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Case Report

Critical Newborn Screens in Double Heterozygotes of Inborn Errors of Metabolism—A Clinical Report and Recommendations

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Abstract: The practice of newborn screening has been in place in the USA since the 1960s, with individual states initially screening for different numbers of disorders. In the early 2000s many efforts were made to standardize the various disorders being screened. Currently, there are at least 34 disorders that each state is mandated to include on their screening panel. Of those 34 disorders, the majority are inborn errors of metabolism (IEM) which include urea cycle disorders (UCD), citrullinemia (CIT) and argininosuccinic aciduria (ASA), as well as a number of fatty acid oxidation disorders. We present here four cases of infants who had critical newborn screens (NBS) in the Commonwealth of Virginia and underwent genetic testing because their clinical presentation and follow-up laboratory studies were not consistent with the disorder that was flagged by NBS. These newborns were found to be carriers for two different IEMs (in three cases) or compound heterozygotes (in one case). Currently no guidelines exist with respect to the appropriate way to manage these children who may or may not be symptomatic in the newborn period. We propose some general recommendations for management based on our experience with these four probands, and discuss the necessity for further conversation and collaboration between physicians encountering these not-so-infrequent presentations.

Keywords: newborn screening; inborn errors of metabolism; hyperammonemia; fatty acid oxidation defect; urea cycle disorder

1. Introduction

Newborn screening (NBS) in the United States has been in place for over 50 years. Individual states were responsible for choosing the disorders they screened for until 2005, when the American College of Medical Genetics and Genomics (ACMGG) and the Secretary's Advisory Committee on Heritable Disorders in Newborns and Children defined a recommended uniform screening panel (RUSP) that defines conditions that all screening programs should include [1–3]. Today, all states must screen for at least 34 conditions including metabolic and endocrine disorders, hemoglobinopathies, critical congenital heart disease, cystic fibrosis, hearing loss, and severe combined immunodeficiency (SCID). Metabolic disorders include inborn errors of metabolism (IEM) such as urea cycle disorders

(UCD), organic acidurias, disorders of cobalamin metabolism, and a number of fatty acid oxidation disorders (FAOD) [2,4]. Infants are screened between 24 and 48 h of life using a small amount of blood, usually from a heel stick, which is placed on a Guthrie blood spot card and mailed to the state lab. Typically, state-appointed centers review critical NBS results and follow up with the infants, often performing additional laboratory studies, which may include evaluating the children in the clinic and obtaining DNA or other confirmatory testing. Not all critical NBSs result in a specific diagnosis, and often carrier status is revealed when follow-up laboratory tests are inconclusive and molecular testing is required.

UCDs are IEMs that result from defects in the breakdown of protein and other nitrogen-containing molecules [5]. Argininosuccinic aciduria (ASA; MIM 207900) and citrullinemia (CIT; MIM 215700) are included on the RUSP. It is important to note that there are several other UCDs that are not on the RUSP, but individual states may still choose to screen for them.

FAODs result from a deficiency in enzymes that play an important role in mitochondrial fatty acid β-oxidation [6]. While there are many FAODs, five are on the RUSP including carnitine uptake defect/carnitine transporter defect (CUD; MIM 212140), medium-chain acyl-CoA dehydrogenase deficiency (MCADD; MIM 607008), very-long-chain acyl-CoA dehydrogenase deficiency (VLCADD; MIM 201475), long-chain 3-Hydroxyacyl-CoA dehydrogenase deficiency (LCHADD; MIM 609576), and trifunctional protein deficiency (TFP; MIM 609015).

The UCD and FAOD on the mandated NBS panel are typically inherited in an autosomal recessive manner, and affected individuals are either homozygotes or compound heterozygotes. We present four cases of infants who had critical NBSs and underwent genetic testing because their clinical presentation and follow-up studies were not consistent with the disorder flagged on the NBS. Of these four cases, two of the newborns had critical newborn screens positive for CIT and were found to be carriers for two different IEMs. The other two newborns had critical newborn screens for different FAODs; one child was a carrier for two different FAODs and one child was a compound heterozygote for one FAOD with acylcarnitine levels on fibroblasts consistent with another FAOD. In this report, we use the term "double heterozygotes" to describe these individuals who are carriers for two different inborn errors of metabolism. Currently there are no guidelines on how to manage newborns with this presentation of critical NBS who are mildly symptomatic or asymptomatic in the newborn period. The need for long-term follow-up of these infants is unclear at this time and guidelines need to be established. With the increasing use of universal carrier screening in the prenatal population, we may be faced with identifying more infants who are carriers of multiple IEMs and may or may not be symptomatic at birth.

