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Short communication

Hypocitrullinemia in expanded newborn screening by LC–MS/MS is not a reliable marker for ornithine transcarbamylase deficiency

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ABSTRACT

In an expanded newborn screening program for inborn errors of metabolism by LC–MS/MS in Tuscany, six newborns out of 169,000 showed decreased blood citrulline levels. In one of them, molecular analysis of the OTC gene identified the known p.Trp265Leu mutation, which is correlated with late-onset ornithine transcarbamylase deficiency (OTCD). Hypocitrullinemia is not a reliable marker for OTCD newborn screening, especially for late-onset forms that may exhibit normal citrulline levels. However, when hypocitrullinemia is detected in a newborn in whom intestinal dysfunction and prematurity have been excluded, OTCD should be investigated first because of the OTCD incidence (1:14,000) and the small size of the OTC gene coding sequence.

1. Introduction

Screening programs exploring acylcarnitine and amino acid profiles through tandem mass spectrometry (LC–MS/MS) in newborns can detect more than 30 inborn errors of metabolism.

Citrulline, one of the amino acids checked in LC–MS/MS newborn screening, is synthesized mainly in the mitochondrial matrix of hepatocytes and to a lesser extent in the small intestine and kidney as an intermediate in the conversion of ammonia in the urea cycle and during nitric oxide production [1].

High blood citrulline levels may be suggestive of citrullinemia type I (MIM 215700), citrullinemia type II (MIM 603471) or argininosuccinic aciduria (MIM 207900). On the other hand, low blood citrulline levels can be detected in defects in the first two steps of the urea cycle, such as ornithine transcarbamylase (OTC; MIM 311250) and carbamoyl phosphate synthetase I (CPSI; MIM 237300) deficiencies [1] and in N-acetylglutamate synthetase deficiency (NAGS; MIM 237310) [2,3], in some mitochondrial disorders, such as NARP (MIM 551500) [4] and MELAS (MIM 540000) [5] or in delta-1-pyrroline-5-carboxylate synthetase deficiency (P5CS; MIM 138250) [6]. These inborn errors are not currently included in conventional newborn screening programs. However, the commonest causes of low citrulline are detected in premature infants or in sick babies such as those with pathological conditions involving the small intestine, i.e. short-bowel syndrome [7,8].

Ornithine transcarbamylase deficiency (OTCD) is the most common urea cycle disorder with an X-linked inheritance (locus Xp21.1) and variable severity affecting both males and females [1]. The *OTC* gene, containing 10 exons, codes a 1062 nt mRNA expressed only in the liver and intestine. So far, about 370 mutations, leading to neonatal or late-onset forms, have been identified in the OTC gene. Early diagnosis and treatment can improve prognosis by preventing possible acute metabolic decompensation and irreversible damage induced by hyperammonemia.

Some reports have described OTCD patients identified through a selective screening program for inborn errors of metabolism [9,10] or born to a mother with known risk [11] or infants in whom results of newborn screening became available after clinical presentation [12,13].

Mass spectrometry is one the most effective method in newborn screening for inborn errors of metabolism. Herein, we report our experience in the monitoring of low citrulline levels in expanded newborn screening. During the last 6 years, 169,000 newborns were screened by LC–MS/MS in Tuscany and six babies were recalled for low blood citrulline levels. In one of them, OCTD was confirmed by molecular analysis. To our knowledge, this is the first case of

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OTCD identified through LC–MS/MS expanded newborn screening in a male with mild and likely unrelated signs.

2. Materials and methods

2.1. LC–MS/MS analysis

Newborn screening by LC–MS/MS has been carried out in Tuscany since November 2001. Blood samples were collected by heel stick between 48 and 72 h of life, spotted on filter paper (903, Whatman), dried and sent by courier to the screening centre. Sample preparation for LC–MS/MS analysis required derivatization for acylcarnitines and amino acids, as previously described [14]. The mass spectrometer was an API 4000 equipped with a TurboIonSpray source from Applied Biosystems-MDS Sciex (Toronto, Canada). In our control population of healthy newborns the normal range for citrulline was 3–25 μmol/L, median value 7.7 μmol/L, 1st centile 3.8 μmol/L and 99th centile 19 μmol/L.

