



Reducing the False-Positive Rate for Isovalerylcarnitine in Expanded Newborn Screening: The Application of a Second-Tier Test

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Abstract

The isodecyl neopentanoate is an ingredient used in the cosmetic industry to prepare a nipple fissure balm. We report on 12 newborns that showed elevated C5-acylcarnitine levels upon newborn screening following treatment with balm. The first 3 neonates were immediately recalled for confirmatory tests and resulted negative for both isovaleric acidemia and short/branched chain acyl-CoA dehydrogenase deficiency. In the other 9 cases, the immediate recall was avoided by applying a new second-tier test able to confirm the presence of pivaloylcarnitine. The concentration of C5-acylcarnitine was measured in the days following the suspension of balm application. Abnormal concentrations of C5-acylcarnitine did not seem to be associated with free carnitine deficiency, probably due to the short time of exposure. A direct correlation between balm ingestion and the elevation in pivaloylcarnitine has been demonstrated in 10 adult volunteers. The commercial balm containing a pivalic acid derivative is causal of false-positive results during newborn screening, and it could have the potential to cause secondary carnitine deficiency when used chronically.

Keywords

newborn screening, tandem mass spectrometry, false positive, pivalic acid, second-tier test

Introduction

Acylcarnitine profile analysis using the flow injection analysis–tandem mass spectrometry (MS/MS) method is commonly used in newborn screening programs for the biochemical screening of fatty acid oxidation and organic acid metabolism disorders.

The profile is analyzed in positive-ion mode, usually using a precursor ion scan experiment or a multiple reaction monitoring (MRM) experiment. A common fragment (85 *m/z*) containing the quaternary ammonium function could be detected with high sensitivity and specificity. Flow injection analysis–MS/MS methods cannot distinguish between isobaric compounds, making chromatographic separation essential when the separation of isomers is required for the differential diagnosis of some inborn errors of metabolism.^{1,2}

Several authors have developed methods for the determination of C4-acylcarnitine and C5-acylcarnitine isomers in plasma and dried blood spots (DBSs) by ultra-performance

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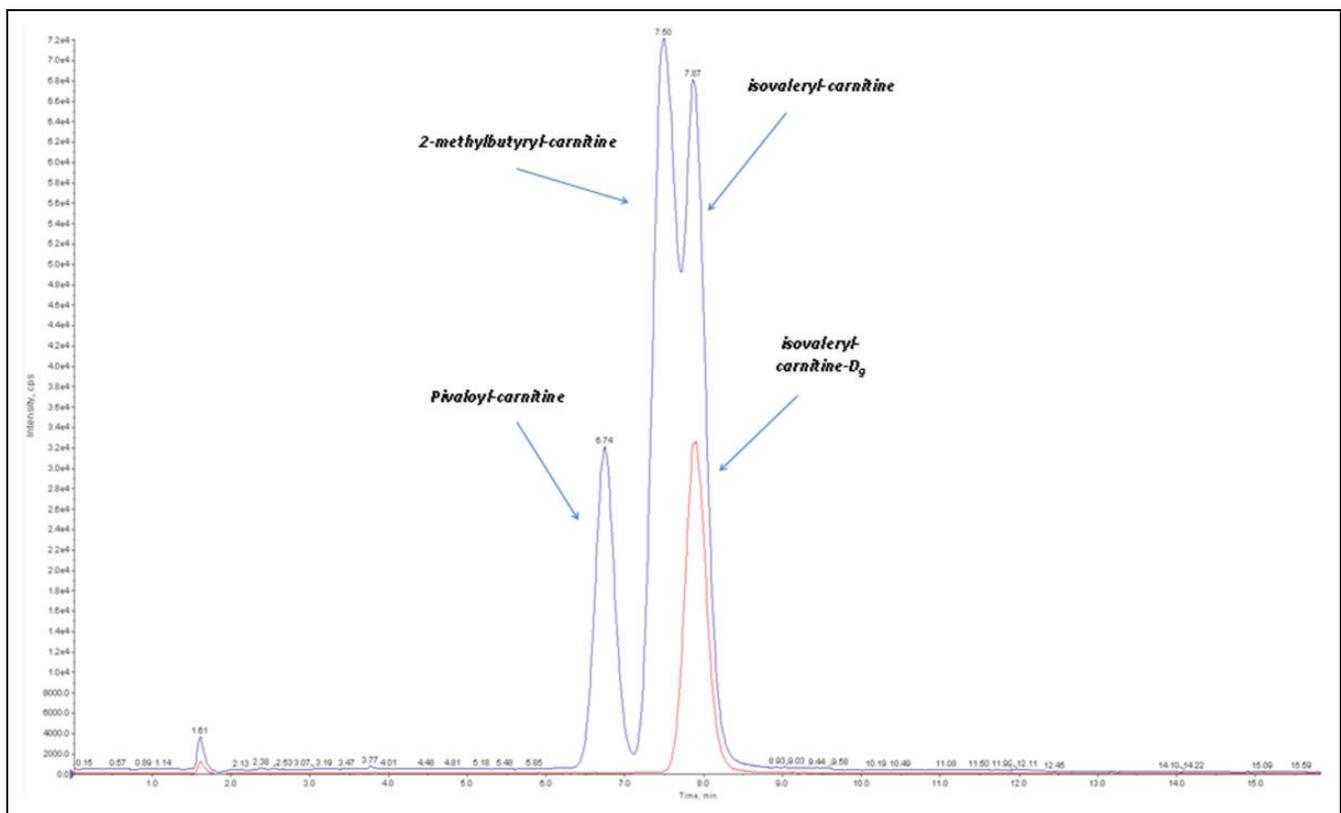