2. Case Reports

2.1. Patient A

Patient A was a 39-week female born following an uncomplicated pregnancy. There were no immediate complications in the neonatal period and the baby was discharged home with her mother on day 2 of life. Please see Table 1 for specifics regarding the patient's initial NBS labs and follow-up values. Patient A's initial NBS was collected after 24 h of life and was abnormal with increased citrulline. A repeat newborn screen was sent, per state protocol, on day of life 16 and was now critical for increased citrulline. This result was reported to our Medical Genetics department by the state newborn screening program. Lab recommendations included an ammonia level which returned normal at 33 μmol/L (≤49 μmol/L). The infant was evaluated and looked clinically well, was feeding well with standard infant formula, and the mother was instructed to follow up with the primary care physician as well as with Medical Genetics.

Table 1. Four probands and their newborn screening lab values as well as follow-up labs.

	Patient A	Patient B	Patient C	Patient D

Pregnancy/ Birth History	• Term female born via C-section to a 32-year-old G3P2-3 mother
	• Uncomplicated pregnancy
	• Birth parameters:
	  • weight and length: 25th percentile
	• No complications in the immediate neonatal period
	• Baby discharged home at two days of life
		• 36-Week female born via vaginal delivery to a 24-year-old G5P3-4 mother
		• Uncomplicated pregnancy
		• Birth parameters:
		  • weight and length: 75th percentile
		• No complications in the immediate neonatal period
		• Baby discharged home at two days of life
			• Term male born via C-section to a 31-year-old G1P0-1 mother
			• Pregnancy complicated by hydronephrosis, pelviectasis, arachnoid cyst and concern for ventriculomegaly
			• Birth parameters:
			  • weight and length: 50th percentile
			• Complications in the neonatal period included low temperatures, weight loss and evaluation for sepsis
			• Discharged home at four days of life with appointments with multiple specialists
				• Term male born via C-section to a 32-year-old G5P1-2 mother
				• Uncomplicated pregnancy
				• Birth parameters:
				  • weight: 25th percentile; length: 75th percentile
				• At day 2 of life baby developed poor feeding and tachypnea, and was transferred to CHKD NICU for evaluation of elevated NH_3 and concern for UCD

First NBS (collected >24 h of life)	• Abnormal for:
	  • citrulline: 80 (normal <65; abnormal 65 to <100)
		• Abnormal for:
		  • citrulline: 72 (normal <65; abnormal 65 to <100)
			• Critical for:
			  • C14 was 1.35 (normal <0.70; critical ≥0.92)
			  • C14:1 was 1.15 (normal <0.66; critical ≥ 1.5)
			  • C16-OH was 0.52 (normal <0.10; critical ≥0.19)
			  • C18:1-OH was 0.22 (normal <0.11; abnormal 0.11 to <0.5)
				• Critical for:
				  • C8 at 0.67 (normal <0.50; abnormal 0.50 to 0.99)
				  • C10 at 1 (normal <0.55; abnormal 0.55 to <0.9; critical ≥0.9)

Table 1. *Cont.*

	Patient A	Patient B	Patient C	Patient D

Second NBS	• Sent at 16 days of life:
	  • critical for citrulline: 178 (≥100)
		• Sent at eight days of life:
		  • critical for citrulline: 111 (≥100)
			• Second card not sent due to critical result	• Sent at five days of life: normal

