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ORIGINAL ARTICLE

Primary and maternal 3-methylcrotonyl-CoA carboxylase deficiency: insights from the Israel newborn screening program

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Abstract
Background 3-Methylcrotonyl-CoA carboxylase deficiency (3MCCD) is an inborn error of leucine catabolism. Tandem mass spectrometry newborn screening (NBS) programs worldwide confirmed 3MCCD to be the most common organic aciduria and a relatively benign disorder with favorable outcome. In addition, several asymptomatic 3MCCD mothers were initially identified following abnormal screening of their healthy babies and were appropriately termed maternal 3MCCD.
Methods This is a retrospective study that summarizes all the clinical, biochemical, and genetic data collected by questionnaires of all 3MCCD individuals that were identified by the extended Israeli NBS program since its introduction in 2009 including maternal 3MCCD cases.
Results A total of 36 3MCCD subjects were diagnosed within the 50-month study period; 16 were classified primary and 20 maternal cases. Four additional 3MCCD individuals were identified following sibling screening. All maternal 3MCCD cases were asymptomatic except for one mother who manifested childhood hypotonia. Most of the primary 3MCCD individuals were asymptomatic except for two whose condition was also complicated by severe prematurity. Initial dried blood spot (DBS) free carnitine was significantly lower in neonates born to 3MCCD mothers compared with newborns with primary 3MCCD (p=0.0009). Most of the mutations identified in the *MCCC1* and *MCCC2* genes were missense, five of them were novel.
Conclusions Maternal 3MCCD is more common than previously thought and its presence may be initially indicated by low DBS free carnitine levels. Our findings provide additional confirmation of the benign nature of 3MCCD and we suggest to exclude this disorder from NBS programs.

Introduction

3-Methylcrotonyl-CoA carboxylase deficiency (3MCCD, OMIM #210210) is an autosomal recessive inborn error of leucine catabolism. Initial reports described patients who

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suffered from metabolic crises including Reye-like episodes, hypoglycemia with or without acidosis, ketosis, and fasting intolerance. Affected children are also thought to be at risk for suffering from hypotonia (most probably due to secondary carnitine deficiency), seizures, and varying degrees of developmental and psychomotor delay (Bannwart et al 1992; Elpeleg et al 1992; Ficicioglu and Payan 2006; Grunert et al 2012; Layward et al 1989). The disorder is associated with mutations in either *MCCC1* (MIM #609010) or *MCCC2* (MIM #609014) genes, which encode for the α and β subunits of the mitochondrial enzyme methyl crotonyl-CoA carboxylase (MCC, EC 6.4.1.4) respectively (Baumgartner et al 2001).

Since the introduction of 3MCCD into the panel of extended newborn screening (NBS) by measuring bloodspot levels of 3-hydroxyisovalerylcarnitine (C5OH), it turned out to be one of the most common organic acidemias with a prevalence variably ranging between 1:2400 (Thomsen et al 2015) and 1:68,333 (Yang et al 2014). Yet despite the increasing numbers of newly diagnosed subjects, mostly due to national NBS programs, a growing number of reports published in recent years have shown that most individuals diagnosed by NBS are in fact asymptomatic (Arnold et al 2008, 2012; Jung et al 2012; Lam et al 2013; Ye et al 2014). Moreover a significant number of newborns that initially screened positive for 3MCCD were actually reflecting a deficiency of the enzyme in their mothers, who were typically asymptomatic (Gibson et al 1998; Gong et al 2013; Koeberl et al 2003; Lee and Hong 2014). This phenomenon, which further emphasizes the benign nature of the disorder, was termed "maternal 3MCCD" as opposed to primary 3MCCD.

As evidence grows, considerable doubts have been raised as to the real nature of 3MCCD, suggesting it merely features a biochemical rather than a clinical phenotype and therefore should be excluded from extended NBS programs. Furthermore, evidence-based clinical guidelines regarding the optimal management and treatment are lacking (Arnold et al 2008; Grunert et al 2012).

In Israel 3MCCD was introduced into the national extended NBS in October 2009 (Department of Community Genetics 2012). In the current study we reviewed the relevant available data of all the subjects who were diagnosed by NBS with either primary or maternal 3MCCD in Israel. Our primary objective was to characterize the epidemiological, clinical, biochemical, and molecular features of the disorder in the Israeli population. In addition, we aimed to reach an evidence based recommendation as to whether to keep 3MCCD among the disorders included in our national NBS panel.