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Table 1. Free Carnitine and C5-Acylcarnitine Concentrations Detected During Expanded Newborn Screening Program in 12 Infants Whose Mothers Used the Nipple Fissure Balm.

Case	C5 (nv 0-0.56) Concentration Expressed in $\mu\text{mol/L}$			C0 (nv 5.5-45) Concentration Expressed in $\mu\text{mol/L}$		
	Newborn Screening	Retesting (Without Suspension)	Retesting (Days After Suspension)	Newborn Screening	Retesting (Without Suspension)	Retesting (Days After Suspension)
1 ^a	1.18	3.63	0.29 (7)	24.4	24.1	26.9 (7)
2 ^a	0.82	0.85	0.11 (10)	13.02	12.02	23.6 (10)
3 ^a	1.13	1.32	0.30 (4)	20.38	18.11	29.5 (4)
4	0.86	-	0.44 (3)	22.33	-	29.4 (3)
5	0.62	-	0.22 (2)	25.62	-	26.7 (2)
6	0.91	-	0.64 (3)	20.74	-	29.1 (3)
7	1.02	-	0.34 (4)	22.9	-	21.2 (4)
8	0.66	-	0.19 (4)	31.9	-	31.6 (4)
9	0.89	-	0.22 (2)	33.37	-	26.5 (2)
10	0.73	-	0.15 (10)	20.51	-	20.5 (10)
11	1.8	-	0.29 (5)	29	-	27.5 (5)
12	1.1	-	0.17 (4)	10.7	-	15.0 (4)

Abbreviation: nv, normal value.

^aNewborns immediately recalled for clinical evaluation.**Figure 1.** Chromatographic separation of the selected transition 302 \rightarrow 85.0 m/z. The chromatogram corresponds to a solution spiked with 0.05 $\mu\text{mol/L}$ of pivaloylcarnitine, 0.1 $\mu\text{mol/L}$ of 2-methylbutyrylcarnitine, and 0.1 $\mu\text{mol/L}$ of isovalerylcarnitine in the presence of D₉-isovalerylcarnitine (311 \rightarrow 85.0 m/z). The separate peaks show enough chromatographic resolution of C5-acylcarnitine isomers.

liquid chromatography (LC)–MS/MS as a second-tier test following flow injection–MS/MS acylcarnitine profile analysis.³ In particular, an increase in C5-acylcarnitine in a newborn screening profile indicates a possible isovaleric acidemia

(IVA) with elevated isovalerylcarnitine (IC) or a possible short/branched chain acyl-CoA dehydrogenase deficiency (SBCADD) with elevated 2-methylbutyrylcarnitine (2MBC).