Initial follow-up labs	• At 20 days of life:
	  • ammonia level: 33 (≤49)
	• PAA:
	  • citrulline: 242 (10–45)
	  • glutamine: 760 (376–709)
	• UAA:
	  • citrulline: 302 (3–20)
	  • leucine: 44 (9–22)
	  • glutamine: 114 (44–118)
	• UOA:
	  • trace excretion of uracil, no orotic acid
	• PerkinElmer© expanded NBS:
	  • citrulline: 193 (<76)
		• At 14 days of life:
		  • ammonia level: 11 (≤49)
		• PAA:
		  • citrulline: 151 (10–45)
		  • tyrosine: 194 (55–147)
			• At 8 days of life:
			• ACP: normal
			• UOA: normal
			• PerkinElmer© expanded NBS: normal
			• Medical Genetics continued to see due to unrelated NBS concerns
				• At 2 days of life:
				  • ammonia at birth hospital: 340 (20–57)
				• Ammonia at CHKD: 112 (≤49)
				• Carnitine total 24.1 (20–70) and free 10.7 (18–58)
				• PAA:
				  • ornithine 1606 (48–211)
				  • arginine 1129 (6–140)
				  • tyrosine 454 (55–147)
				  • citrulline: normal
				• UOA: abnormal
				  • disruption of FAO either due to a primary defect or secondary to mitochondrial dysfunction
				• ACP
				  • C5DC: 0.27 (<0.04)
				  • C6-C10: multiple elevations
				  • C12-C18: multiple elevations

Table 1. *Cont.*

	Patient A	Patient B	Patient C	Patient D

Second follow-up labs	• At 3 weeks of age:
	  • ammonia level: 27 (≤49)
	• PAA:
	  • citrulline: 215 (10–45)
	  • glutamine: 731 (376–709)
		• At 16 days of life:
		  • ammonia level: <9 (≤49)
		• PAA:
		  • citrulline: 192 (10–45)
		  • glutamine: 760 (376–709)
		• UAA:
		  • citrulline: 69 (3–20)
		  • glutamine: 166 (44–118)
		• UOA: normal
		• PerkinElmer© expanded NBS: citrulline: 132 (<76)
			• During intercurrent illnesses over the next several years:
			  • ACP revealed elevations in:
			  • C14-OH
			  • C16:1-OH
			  • C18-OH, C18:1-OH, C18:2-OH
			• In a well state ACP is normal
			• On levocarnitine for low carnitine levels:
			  • total carnitine: 14 (25–69)
			  • free carnitine: 12 (16–60)
				• At 4 days of life:
				  • ammonia: 32 (≤49) (after arginine and Ammonul)
				• At 6 days of life:
				• PAA:
				  • several low values, not suggestive of an IEM and reflected decreased protein intake.
				• UOA: normal
				• PerkinElmer© expanded NBS: normal

Third follow-up labs	• At 5 weeks of age:
	  • ammonia level: 9 (≤34)
	• PAA:
	  • citrulline: 189 (3–35)
	  • glutamine: normal
		• At 3 weeks of age seen in ED due to congestion and fever:
		  • ammonia level: 28 (≤49)
		• PAA:
		  • citrulline: 111 (10–45)
		  • glutamine: normal
		• UAA:
		  • interfering substances, unable to interpret
		• UOA:
		  • abnormal secondary to bacterial metabolism and antibiotics
			• At 3 years old, after WES was complete, patient underwent a skin biopsy for FAOD testing.
			• FAOD testing on fibroblasts:
			  • C12 0.052 nmol/mg/protein (control <0.034)
			  • C14 0.033 nmol/mg/protein (control <0.034)
			  • C16 0.154 nmol/mg/protein (controls <0.269)
			  • these findings were consistent with a late-onset or mild variant of Very long chain acyl-CoA dehydrogenase deficiency (VLCADD)
				• At 1 week of age:
				  • while on levocarnitine: total: 67 (32–62) and free: 50 (25–54)
				• UOA: normal
				• ACP:
				• C6: 0.19 (<0.10)
				• C8DC: 0.04 (<0.02)
				• PAA: normal
				• At 3 months old:
				  • off levocarnitine: total: 38 (32–62) and free: 27 (25–54)