2.2. Confirmatory testing

Babies with a positive newborn screening result for hypocitrullinemia were recalled for a second blood spot (DBS).

In our protocol, confirmatory testing are carried out when also the second DBS is positive, except for premature babies and sick infants, particularly those with intestinal dysfunctions. Patients with a second negative DBS are not further investigated unless they exhibit a positive clinical picture and/or family history.

Confirmatory testing for OTCD included a plasma amino acid analysis by LC–MS/MS and by Biochrom 30 amino acid analyzer (Cambridge, UK), quantification of orotic acid in urine by LC–MS/MS [15] and molecular analysis of the OTC gene. Genomic DNA was isolated using QIAamp DNA kit (Quiagen, Hilden, Germany) from peripheral blood of Newborn 6 and related family members after informed consent had been obtained. Exonic and flanking intronic regions of the OTC gene were amplified as previously described [16]. Mutation detection was performed by direct sequencing on an ABI PRISM 310 genetic analyzer using BigDye terminator chemicals (Applied Biosystems, Foster City, CA).

3. Results and discussion

About 48,000 newborns were screened by LC–MS/MS in Tuscany during a pilot project (November 2001 to October 2004) and 121,000 during the mandatory expanded newborn screening program (November 2004 to June 2008) [17]. Only six babies showed decreased blood citrulline levels. A second DBS, clinical information and family history were obtained for each neonate. The main clinical and biochemical findings for the six newborns are summarised in Table 1. When the clinical picture and/or family history were positive, plasma citrulline was investigated, even if citrulline fell into the normal range on the second DBS.

Four recalled newborns (Newborns 1, 2, 3 and 4) were premature (27th to 37th week) with low birth weights (<2500 g); clinical findings and family history were negative for metabolic diseases. In Newborns 1 and 2 citrulline values were within the normal range on the second DBS. In premature infants citrulline levels are reported to be 25% lower than full term newborns [18], thus the transitory alterations detected may have been due to the newborns' immaturity.

Newborns 3 and 4 showed decreased citrulline levels on both the second DBS and plasma sample (plasma values: 6.6 and 5.9 μmol/L, respectively, n.v. 9–33); they presented intestinal malrotation. Since the small intestine releases large amounts of citrulline into circulation [19], the detected hypocitrullinemia might reflect the pathologic intestinal condition. In these two patients plasma citrulline levels that were investigated to point out the possible role of intestine dysfunction in determining hypocitrullinemia, were normalized at 1 month of life after surgery for intestinal problems (26 and 21 μmol/L, respectively, n.v. 17–53).

Two recalled newborns (Newborns 5 and 6) were born at term with normal weights (>3200 g). In Newborn 5, with negative clinical and family histories, citrulline levels on 2nd DBS were normal and thus a plasma sample was not investigated.

Newborn 6 showed at the neonatal screening a citrulline level of 2.4 μmol/L (n.v. 3–25) (Fig. 1). The boy was born at term after an uneventful pregnancy and delivery (Apgar score 9/10, birth weight 3240 g, body length 50 cm, head circumference 34.5 cm) to healthy non-consanguineous Italian parents. No obvious clinical abnormalities were reported at birth. On the second day of life feeding difficulties, tremors and irritability appeared. The baby was discharged from the hospital on the third day of life. The second DBS, collected on the 8th day of life, showed normal citrulline levels (3.7 μmol/L, n.v. 3–25) though below the 1st centile. However, the clinical picture in the second day of life, which was unlikely related to OTCD, and a family history of hypertransaminasemia in the patient's sister and maternal aunt, led us to re-examine the baby. Hypocitrullinemia was detected on the plasma sample by LC–MS/MS (4 μmol/L, n.v. 9–33) and by amino acid analyzer (5 μmol/L, n.v. 17–53). Except for hypocytrullinemia, no biochemical abnormalities were detected. Based on the low citrulline levels in plasma and the family history, molecular analysis of the OTC gene was carried out. Direct sequencing on genomic DNA of OTC gene identified the c.794G>T (p.Trp265Leu) mutation in exon 8 at a hemizygous level. After genetic counselling, the molecular analysis extended to the family members identified the patient's mother, sister, maternal aunt and first cousin as carriers for this mutation. Citrulline supplementation (170 mg/(kg day)) was started. At present the baby (at 3 years old) is healthy and exhibits normal psychomotor development. Citrulline fluctuates between normal and slightly decreased values.