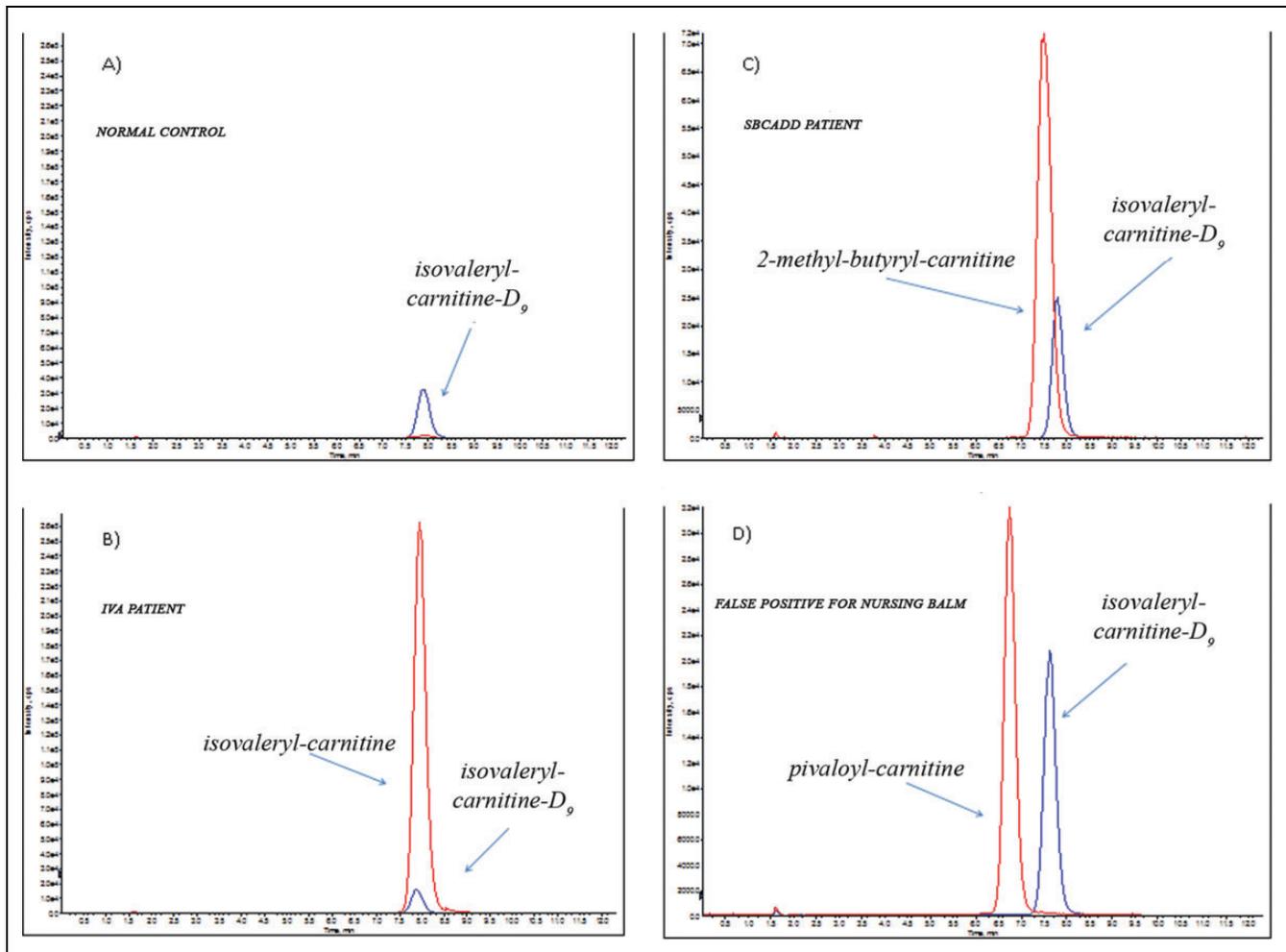


Figure 2. Extracted ion chromatogram of the monitored mass transitions 302→85 m/z and 311→85 m/z (used as internal standard) in a normal newborn (A), in a patient with isovaleric acidemia (IVA; B), in a patient with short-branched chain acyl-CoA dehydrogenase deficiency (SBCADD; C), and in a breast-fed neonate whose mother was using nipple cream (D).