Table 1. *Cont.*

	Patient A	Patient B	Patient C	Patient D

Genetic testing follow-up	• Gene sequencing panel of 41 genes associated with hyperammonemia/UCD:
	  • one known disease causing mutation in ASS1 (citrullinemia), R363Q
	  • one variant, likely disease causing in CPS1 (carbamoylphosphate synthetase I deficiency), T550S
	  • one variant of unknown significance in NAGS (N-acetylglutamate synthase deficiency), E61G
		• Gene sequencing panel of 41 genes associated with hyperammonemia/UCD:
		  • one variant, likely disease causing in ASS1 (citrullinemia), L195P
		  • one variant of unknown significance in ACADVL (VLCADD), E534K
			• Whole exome sequencing was performed due to other issues including developmental delay, central hypotonia, renal pelviectasis, an arachnoid cyst, and macrocephaly:
			  • one disease causing mutation in HADHA (LCHADD/TFP), G1528C
			  • one variant, likely damaging in HADHA (LCHADD/TFP), G1748A
				• Common MCADD mutation testing by PerkinElmer©:
				  • one copy of A985G common mutation
				• Fatty Acid Oxidation Disorder Panel of 15 genes with sequencing and deletion/duplication
				  • one disease causing mutation in ACADM (MCADD) A985G
				  • one disease associated variant in ACADS (SCAD) C511T

Current clinical findings	• Last seen at 13 months old and doing well • Given metabolic sick letter • Follow up in outpatient clinic in one year	• Last seen at four months old and doing well • Given metabolic sick letter • Follow up in outpatient clinic in one year	• Last seen at 4.5 years old and doing well from a metabolic standpoint. Still has unrelated developmental delays • Given metabolic sick letter • Follow up in outpatient clinic every six months	• Last seen at one year old and doing well • Given a metabolic sick letter • Follow up in outpatient clinic every six months

Ammonia levels given in μmol/L; NBS (newborn screening) results given in μmol/L; PAA (plasma amino acids) given in μmol/L; UAA (urine amino acids) given in mmol/molCRT; ACP (acylcarnitine profile) given in μmol/L; carnitine levels given in μmol/L; UOA (urine organic acids); FAOD (fatty acid oxidation disorder).

At day of life 20, the baby was seen by Medical Genetics for follow-up. At this time, plasma amino acids (PAA), urine amino acids (UAA), and urine organic acids (UOA) were collected and sent to The University of Maryland Biochemical Genetics Laboratory and a PerkinElmer© expanded newborn screening test was obtained. PAA showed minimal elevations of several amino acids and significantly elevated citrulline (see Table 1). Glutamine was slightly elevated and UAA showed nonspecific variations of numerous amino acids including a marked elevation of citrulline. There was also a minimal elevation of leucine, which can be suggestive of argininosuccinic aciduria, as arginosuccinate coelutes in this region, but higher values are typically expected. Glutamine was minimally elevated, another finding in possible UCDs. UOA showed trace excretion of uracil; however, no orotic acid was detected. Excretion of uracil can also be associated with UCDs. Results of the PerkinElmer© expanded newborn screening test showed an elevated citrulline. An ammonia level done that day returned again normal at 27 μmol/L.

At the recommendation of The University of Maryland, repeat PAA were obtained two days later at 22 days of age, and again showed elevations of several amino acids including a moderate elevation of citrulline. Glutamine was slightly elevated and there was no evidence of argininosuccinic acid or its anhydrides in this sample.

At five weeks of age, the baby was seen again in the clinic; a repeat ammonia level was obtained and had a value of 9 μmol/L (normal $\leq$ 34 μmol/L). PAA showed minimal elevations of several amino acids including a moderate elevation of citrulline, continuing a downward trend. Glutamine was now within normal limits for the age. At this point, a genetic testing panel for metabolic disorders was sent to GeneDx (Gaithersburg, MD, USA) which included 41 genes associated with hyperammonemia/UCD. Concurrent sequencing revealed the baby was heterozygous for one known disease causing mutation *ASS1*, one variant (likely disease-causing) in *CPS1* (MIM 608307), and one variant, likely benign, of unknown significance in *NAGS* (associated with *N*-acetylglutamate synthase deficiency; MIM 237310). Deletion/duplication testing for *CPS1*, *ASS1* and *NAGS* was negative.