Materials and methods

Study cohort

Samples for the Israeli National Newborn Screening Program are collected at 36–72 h from birth. Concentrations of C5OH and free carnitine were measured from dried blood spots (DBS) extract by a non-derivatized method using tandem mass spectrometry (Waters micromass MS technologies, Quattro Premier XE, Elstree, UK).

The study group consisted of all individuals diagnosed with 3MCCD since the disorder was introduced into the Israeli extended NBS test panel in October 2009. All the newborns who were screened positive for 3MCCD, as defined by elevation of C5OH above 1.0 μM, underwent a confirmatory analysis that included a follow-up acylcarnitine profile by tandem mass spectrometry to test the level of C5OH and urinary organic acid analysis using gas chromatography/mass spectrometry to test for the metabolites 3-hydroxyisovaleric acid (3-HIVA) and 3-methylcrotonylglycine (3-MCG). Only when primary 3MCCD was ruled out in the babies, acylcarnitine profile and urinary organic acids analysis was assessed in their mothers for the possibility of maternal 3MCCD. Also included in the study group were older siblings of subjects diagnosed by the NBS. Consequently, we divided the study cohort into two subgroups as follows: infants with primary 3MCCD and mothers with maternal 3MCCD who were initially diagnosed following abnormal screening of their newborns.

Data collection

The study was approved by the local ethics review board (EMC-148-12). We collected the relevant epidemiological, clinical, and genetic data by means of reviewing questionnaires that were completed by the treating physicians of the subjects included in the study group. Clinical data regarding maternal 3MCCD subjects was obtained by retrospective review of their medical records.

Statistical analysis

We used the Wilcoxon rank-sum test in order to compare initial free carnitine (C0) and C5OH measured in neonatal DBS between the two subgroups of "primary 3MCCD" and "maternal 3MCCD". We considered $p<0.05$ statistically significant. The statistical analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

3MCC genetic analysis

Molecular analysis was offered for all families with a confirmed diagnosis of 3MCCD as defined above. After obtaining written informed consent from affected individuals (age>18y) or their parents or legal guardians (age<18y), DNA was extracted from EDTA-blood samples using the FlexiGene DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Direct sequencing of the two genes *MCCC1* and *MCCC2* was performed on an ABI 3130 capillary sequencer (Applied Biosystems, Foster City, CA) after

PCR amplification of exons 1–19 and the flanking intronic regions of *MCCC1* and exons 1–17 and the flanking intronic regions of *MCCC2* (detailed conditions and primer sequences are available on request).

Results

Epidemiology

Between October 2009 and December 2013, 36 new cases of 3MCCD were identified in Israel by NBS. The diagnosis was further confirmed by biochemical studies (i.e., urinary organic acids and repeated DBS acylcarnitine profile) and when available by molecular studies of *MCCC1* and *MCCC2*. In two cases (A10 and A11), diagnosed with primary 3MCCD by NBS, further evaluation of first degree family members identified four additional subjects with 3MCCD. Three siblings of individual A10 were diagnosed at the age of 4, 8, and 9 years and one sibling of individual A11 was diagnosed at the age of 5 years. Altogether 40 new cases of 3MCCD were identified during the study time period.

Among the subjects diagnosed by NBS, 16 were subsequently found to have primary 3MCCD (ten males and six females) and are designated and numbered A1-A16 accordingly, while the other 20 who tested positive are designated and numbered B1-B20 accordingly, were eventually diagnosed with maternal 3MCCD (i.e., their mothers are the affected individuals). The affected mothers are designated M(B1-B20) accordingly.

Throughout the study period a total of 694,018 babies were born in Israel making the calculated prevalence of primary 3MCCD in Israel 1:43,000. The ethnic distribution included 20 subjects of Jewish origin (56 %), nine subjects of Moslem Arab origin (25 %), six Bedouin subjects (17 %), and a single subject of Israeli Druze origin (2 %).