Moreover, an increase in C5-acylcarnitine due to the presence of pivaloylcarnitine (PC) originating from pivalic acid in some antibiotic preparations and generating many false-positive results in newborn screening programs has been reported in the literature.^{4,5} More recently, Boemer and collaborators described a pivalic acid derivative “neopentanoate” used in the cosmetic industry to formulate a nipple fissure unguent that leads to false-positive increases in C5-acylcarnitine.⁶ In 2015, Yamada and colleagues reported on 2 cases that developed elevated C5-acylcarnitine levels following treatment with sivelestat, a neutrophil elastase inhibitor used to treat acute respiratory distress syndrome.⁷

Patients with SBCADD are generally asymptomatic, and its clinical consequences are not yet known.^{8,9} On the contrary, IVA is a branched-chain amino acid metabolism disorder characterized by severe episodes of ketoacidosis, vomiting, and lethargy, progressing to coma.¹⁰

It is therefore very important to differentiate between IVA, SBCADD, and increased levels of C5 due to PC. In 2014, an increased false-positivity rate due to elevated C5-acylcarnitine

was observed in the newborn screening programs of Tuscany and Northern Sicily.

Here, we report 12 cases presenting elevated C5-acylcarnitine due to nipple fissure balm, and we describe the second-tier method developed to identify specific isobars on DBS from newborns. Moreover, a direct correlation between the ingestion of nipple fissure balm and growing values of C5-acylcarnitine has been demonstrated in 10 adult volunteers.

Materials and Methods

Standards and Solvents

D₉-isovalerylcarnitine (D₉-IC) was purchased from Cambridge Isotope Laboratories (Andover, Massachusetts) to be used as an internal standard. The PC, 2MBC, and IC standard solutions were a gift from the Department of Chemistry of the University of Florence. Stock solutions of chemical and labeled standards at 100 mmol/L were prepared in methanol and stored at -20°C. Other reagents such as LC/MS-grade water, formic acid (purity

98%-100%), and LC/MS grade acetonitrile were purchased from Panreac (Barcelona, Spain). *N*-butanol containing 3N HCl, used as a derivatizing agent for the second-tier test, was purchased from Regis Chemical (Morton Grove, Illinois).

Sample Preparation and Chromatography

For the sample extraction, 200 μL of methanol solution containing $\text{D}_9\text{-IC}$ as an internal standard at a concentration of 40 nmol/L was added to a DBS punch (3.2-mm diameter, about 3.3-3.5 μL) and kept at 37°C for 25 minutes in an orbital shaker. The extracted samples were completely dried under nitrogen flow at room temperature (Evaporator Porvair Micro-DS 96; Stepbio, Bologna, Italy) and were then derivatized using 75 μL of 3N HCl in *n*-butanol and kept at 65°C for 20 minutes. The derivatized samples were completely dried under nitrogen flow at 40°C and finally reconstituted with 200 μL of water/acetonitrile 30:70 (vol/vol) + 0.1% of formic acid to prepare them for analysis.

The chromatographic separations were performed on an Agilent 1260 Quaternary Capillary Pump with a Synergi Polar-RP 80A, 4 μm 150 \times 2 mm high-performance liquid chromatography column (Phenomenex, Anzola, Bologna, Italy) and 4 \times 2 mm precolumn cartridge (Phenomenex) for separation.

The mobile phase was composed of water (eluent A) and methanol (eluent B) with a flow rate of 200 $\mu\text{L}/\text{min}$. Gradient elution was achieved using a program with mobile phase A and mobile phase B as follows: 5% B to 50% B in 3 minutes and maintained for 0.1 minute, then from 50% B to 80% in 0.1 minute and maintained for 4.5 minutes, then back to 5% B in 0.1 minute and reequilibration for 4.2 minutes. The total analysis run time was 12 minutes. One microliter of the extracted sample was injected for the LC-MS/MS experiments.

An API 4000 Triple-Quad Mass Spectrometer equipped with the Turbo V-spray source with the turbo gas temperature set at 400°C was used for the MS/MS tests. The source operates in positive ionization polarity at a potential of +5500 V. The transitions for the MRM experiments were 302.2 \rightarrow 85 m/z for C5 isomers and 311.2 \rightarrow 85 m/z for the labeled internal standard $\text{D}_9\text{-IC}$. Optimal Collision Energy (CE), Cell exit Potential (CXP) and Declustering Potential (DP) were found at 25, 7, and 40 V, respectively, for both transitions.

Experimental Design and Sample Collection

About 300 mg of a nipple fissure balm containing neopentanoate as a cosmetic ingredient was added to 50 mL of commercial fresh pasteurized cow milk and administered to 3 males and 5 females, all volunteers from the NBS laboratory of Meyer Children's Hospital.