The patient was seen again in the Medical Genetics clinic at three months of age. She had not had any intercurrent illnesses or fevers, was thriving, and was developmentally appropriate. It is clear at this time that while Patient A does not have classic citrullinemia, her carrier status for an UCD appears to be responsible for her abnormal NBS and other abnormal test results. The mother has been given a metabolic emergency letter and was instructed to be extra vigilant during times of illness. Our office will follow up yearly until a time when further recommendations are established.

2.2. Patient B

Patient B was a female infant born at 36 weeks of gestation. The initial NBS was collected was abnormal for citrulline. A repeat newborn screen was sent at day of life 8, and was critical for citrulline. Medical Genetics was contacted by the state and the critical lab value was reported. An ammonia level was obtained which was found to be normal with a value of 11 μmol/L (normal $\leq$49 μmol/L). PAA were collected and sent for immediate analysis. Since the baby was stable and had a normal ammonia level, she was discharged home with no change to her diet and instructed to follow up with the primary care physician and Medical Genetics outpatient clinic.

Two days later, at two weeks of age, Patient B was seen in the outpatient Medical Genetics clinic. At that time, the initial PAA had demonstrated minimal variation of several amino acids including citrulline and tyrosine (see Table 1). Higher levels of citrulline are typically seen in classic citrullinemia and elevated tyrosine can be see secondary to diet and/or clinical status. Given the elevation of citrulline, an ammonia (which was normal at a value of <9 μmol/L), PerkinElmer© expanded newborn screening card, UAA, UOA and follow-up PAA were ordered. The PAA again showed minimal to moderate elevation of citrulline, UOA were normal, and UAA showed nonspecific variations of numerous amino acids including a moderate elevation of citrulline and glutamine. The PerkinElmer© expanded newborn screening test was also presumptive positive with an elevated citrulline.

At three weeks of age, Patient B presented to an outside emergency room with a fever and congestion. She was transferred to our center where a complete sepsis workup was initiated. An ammonia level was immediately drawn and had a normal value of 18 μmol/L (normal ≤49 μmol/L). PAA, UOA, and UAA were also obtained. Results of the PAA demonstrated a minimal elevation of citrulline, and glutamine was now within normal limits for the age. No argininosuccinic acid was detected. The UAA profile was not able to be interpreted due to the presence of antibiotic metabolites and UOA was notable for bacterial metabolites and antibiotic artifacts. During Patient B's inpatient stay, a hyperammonemia/UCD panel with 41 genes was sent for sequencing and deletion/duplication studies to GeneDx (Gaithersburg, MD, USA). Sequencing revealed the baby was heterozygous for one *ASS1* (MIM 603470) variant, likely disease-causing, and one variant of unknown significance in the *ACADVL* (MIM 609575) gene. As with Patient A, mutations in the *ASS1* gene are associated with the autosomal recessive disorder classic citrullinemia. Mutations in the *ACADVL* gene are associated with the autosomal recessive disorder very-long-chain acyl-CoA dehydrogenase deficiency (VLCADD). Deletion/duplication testing of *ASS1* and *ACADVL* was normal.

Patient B was seen for a follow-up visit in the Medical Genetics clinic at four months of age. In the interim time, she had been hospitalized for a sepsis work-up secondary to fever, with her ammonia levels remaining normal. Since that hospitalization she has done well with no changes to diet and had normal development. She has since had several intercurrent illnesses, but through each her ammonia levels have remained normal. During a hospitalization at seven months of age for viral pneumonia, her ammonia was again normal and PAA were ordered. Citrulline remained slightly elevated (see Table 1). No other elevations were seen. It is clear that Patient B does not have classic citrullinemia. She is a carrier for a UCD which may be responsible for her abnormal NBS and other abnormal test results. She is currently doing well and continues to be seen yearly.