The c.794G>T (p.Trp265Leu) mutation was previously reported in two Italian patients with late-onset OTCD, 12 months and 4 years, respectively [20]. So far, other genetic lesions mapped in the same

Table 1
Babies recalled for hypocitrullinemia at newborn screening.

Newborn	Sex	Weeks of gestation	Birth weight (g)	Citrulline in NBS (n.v. 3–25 μmol/L)	Citrulline in 2nd DBS (n.v. 3–25 μmol/L)	Citrulline in plasma (n.v. 9–33 μmol/L)	Clinical picture and family history
1	M	31	1940	2.8	3.6	–	Negative
2	F	27	550	2.7	3.6	–	Negative
3	F	34	2230	2.4	2.6	6.6	Intestinal malrotation
4	F	37	1890	1.6	1.9	5.9	Intestinal malrotation
5	M	40	3300	2.8	6.2	–	Negative
6	M	39	3240	2.4	3.7	4	Feeding difficulties, tremors and irritability. Hypertransaminasemia in sister and maternal aunt

M: male; F: female; NBS: newborn screening spot; DBS: dried blood spot.

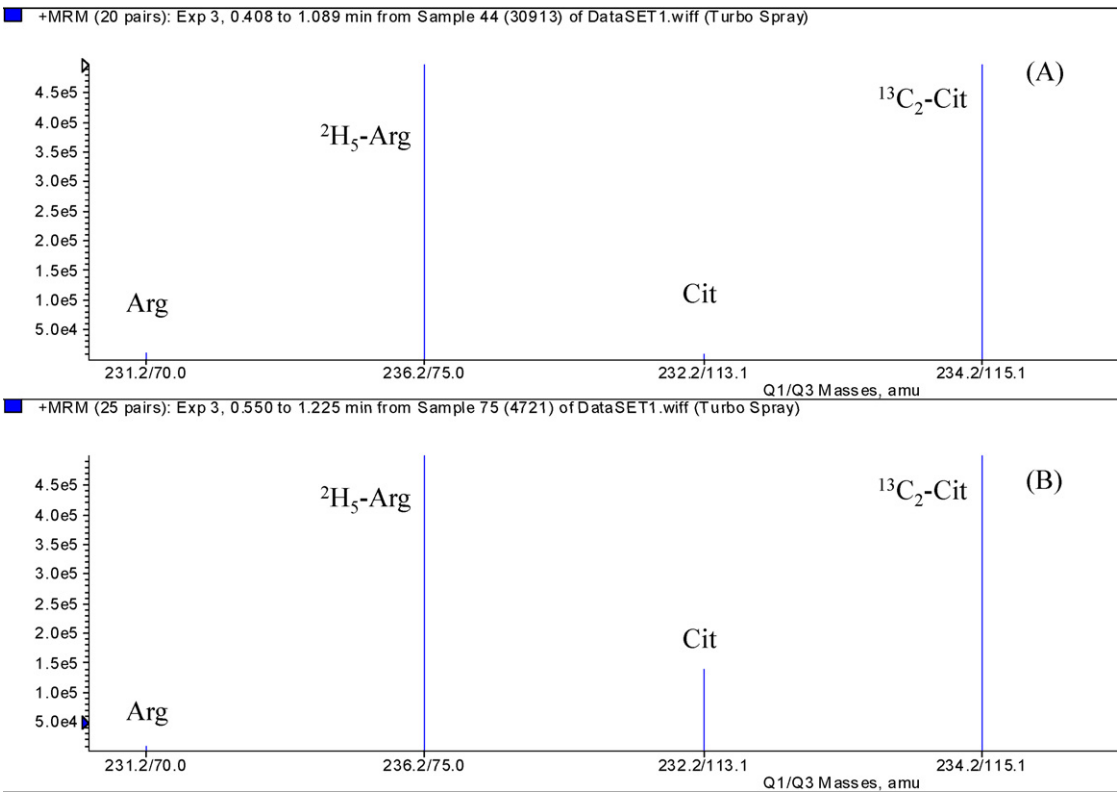

Fig. 1. Citrulline levels from newborn screening spot of the OTCD patient (A) vs. a normal control (B). The amino acid citrulline is detected by the ion 232/113 transition using the Multiple Reaction Monitoring mode (MRM).

OTC protein region (codons 264, 267, 268, 273 and 277) have been correlated with late-onset of the disease [21]. The PolyPhen server (http://www.bork.embl-heidelberg.de/PolyPhen) predicts that the p.Trp265Leu is probably damaging because it creates a cavity at buried site. Trp265 is part of the flexible SMG loop (residues 263–286) of the OTC enzyme that swings towards the active site and interacts with ornithine when both substrates bind [22]. Alteration of this loop (due to the p.Trp265Leu mutation) seems to destabilize, although not completely abolish the ornithine binding, and may therefore be expected to cause a partial deficiency and late-onset phenotype.

Among the urea cycle disorders associated with hypocitrullinemia, both OTC and CPSI deficiency are not currently included in conventional newborn screening programs. A scoring system has been developed by the American College of Medical Genetics (ACMG) to evaluate the conditions that have an acceptable newborn screening test. Conditions scoring <1000 are considered inappropriate for inclusion in a newborn screening panel. OTC and CPSI deficiency scored 942 and 833, respectively and therefore cannot be recommended for inclusion in newborn screening programs [23]. Compared to OTCD, CPSI deficiency is less frequent and the causative gene is considerably