Additionally, 2 volunteers, 1 male and 1 female, drank only 50 mL of commercial fresh pasteurized cow milk (vehicle) without balm. Dried blood spot samples from each volunteer were obtained by finger prick before and after balm ingestion (T0, T1, T2, T4, T8, T24 hours). A fully automated finger

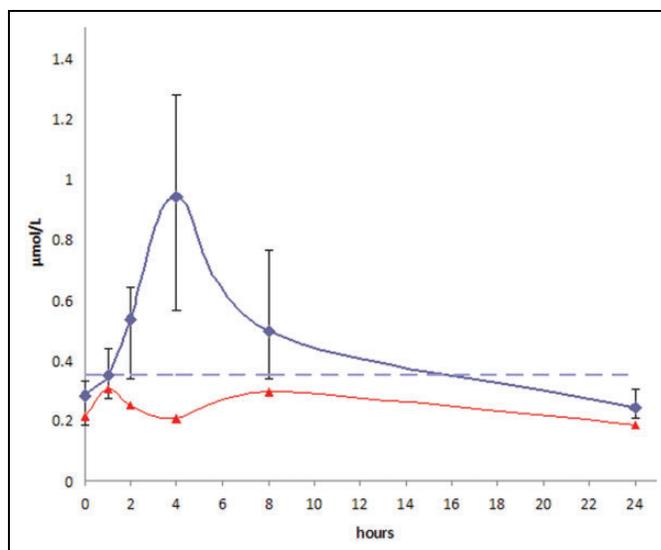


Figure 3. Graphic representation of the effect of balm ingestion on blood C5-carnitine levels. Blood samples were collected sequentially over a 24-hour period from 10 healthy volunteers, 8 who received an oral dose of nursing balm (purple line) and 2 monitored as controls (red line). The horizontal dashed line represents the higher cutoff of C5 (0.35 $\mu\text{mol}/\text{L}$) on blood for normal adults.

incision device for blood sampling was used (Tenderfoot Preemie; ITC, Edison, New Jersey). Dried blood spots were prepared by spotting whole blood onto filter paper cards (903; Whatman GmbH, Dassel, Germany), which were then left to dry and stored at -20°C in a sealed plastic bag containing desiccant until analysis. Samples were prepared in triplicate for routine screening tests, as previously described.¹¹

Results

Between January and December 2014, the newborn screening programs of Tuscany ($n = 37111$) and Northern Sicily ($n = 16020$), limited to LC-MS/MS testing, had a detection rate of 1:2062 and 1:2620, respectively. Of 91 total recalls in Tuscany, 5 (5.5%) were for abnormal values of isolated C5-acylcarnitine (normal values $\leq 0.56 \mu\text{mol}/\text{L}$). Of 529 total recalls in Sicily, 27 (5.1%) were for abnormal values of isolated C5 (normal values $\leq 0.56 \mu\text{mol}/\text{L}$). Only 2 of the 32 were true IVA positives—1 from Tuscany and 1 from Sicily.

Twelve of the 32 neonates showed abnormal C5-acylcarnitine concentrations due to indirect absorption of isodecyl neopentanoate originating from a nipple fissure balm used by nursing mothers to prevent rhagades. Three of them were from the Tuscany Newborn Screening Center and 9 were from the Northern Sicily Newborn Screening Center. The remaining 18 false positives from newborn screening resulted negative upon the second-tier test. The C5-acylcarnitine and free carnitine concentrations of the 12 false-positive newborns for balm are reported in Table 1.

As IVA can lead to acute metabolic decompensation, in the period preceding the setup of the second-tier test, newborns 1