2.3. Patient C

Patient C was a male born at 39 weeks of gestation after a prenatal course complicated by hydronephrosis, pelviectasis, an arachnoid cyst and concern for ventriculomegaly. Complications in the neonatal period included low temperatures and weight loss. The infant was transferred to the level II nursery and evaluated for a ventricular septal defect, neonatal sepsis, an episode of hypocalcemia (which normalized after adequate oral intake) and mildly dysmorphic features including macrocephaly, frontal bossing and low-set ears. Patient C was discharged home on day of life 4 with appointments to follow up with multiple specialists, including Medical Genetics.

Patient C's first NBS was collected after 24 h of life and was flagged critical for multiple elevations in the FAOD profile, including C14, C14:1, C16-OH, and C18:1-OH (see Table 1). These elevations are most consistent with a diagnosis of VLCADD or LCHADD/TFP. The Medical Genetics division was contacted by the state and the critical lab values were reported. Patient C was immediately seen by his primary care physician and UOA, acyl carnitine profile (ACP) and a PerkinElmer© expanded newborn screening card were collected. The baby was appeared well on the exam; the parents were advised to continue frequent feeds Results of the UOA were normal, as were the results for the ACP and PerkinElmer© card.

Several years later, at three years of age, Patient C returned to the Medical Genetics outpatient clinic. During the interim period he had been seen by another institution that ordered whole exome sequencing (WES) due to developmental delay, central hypotonia, a ventricular septal defect, macrocephaly, and the history of the arachnoid cyst and renal pelviectasis. WES found Patient C was a carrier for two FAOD gene changes as well as other variants unrelated to metabolic conditions. He was found to be a compound heterozygote for two variants of unknown significance in *HADHA* (MIM 600890), both of which are predicted to be damaging. Mutations in the *HADHA* gene are associated with the autosomal recessive disorder LCHADD/TFP. Patient C, who has a history of other clinically significant medical problems, has never been formally diagnosed with LCHADD/TFP. Follow-up ACPs had shown slight elevations for C14-OH, C16:1-OH, C18-OH, C18:1-OH and

C18:2-OH when he has had an intercurrent illness. When well, his ACP is normal. He continues to takes levocarnitine daily (see Table 1). In addition to the above changes, the proband was found to be heterozygous for a novel variant in the *SYNGAP1* gene (p.S955F) associated with Autosomal Dominant Mental Retardation syndrome type 5 (MIM 612621); this change was maternally inherited. This novel variant is not felt to be significant as it has not been previously reported in association with disease. Pathogenic variants in the *SYNGAP1* gene have been reported as mostly de novo truncating heterozygous variants. Individuals who inherited variants in the *SYNGAP1* gene did so from an affected parent (MIM 612621). Patient C's mother is felt to be healthy, without any cognitive or developmental concerns.

After receiving the WES results, Patient C underwent a skin biopsy for FAOD testing to see if a diagnosis of LCHADD/TFP had been missed. His results showed only elevated values of C12 with normal values of C14 and C16. These findings were consistent with a late-onset or mild variant of VLCADD. At this time, we acknowledge that Patient C's carrier status for the two mutations in *HADHA* is likely responsible for his critical NBS. What is unusual is that his enzyme activity is consistent with a mild variant of VLCADD; however, his genetic testing shows variants in *LCHADD/TFP* gene. We are following Patient C every six months in clinic and the family was provided a metabolic emergency letter.

2.4. Patient D

Patient D was a male infant born at 39 weeks of gestation after an uncomplicated pregnancy. At day of life 2, while still at the birth hospital, the baby developed poor feeding and tachypnea. An ammonia level was drawn and was elevated at 476 μmol/L (normal <49 μmol/L). He was started on dextrose-containing fluids due to concerns of a possible UCD. His ammonia level upon arrival to our center was 112, after 2–3 h of dextrose-containing IV fluids, and continued to decrease after starting Ammonul and IV arginine. The ammonia levels remained in the 70–80 s·μmol/L range over the next two days and then normalized by day of life 4.

The state NBS, collected after 24 h of life, was flagged as critical for C8 and C10, consistent with a critical screen for MCAD. A repeat NBS at day of life 5 was normal.