Clinical features

Sixteen of 20 individuals with primary 3MCCD are in regular follow up by means of clinical visits. The age distribution of these 16 subjects at last clinical visit ranges between 2.5 and 5.3 years with a median follow-up period of 4.2 years. The remaining four individuals were withdrawn from regular follow as their parents chose to discontinue clinical visits. Apart from three subjects, all have normal development and have had no metabolic crises. One patient is severely disabled following a car accident not related to the metabolic defect. Two individuals born extremely premature (below 30 weeks gestation) suffer from moderate (A15) and severe (A11) developmental delay that is attributed to the sequelae of their premature birth. Two siblings (A10 and his brother) both had consistently elevated serum levels of creatine kinase (CK) (around 400 IU/L, reference range <200 IU/L). They are clinically asymptomatic and had no skeletal muscular complaints or manifestations such as exercise intolerance or fatigue. Both have low levels of serum C0 for which they receive regular carnitine supplementation. A total of eight primary 3MCCD individuals were treated with oral carnitine after the diagnosis. The therapy in four of them was later discontinued and the rest are still receiving carnitine supplementation. Of the 20 mothers diagnosed with maternal 3MCCD 19 are completely asymptomatic. One mother who currently considers herself healthy was diagnosed with childhood hypotonia due to 3MCCD in the past (Elpeleg et al 1992). As she did not report this in the delivery room, she was re-diagnosed when her baby screened positive. One of the asymptomatic mothers with very low C0 (M(B7)) is receiving carnitine supplementation, whereas all the other mothers are not being supplemented despite low C0 levels.

Comparison of initial carnitine levels

Elevated concentration of C5OH is the prime biochemical biomarker of 3MCCD on NBS. It is usually associated with significantly low concentrations of C0. In keeping with the high prevalence of maternal 3MCCD observed in the current study, we aimed to differentiate between the neonates who suffer from primary vs. maternal 3MCCD as early as possible. Hence, in our study, we compared the concentrations of the initial C0 and C5OH as measured in the initial DBS of neonates from both groups (maternal and primary 3MCCD). The results are summarized in Tables 1 and 2, respectively:

The mean concentration of initial neonatal C0 in maternal 3MCCD was significantly lower compared with the group of newborns with primary 3MCCD (7.27 μM vs 18.97 μM, p= 0.0009). Additionally, the ratio of C0/(C0+C5OH) was significantly higher in the primary 3MCCD compared with the maternal 3MCCD (71.42 vs.50.69, p=0.0012). However, there was no significant difference in the initial levels of C5OH between both groups (6.16 μM vs. 6.01, p=0.84). Accordingly, the initial levels of bloodspot C0 and the C0/C0+C5OH ratio are probably helpful in distinguishing between primary and maternal 3MCCD.

Molecular studies

A total of 13 subjects who were diagnosed with 3MCCD following NBS had complementary molecular studies of *MCCC1* and *MCCC2* genes (Table 3). In the other 23 cases the families did not consent to further genetic analysis. Additionally, 3MCCD was confirmed molecularly in a further three siblings of an affected subject and in a sister of another affected subject. Altogether, all 17 subjects who had diagnostic genetic testing, carried bi-allelic mutations in either *MCCC1* (11 individuals) or *MCCC2* (six individuals), compatible with

Table 1 Summary of initial DBS carnitine analysis in children with primary 3MCCD

Subject	Gender	Average free carnitine level (Co) μM	Average conjugated carnitine level (C5OH) μM	Co/(Co+C5OH) %
A1	F	7.88	8.58	47.87
A2	F	10.30	6.00	63.18
A3	F	19.27	2.01	90.55
A4	M	23.91	10.45	69.58
A5	M	30.15	10.81	73.62
A6	F	17.13	10.39	62.24
A7	M	18.07	8.67	67.58
A8	M	14.57	1.99	88.01
A9	M	10.09	5.52	64.63
A10	M	25.13	8.46	74.81
A11	M	3.26	5.26	38.26
A12	F	12.88	9.24	58.22
A13	M	19.85	1.50	92.97
A14	F	9.50	4.54	67.66
A15	M	73.38	1.32	98.23
A16	M	8.21	1.42	85.30
Total average		18.97	6.01	71.42
Mean		15.85	5.76	68.62
SD		16.20	3.56	16.52
Range		3.26–73.38	1.32–10.81	38.26–98.23

M male, *F* female, *SD* standard deviation

Table 2 Summary of initial DBS carnitine analysis in newborns with maternal 3MCCD

