5		
		Disease	GA1 (n=7)	MADD (n=1)	MCADD (n=31)	IBCDD (n=4)	SCADD (20)	IVA (n=2)	2-MBDD (n=1)	Antibiotic treatment (n=10)
GLUT	Cut-off	SE, %	100	100	–	–	–	–	–	–
	(4 µmol/L)	SP, %		36	–	–	–	–	–	–
		PV, µmol/L	14.6 (4.3–22.1)	38.6	–	–	–	–	–	–
3OHGLUT	Cut-off	SE, %	29	–	–	–	–	–	–	–
	(12 µmol/L)	SP, %	88	–	–	–	–	–	–	–
		PV, µmol/L	10.8 (6.8–13.2)	–	–	–	–	–	–	–
2OHGLUT	Cut-off	SE, %	–	100	–	–	–	–	–	–
	(43 µmol/L)	SP, %	–	78	–	–	–	–	–	–
		PV, µmol/L	–	105.3	–	–	–	–	–	–
EMA	Cut-off	SE, %	–	100	–	–	90	–	–	–
	(3.7 µmol/L)	SP, %	–	98	–	–	98	–	–	–
		PV, µmol/L	–	5.3	–	–	11.9 (1.8–43.8)	–	–	–
IBC	Cut-off	SE, %	–	100	–	100	–	–	–	–
	(0.2 µmol/L)	SP, %	–	99	–	99	–	–	–	–
		PV, µmol/L	–	4.9	–	0.45 (0.3–0.9)	–	–	–	–
IBG\BG	Cut-off	SE, %	–	100	–	75	50	–	–	–
	(0 µmol/L)	SP, %	–	79	–	79	79	–	–	–
		PV, µmol/L	–	7.8	–	0.1 (0–0.2)	0 (0–0.3)	–	–	–
BC	Cut-off	SE, %	–	100	–	–	35	–	–	–
	(0.5 µmol/L)	SP, %	–	99	–	–	99	–	–	–
		PV, µmol/L	–	0.5	–	–	0.2 (0–2.4)	–	–	–
IVC	Cut-off	SE, %	–	100	–	–	–	100	–	–
	(0.5 µmol/L)	SP, %	–	99	–	–	–	99	–	–
		PV, µmol/L	–	10.1	–	–	–	26 (11.2–40.8)	–	–
IVG	Cut-off	SE, %	–	100	–	–	–	100	–	–
	(0.5 µmol/L)	SP, %	–	99	–	–	–	99	–	–
		PV, µmol/L	–	1	–	–	–	16.9 (16.1–17.6)	–	–
2MBC	Cut-off	SE, %	–	0	–	–	–	–	100	–
	(0.1 µmol/L)	SP, %	–	91	–	–	–	–	91	–
		PV, µmol/L	–	0.1	–	–	–	–	0.3	–
2MBG	Cut-off	SE, %	–	0	–	–	–	–	0	–
	(0.3 µmol/L)	SP, %	–	98	–	–	–	–	98	–
		PV, µmol/L	–	0	–	–	–	–	0.1	–
PVC	Cut-off	SE, %	–	–	–	–	–	–	–	80
	(0.04 µmol/L)	SP, %	–	–	–	–	–	–	–	93
		PV, µmol/L	–	–	–	–	–	–	–	0.35 (0–3.2)
SG	Cut-off	SE, %	–	0	74	–	–	–	–	–
	(0.1 µmol/L)	SP, %	–		99	–	–	–	–	–
		PV, µmol/L	–	0	0.7 (0–28.1)	–	–	–	–	–

For the calculation of the specificity we included the positive samples for other diseases that don't share the specific 2TT as key metabolite. PV are expressed as median (minimum value-maximum value). ACN, acylcarnitine; BC, butyryl-carnitine; C4, butyryl-carnitine\isobutyryl-carnitine; C5, isovaleryl-carnitine\2-methylbutyryl-carnitine\pivaloyl-carnitine; C5DC, glutaryl-carnitine; EMA, ethylmalonic acid; GA1, glutaric aciduria type 1; GLUT, glutaric acid; 2OHGLUT, 2-hydroxyglutaric acid; 3OHGLUT, 3-hydroxyglutaric acid; IBG\BG, isobutyrylglycine\butyrylglycine; IBC, isobutyryl-carnitine; IBCDD, isobutyryl-CoA dehydrogenase deficiency; IVA, isovaleric acidemia; IVC, isovalerylcarnitine; IVG, isovalerylglycine; MADD, multiple acyl-CoA deshydrogenase deficiency; 2MBC, 2-methylbutyrylcarnitine; 2MBG, 2-methylbutyrylglycine; 2-MBDD, 2-methylbutyryl-CoA dehydrogenase deficiency; MCADD, medium chain acyl-CoA dehydrogenase deficiency; PV, pathological values; PVC, pivaloyl-carnitine; SCADD, short-chain acyl-CoA dehydrogenase deficiency; SE, sensitivity; SG, suberylglycine; SP, specificity; 2TT, second tier-test.

new 2TT strategy to detect MCADD because neither AD nor hexanoylglycine, the key metabolites, have shown good analytical performance.