Additional labs were ordered upon arrival to our medical center including total and free carnitine levels as well as PAA, which showed significant elevations of ornithine, arginine, and a moderate to marked elevation of tyrosine (see Table 1). Citrulline was within normal limits. The overall pattern was not consistent with a UCD. UOA were abnormal and suggested a disruption in fatty acid oxidation either due to a primary defect or secondary to mitochondrial dysfunction. ACP results demonstrated a moderate to marked elevation of several acylcarnitine species including C5DC, C6–C10 and long- and very-long-chain acylcarnitines (C12–C18). Follow-up PAA drawn at day of life 6 showed several low values, possibly reflecting decreased protein intake, but was not suggestive of an IEM. A repeat UOA and a PerkinElmer© expanded NBS card were essentially normal and were not suggestive of an IEM. DNA testing of the common mutations in *ACADM* (MIM 607008), the gene associated with autosomal recessive MCADD, revealed one copy of the common A985G mutation.

Patient D was seen in the Medical Genetics outpatient clinic when he was two weeks of age; he was feeding and growing well. At discharge, Patient D was started on levocarnitine for low carnitine levels. Additional labs were obtained at the outpatient visit including total and free carnitine levels and UOA, both of which were within normal limits, and an ACP which showed several minor elevations in C4DC, C4-OH, C5DC, C5-OH, C6, C8DC, C12-OH, C14-OH, C14:1-OH and C16:1 but which were not consistent with a specific disorder. PAA were within normal limits for age.

Given the non-specific test results, a genetic testing panel of 15 genes associated with FAODs was sent to GeneDx (Gaithersburg, MD, USA). Sequencing revealed the same disease-causing mutation in *ACADM* that was found previously by PerkinElmer©. In addition, another disease-associated variant in *ACADS* (MIM 606885), the gene associated with autosomal recessive short-chain acyl-CoA dehydrogenase deficiency (SCADD), was identified. Deletion/duplication testing was normal.

The infant was followed up in the Medical Genetics clinic at four months of age. He had continued to do well with no significant intercurrent illnesses. Lab studies ordered at that visit were an ACP which showed increases in several long- and very-long-chain acylcarnitine species (C12–C18), which was more suggestive of VLCADD. Acylcarnitine C6, C8DC and C10 were also mildly elevated but not as high as those typically seen with classic MCADD. Total and free carnitine levels were normal off of levocarnitine supplementation. At his last follow-up, at a little over a year of age, he was walking, talking, and was felt to have completely normal development and health.

While it is clear that Patient D does not have an IEM, he is a carrier of mutations of two separate FAODs. His carrier status is likely responsible for his critical NBS and hyperammonemia in the newborn period. Our office continues to follow up yearly until a time when further recommendations are established. The family was also given a metabolic emergency letter.

3. Methods

The Virginia Newborn Screening Program utilizes three tiers of reference ranges: normal, abnormal and critical. Typically, abnormal screening results require a repeat NBS. Critical metabolic screening results are called to both the PCP and regional metabolic specialist. Diagnostic follow-up is recommended as soon as possible. The newborn screening program will follow-up on abnormal and critical results with the PCP and specialists at one, three, and five months or until diagnosis is obtained.

A chart review of the four probands was undertaken and pertinent past medical history, lab trends, and clinical follow-up was obtained and reviewed.

Plasma amino acids were measured at the University of Maryland Biochemical Genetics laboratory by ion exchange high-performance liquid chromatography with post-column ninhydrin detection [7].

Fibroblast analysis on Patient C was performed by Mayo Medical Laboratory in Rochester, MN. Skin fibroblasts were incubated with cell medium enriched with palmitic acid (C16:0 fatty acid), L-carnitine, and isotopically labeled L-valine (^{13}C-Val) and L-isoleucine (^{13}C-Ile). The medium was separated from the cells following the incubation. The cell pellet was used for protein determination and the medium was be spotted and dried on filter paper. An acylcarnitine analysis was performed by tandem mass spectrometry (MS/MS) using a one-quarter-inch filter paper punch, following the addition of isotopically labeled acylcarnitines as internal standards, extraction and derivatization to methyl esters. The assay was performed in triplicate [8].