Subject	Gender	Average free carnitine level (Co) μM	Average conjugated carnitine level (C5OH) μM	Co/(Co+C5OH) %
B1	F	2.52	7.69	24.66
B2	M	3.35	6.17	35.19
B3	M	3.59	1.75	67.29
B4	M	3.53	6.12	36.57
B5	F	3.32	2.91	53.25
B6	M	9.67	12.21	44.20
B7	M	5.52	4.91	52.90
B8	M	23.04	10.15	69.41
B9	M	3.79	8.66	30.43
B10	M	4.10	11.13	26.90
B11	M	20.64	1.97	91.29
B12	F	8.26	9.02	47.81
B13	F	3.80	4.68	44.81
B14	F	13.33	1.57	89.46
B15	F	9.17	5.88	60.92
B16	F	3.57	4.97	41.83
B17	F	7.29	6.40	53.25
B18	F	3.03	5.17	36.91
B19	F	5.51	6.18	47.13
B20	F	8.45	5.75	59.51
Total average		7.27	6.16	50.69
Mean		4.80	6.00	47.47
SD		5.75	2.98	18.41
Range		2.52–23.04	1.57–12.21	24.66–91.29

M male, *F* female, *SD* standard deviation

autosomal recessive inheritance. Overall, we identified a total of 11 different mutations of which five were novel and six were previously reported pathologic mutations (Table 3). We identified two nonsense mutations: c.994 C>T p.Arg332 stop in *MCCC2* and a deletion of 23 nucleotides in *MCCC1;* both were predicted to result in a premature stop codon and truncation of the encoded relevant MCC subunit. The rest of the mutations were missense and were predicted to be deleterious, except for one variant c.1391A>C in *MCCC1* predicted to be a non-pathologic polymorphism by both MutationTaster and PolyPhen 2. Nevertheless, since both individuals who harbored this variant had classical biomarkers compatible with 3MCCD we decided to regard this variant as a pathologic mutation. Notably, all *MCCC2* missense mutations were predicted to disrupt the carboxyl transferase domain and the missense mutations in *MCCC1* were predicted to disturb the biotin carboxylation site.

Three mutations recurred in more than one family: one in the Arab population (c.1271 A>T in *MCCC1*, p.Asp424Val in two separate individuals) and two in Bukharian Jews (c.1115 A>C p.Gln372Pro and c.1522-1544 del.23 both in *MCCC1* gene). Another mutation (c.463 C>T in *MCCC2* gene, p.Arg155Trp) was found in a Tunisian Jewish patient. The same mutation was previously detected in another Tunisian Jewish patient born in 2002 who was diagnosed clinically with 3MCCD before the introduction of NBS (Grunert et al 2012).

Discussion

This study attempted to evaluate the clinical, biochemical, and molecular characteristics of all the cases identified with 3MCCD following the implementation of extended NBS in Israel in 2009. We specifically focused on the long-term clinical course of the individuals diagnosed in terms of morbidity, metabolic crises, and development. Notably, of the 36 individuals initially identified by the NBS program only 16 were primarily affected newborns and the remaining 20 cases were secondary to affected mothers (maternal 3MCCD), of whom

Table 3 Summary of *MCCC1* and *MCCC2* molecular findings

Subject	Ethnic origin	Affected gene	Mutation
M(B17)	Arab	*MCCC1*	Homozygote **c.1271 A>T p.Asp424Val**
(A11)	Druze	*MCCC2*	Homozygote c.994 C>T p.Arg332 stop
S(A11)	Druze	*MCCC2*	Homozygote c.994 C>T p.Arg332 stop
M(B7)	Arab	*MCCC2*	Homozygote **c.1123 G>A p.Val375Glu**
A9	Arab	*MCCC1*	Homozygote **c.1271 A>T p.Asp424Val**
A10	Arab	*MCCC2*	Homozygote c.994 C>T p.Arg332 stop
S(A10)	Arab	*MCCC2*	Homozygote c.994 C>T p.Arg332 stop
S(A10)	Arab	*MCCC2*	Homozygote c.994 C>T p.Arg332 stop
S(A10)	Arab	*MCCC2*	Homozygote c.994 C>T p.Arg332 stop
M(B10)	Jewish (Bukharian)	*MCCC1*	Homozygote **c.1522–1544 del.23**
M(B5)	Jewish (Tunisian)	*MCCC2*	Homozygote c.463 C>T p. Arg155Trp
A14	Arab	*MCCC2*	Homozygote **c.848 C>T p. Ala283Val**
A1	Arab	*MCCC2*	Homozygote c.652 G>A p. Ala218Thr
A13	Jewish (Morrocan)	*MCCC1*	Compound heterozygote: c.1331 G>A, p. Arg444His, c.1391 A>C p.His464Pro
M(B18)	Jewish (Bukharian)	*MCCC1*	Compound heterozygote: c.1115 A>C p.Gln372Pro, **c.1522–1544 del.23**
A3	Jewish (Bukharian)	*MCCC1*	Compound heterozygote: **c.608 T>C p.Met203Thr,** c.1115 A>C p.Gln372Pro
A8	Jewish (Bukharian)	*MCCC1*	Heterozygote c.1115 A>C p.Gln372Pro, homozygote c.1391 A>C p.His464Pro