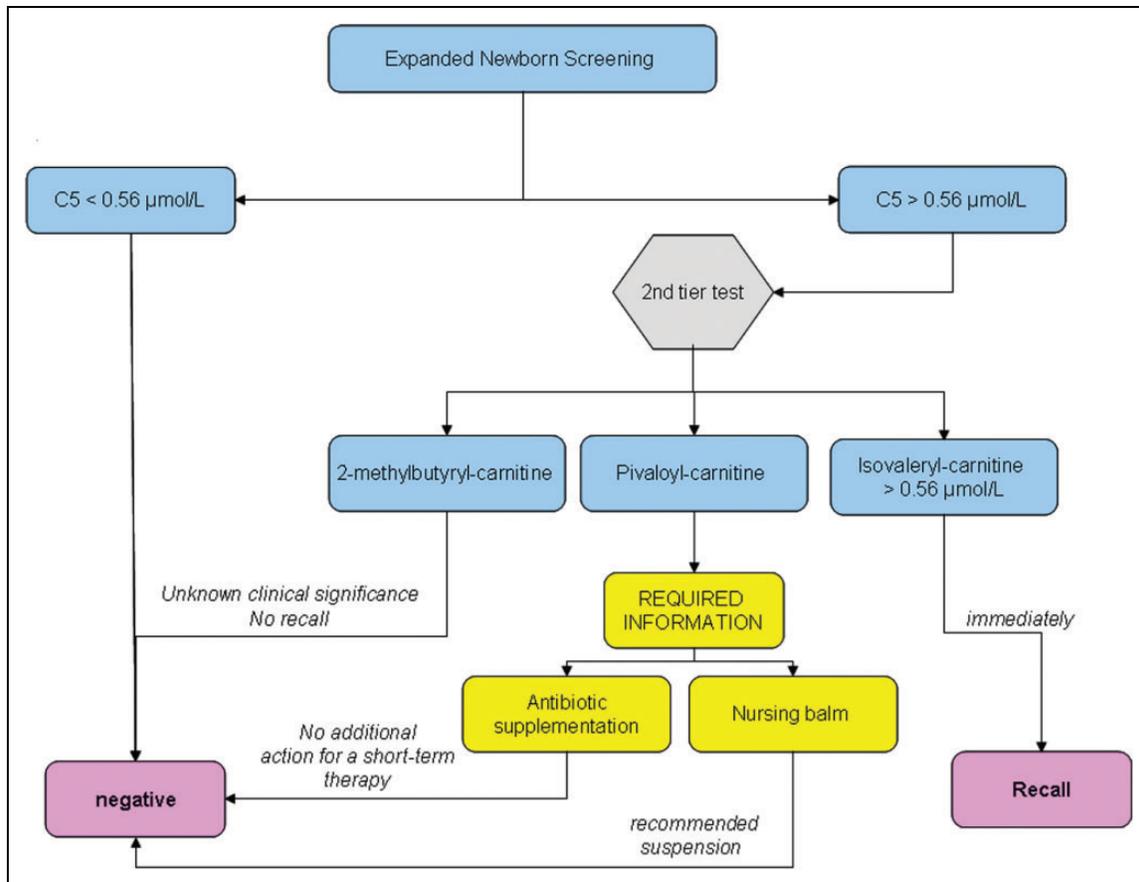


Figure 4. Flow diagram for the C5-carnitine evaluation in the newborn screening.

(from Tuscany), 2, and 3 (from Sicily) were immediately recalled following the newborn screening results for clinical evaluation and second-level confirmatory testing according to internal protocol. However, IVA was considered to be unlikely because isovaleryl-glycine and 3-OH-isovaleric acid were not detected by urinary organic acid analysis. Moreover, no abnormal findings such as body smell or hypoglycemia, which are typical symptoms of IVA, were noted in these patients. During the anamnestic evaluation, their mothers referred to the use of the same nipple fissure balm (to prevent rhagades) at the time that blood was drawn (as preventive of rhagades) without concomitant drug supplementation. After these first 3 cases, we decided to develop a second-tier test able to separate C5-acylcarnitine isomers.

Figure 1 shows the chromatographic separation of butylated C5 isomers at 0.05 $\mu\text{mol/L}$ (D₉-IC and PC) and at 0.1 $\mu\text{mol/L}$ (2MBC and IC) in acetonitrile/water 70:30 + 0.1% formic acid. The corresponding retention times were 7.83, 6.74, 7.50, and 7.87 minutes, respectively.

To assess whether second-tier testing could be effective, we applied it to 100 blood spots reported as normal during newborn screening by LC-MS/MS, and 32 spots recalled for abnormal values of C5 (newborns 1, 2, and 3 were retrospectively analyzed). In addition, we tested 4 truly positive newborn screening blood spots (2 IVA and 2 SBCADD). No quantifiable

signal corresponding to IC or 2MBC was detected for controls or false positives. Figure 2 shows the extracted ion chromatogram from a normal neonatal DBS (panel A), patient with IVA (panel B), patient with SBCADD (panel C), and false positive due to PC (panel D).