4. Discussion

Newborn screening, one of the greatest public health initiatives in the last century, has the potential for creating more questions than answers, as evidenced by the current state of genetic testing. Through the ACT flowsheets, the ACMGG has created clear protocols for management of newborns with critical newborn screening [2]. How to manage infants with newborn screens that are critical, yet never yield a definitive diagnosis, however, is less clear. We report these four patients to illustrate the complexity of uncovering carriers for multiple IEMs, two who were symptomatic at the time around birth, and two who never manifested any clinical features of an inborn error.

The purpose of NBS is to provide information to help clinicians better manage patients in the newborn period [4]. Molecular testing, which is promoted as leading to a better understanding of genetic disease in cases where the answer is not clear, can, in actuality, lead to conflicting results.

It is yet unclear if individuals who are found to be double heterozygotes for two different IEMs have the potential to become symptomatic at times of illness or metabolic stress. This may be less of a possibility in individuals who are carriers for disorders in two different pathways (i.e., a UCD and a FAOD, as seen with patient B); there may be more concern for individuals such as patients A, C, and D, who were all found to carry mutations in genes within the same metabolic pathway. It is also possible that a mother's disease status, the stress surrounding the delivery, poor feeding, prematurity or other complications may contribute to the critical levels seen on the newborn screen.

These circumstances may not be replicated in the same way as the child grows, and the impact of the double heterozygote status may not be an issue later on.

We propose that providers should take caution when ordering molecular testing on newborns with critical newborn screens. This is not to say that molecular testing is not warranted, but rather that when counseling the families and anticipating results, one should be prepared for a less-than-diagnostic result. Infants who are found to be double heterozygotes following a critical newborn screen for analytes consistent with one or more conditions should be monitored closely for any signs of metabolic decompensation, including lethargy, poor feeding, hypothermia, hypoglycemia, or seizures. In instances where the urea cycle pathway is at play, a baseline ammonia level should be obtained, and repeated if there are signs of illness. It is not unreasonable to provide the family with a metabolic emergency letter should the child present to the emergency room with an intercurrent illness so that the appropriate labs can be drawn and the metabolic provider can be notified.

All four of these patients have continued to grow and develop well despite intercurrent illnesses and routine outpatient surgeries. Fortunately, none of the patients have had episodes of metabolic decompensation. Their health may be due to more vigilance by parents or providers, or by chance that they have not had a significant illness that could trigger decompensation. Alternatively, due to their compound heterozygous carrier statuses, they may never have complications from their carrier states. At this time it is impossible to determine which, if any, of these scenarios hold true.

Patient C has some moderate speech delay, but there is evidence to suggest he has some other issues complicating his development apart from the carrier status for the two FAODs. After the first few visits in Medical Genetics, we have continued to follow the other three patients in the clinic annually. We have instructed the families to be vigilant for signs and symptoms out of proportion to what would be expected in instances when the child becomes ill with fever, vomiting, poor oral intake, etc. At this time there are no guidelines for the management and follow-up of individuals who are double heterozygotes for IEMs. We recommend to clinicians who uncover double heterozygotes that a thorough initial work-up is warranted, with close subsequent follow-up, ideally every few months. Finally, families should be provided with an emergency letter and strongly encouraged to contact their metabolic physician with concerns should the child become ill.

Molecular testing, while a valuable tool in the practice of medical genetics and metabolism, may not necessarily lead to a better understanding of metabolic test results in newborns with critical newborn screens, and thus should be ordered with caution, while keeping the clinical picture of the patient at the forefront of decision-making.

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Abbreviations

UCD	urea cycle disorder
CIT	citrullinemia
ASA	arginosuccinic aciduria
NBS	newborn screen
IEM	inborn errors of metabolism
SCID	severe combined immunodeficiency
FAOD	fatty acid oxidation disorder
CUD	carnitine uptake deficiency
MCADD	medium chain acyl-CoA dehydrogenase deficiency

VLCADD	very long chain acyl-CoA dehydrogenase deficiency
LCHADD	long-chain 3-hydroxyacyl-Coa dehydrogenase deficiency
TFP	tri-functional protein deficiency
NAGS	*N*-acetylglutamate synthase
UOA	urine organic acids
UAA	urine amino acids
PAA	plasma amino acids
ACP	acylcarnitine profile

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