more complex to explore (38 exons). One reason because OTCD is considered inappropriate for newborn screening is that the amino acid profile by MS/MS cannot detect this condition consistently because the monitoring of low citrulline levels lacks sensitivity and specificity. However, no patient with OTCD has been missed during the time period considered in this study. Although false positive cases for low citrulline levels are not infrequent, especially in premature babies and in neonates with low protein intake, our recall rate for hypocitrullinemia is very low (0.003%).

In our opinion, when hypocitrullinemia is detected in a neonate, with the exception of premature infants and sick babies as those with pathologies affecting the intestine, OTCD should be the first diagnostic hypothesis to investigate. Such opinion is based on the incidence of this genetic defect (1:14,000) [21] and the small size of the OTC gene coding sequence, which allows easy molecular analysis.

Only scanty data are available in literature concerning OTCD identified through expanded newborn screening. A few cases were identified after a prenatal diagnosis in high-risk families or after the clinical presentation [11–13]. This may be due to missing diagnosis of late-onset OTCD cases because they may have normal citrulline levels, even when sick [24]. To our knowledge, we report the first case of OTCD identified through LC–MS/MS expanded newborn screening by hypocitrullinemia in a male with mild and likely unrelated signs and suspected family history. An early diagnosis of OTCD before the manifestation of typical symptoms associated with hyperammonemia is a decisive factor for improvement of prognosis.

We suggest that when hypocitrullinemia is identified in an expanded newborn screening program and intestinal dysfunctions and prematurity have been excluded, the family history and clinical picture of the newborn should be evaluated bearing in mind the possibility of a OTCD, even if negative data do not exclude a late-onset form. Hypocitrullinemia is not a reliable marker for newborn screening of OTCD, especially for late-onset forms, and only in rare cases a thorough diagnosis by molecular analysis is justified. The diagnostic power of molecular analysis is in turn only partial in that about 20% of patients with enzymatically proven OTCD will escape mutation detection [21].

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