In the period of April to December 2014, the application of the second-tier test allowed the identification of 9 additional cases of false positives due to balm application during breastfeeding. For all of them, we decided to ask for a new DBS after the suspension of balm application to control the C5-acylcarnitine normalization (Table 1).

A new experiment was performed in 10 adult volunteers to demonstrate the direct correlation between balm ingestion and the increase in C5-acylcarnitine blood levels. Eight of them (3 males and 5 females, age 25-46 years) received 50 mL of commercially available fresh cow milk containing about 300 mg of the balm. One male (42 years old) and 1 female (46 years old) drank only 50 mL of commercially available fresh cow milk (vehicle) without balm. Dried blood spot samples were obtained from each volunteer by finger prick over 24 hours.

Figure 3 shows the C5-acylcarnitine trend. The reported C5 acylcarnitine values are the average of 3 replicates for all volunteers at each time point. The maximum C5-acylcarnitine blood concentration was reached 4 hours after balm ingestion in all volunteers.

Considering that the pivalic acid derivative neopentanoic acid conjugates the free carnitine⁴⁻⁶ and that the breast-feeding period could be quite long (generally some months), we cannot exclude a potentially dangerous secondary carnitine deficiency due to a long-term nipple fissure balm application. Exogenous unusual long-term pivalate exposure from pivalic esters of antibiotics to mothers or newborns had previously been reported as responsible for carnitine depletion and hypoglycemic convulsions.¹²

Moreover, Ito and collaborators reported that serum-free and total carnitine levels decreased by about 50% even after short-term administration of these drugs.⁴ In our experience, the free carnitine levels in the 12 false-positive newborns and in adult volunteers were within the normal ranges (Table 1 and Figure 3), probably due to short-term exposure.

The NBS protocol has been modified in daily practice as reported in Figure 4. If an abnormal value of C5-acylcarnitine is present during a routine newborn screening procedure, the second-tier test is immediately performed. If PC is present, we contact the mother by phone and ask if she has been using some antibiotics or the balm. In the first case, no further action is required if a short-term therapy has been recommended by a physician; in the latter, we recommend the suspension of balm application and/or possibly replacement with other creams. If IC is present and the concentration in blood is higher than 0.56 $\mu\text{mol/L}$, we proceed to the immediate recall of the baby for clinical evaluation and second-level confirmatory testing.

Considering that patients with SBCADD are generally asymptomatic,^{8,9} SBCADD is not included in the NBS panel because of its unknown clinical significance; therefore, if 2MBC is present, the newborn is not recalled.

Conclusion

The aim of this work is to declare that the use of the nipple fissure cream containing neopentanoate esters as an ingredient causes false-positive results during newborn screening. Warning nurseries, pediatric facilities, and manufacturing companies about the effects of these compounds is very important in order to decrease diagnostic errors and consequent recalls. Recalls induce stress in the families of newborns and may also cause the loss of milk in mothers or influence the child-parent relationship.¹³

In the experiences of Tuscany and Sicily, the biomarker C5-acylcarnitine is not causative of many recalls during newborn screening (about 5%-6% of total recalls); however, the positive predictive value of the biomarker is not good (2 IVA of the 32 positives corresponding to 6.25%). Based on the conviction that the combination of first- and second-tier testing for many metabolic disorders must be considered the best working practice to achieve the most benefit out of newborn screening programs, in our opinion, the new second-tier test for C5-acylcarnitine can reduce the false-positive rate, improve the positive predictive value, and above all avoid superfluous urgent recalls with a consequent reduction in laboratory analyses, personnel costs for repeat tests, and considerable anxiety for parents. The use of pivalic acid derivatives in drugs or cosmetics is still widespread worldwide, in spite of important

reports. During the preparation of this article, a new false positive for C5-acylcarnitine was identified by the Tuscan newborn screening program because of a newborn's exposure to an antibiotic named "cefditoren pivoxil," a semisynthetic cephalosporin used in pediatric patients with laryngopharyngitis and tonsillitis. New and better strategies should be developed to provide testing options that, applied to newborn screening programs, reduce recall rate, overall expenses, and parental anxiety.

Authors' Note

Sara Poggiali and Daniela Ombrone are contributed equally to this work.

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Declaration of Conflicting Interests

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