There are only a few published reports of 2TT methods to detect C5OH-related disorders in NBS programmes [15, 18, 31, 41]. Two 2TT methods have been reported: they have confirmed that 3OH3MEGLUT and 3MGA are optimal discriminatory biomarkers for HMGCLD [15, 31]. One of these strategies enabled the differential diagnosis of HMGCLD, BKTD, and 3-MCCD by analysing short-chain acylcarnitines and acylglycines [15]. Another study described the development of a specific method to measure 2-methyl-3-hydroxybutyrylcarnitine and 2-methylacetoacetylcarnitine to discriminate BKTD from HSD10D [41]. Our method is based on the same assay and could differentiate between HMGCLD, BKTD, and 3-MCCD, but not between BKTD and HSD10D. It is important to differentiate 3-MCCD from other disorders because it is not an emergency condition and maternal 3-MCCD is frequently detected. Thus, several programmes, including ours, do not screen for 3-MCCD.

Concerning C5DC, some studies have used GLUT and 3OHGLUT as 2TT metabolites to detect GA1 [15, 18, 19]. Shigematsu et al. [15] revealed an overlap in the GLUT levels between affected patients and controls, and the 3OHGLU level was elevated in all patients. In contrast, we detected high GLUT levels in all seven patients with GA1, but this marker showed low diagnostic specificity. Furthermore, we observed increased 3OHGLUT levels in only two patients, despite good recovery using our method (Table 3). This outcome could be explained by the fact that six of our seven patients with GA1 exhibited a low excretory biochemical phenotype. Nevertheless, to avoid false negatives, we decided not to use GLUT and 3OHGLUT as 2TT metabolites to detect GA1.

The inclusion of orotic acid in our 2TT amino acid panel will be useful to detect some UCDs. Other NBS programmes have also developed methods to use orotic acid as a 2TT metabolite in DBS samples [18, 19]. However, it should be noted that in some females with ornithine transcarbamylase deficiency, normal levels of orotic acid may be observed due to X-linked lyonisation.

In summary, we have established a new method for our routine laboratory protocol by performing multiple 2TTs in initial DBS samples using an easy and rapid sample extraction protocol without derivatisation and with short chromatographic separation and detection through UPLC–MS/MS. We could detect the key metabolites of core diseases included in our panel with 100 % sensitivity and 74–99 % specificity, except for 3OHGLUT for GA1 detection. However, it is important to investigate a larger number of patients because certain cases may exhibit variable biochemical alterations. Another limitation of our study is the relatively small number of cases included for certain core conditions, such as such as methylenetetrahydrofolate reductase deficiency (MTHFR), HMGCLD, BKTD, IVA, and MADD. In addition, we did not include cases of holocarboxylase synthase deficiency, BTD, and other conditions.

The implementation of this new method, based on a retrospective analysis, led to the reduction of second sample requests by 43 % per year compared with our initial strategy. This is expected to reduce anxiety in families, although a second dried urine sample is still needed to detect GA1. We expect that this new strategy will decrease the FP rate by an additional 1.89 % per year with respect to the previous 2TT strategy because both strategies incorporate the markers that reduce the major occurrence of FPs: MMA, MCA, and Hcys. Furthermore, the use of a 2TT strategy allows us to reduce the cut-off value and thus reduce the FN rate, as we demonstrated with our first 2TT strategy for an increase in the primary marker C3 [12]. Overall, we expect the new 2TT strategy to increase the PPV of our programme and to reduce costs associated with laboratory instrumentation and equipment. Furthermore, we expect it to significantly reduce the detection time, thereby allowing for rapid clinical intervention.

Conclusions

The simultaneous 31 2TT panel using LC–MS/MS improves the accuracy of detecting various organic acidurias, certain FAODs, and UCDs. The combination of this complex panel of organic acids, acylglycine and acylcarnitine isomers, Hcys, and orotic acid with a complete panel of amino acids will have a positive impact on Catalonia's NBS programme by increasing the PPV as well as reducing the number of FPs, FNs, and second sample requests, thereby allowing for earlier intervention.

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Research ethics: The local Institutional Review Board deemed the study exempt from review. Q6

Informed consent: Informed consent was obtained from all individuals included in this study.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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