M mother of neonate in parentheses, *S* sibling of neonate in parentheses. Novel mutations are in bold

only one mother was previously diagnosed with 3MCCD (Elpeleg et al 1992). Maternal 3MCCD has been previously reported mainly as an incidental finding following abnormal results of NBS (Koeberl et al 2003), but to our knowledge our group containing 20 maternal 3MCCD cases is the largest cohort reported so far. Therefore, the incidence of maternal 3MCCD among screen positive subjects is much higher than previously realized, at least in our population. Most importantly, and similar to previous reports (Gibson et al 1998; Koeberl et al 2003; Gong et al 2013; Lee and Hong 2014), 19 of the 20 affected mothers in our study were completely asymptomatic whereas only one displayed muscle weakness in childhood that later improved following carnitine supplementation (Elpeleg et al 1992). In this regard our results raise the concern of the over diagnosis of healthy women and in addition emphasize the dilemma of following or treating (carnitine supplementation in case of low serum carnitine) or not, these asymptomatic women.

Our results underscore the differential diagnosis of an elevated C5OH in an MS/MS acylcarnitine profile on initial bloodspot as a marker for 3MCCD deficiency, which includes primary and maternal types. In order to differentiate between the two types, urinary organic acid should be analyzed to detect elevated levels of 3-HIVA and 3-MCG in the affected individual. In the current study we show for the first time that the levels of C5OH and free carnitine on initial NBS acylcarnitine bloodspot testing may suggest the identity of the affected individual. The mean C5OH is similar in both types but the mean levels of C0 are markedly decreased in the maternal form compared with the primary form (Tables 1 and 2). This difference can be explained by the massive accumulation of C5OH, which ultimately leads to C0 depletion in both types. However, in the primary form C5OH begins to accumulate only after birth, thus explaining the modest decrease in C0, whereas in the maternal form the neonate reflects longstanding and thus more severe maternal C0 depletion.

Although the discrepancy in mean levels is statistically significant (4.8 μM vs.15.85 μM; $p=0.009$) there is nevertheless marked overlap in the observed ranges (2.52–23.04 μM vs. 3.26–73.38 μM) and therefore the mean C0 level in itself is not a reliable enough diagnostic marker and in practice should not be used by itself to differentiate between the two forms. An acylcarnitine profile and urinary organic acids assay should be obtained in all cases from both the mother and from her infant in order to verify precisely who is the affected individual. Our study clearly emphasizes that maternal 3MCCD should be considered and tested for in the diagnostic evaluation of elevated C5OH on NBS, particularly when the initial C0 level on NBS acylcarnitine bloodspot testing is very low.

Noteworthy, more than half of the individuals diagnosed biochemically in this study (metabolite analysis) were not confirmed molecularly due to either patient or guardian refusal. Although uncommon, some of them may actually reflect asymptomatic carriers (Morscher et al 2012). The birth incidence of primary 3MCCD in Israel based on our study is 1:43,000 making this disorder the most common organic aciduria in Israel. With one exception (Faroe Islands, incidence 1:2400, (Thomsen et al 2015) the Israel incidence falls within

previously reported ranges 1:40,000–1:70,000 (Lam et al 2013; Yang et al 2014). Notably, the disorder is more common in the non-Jewish population compared with the Jewish one, taking into account the ratio of 80:20 Jewish/non Jewish in the population. This may be accounted for by the high consanguinity rate, reaching as high as 45 % in the non-Jewish population, a phenomenon known to be associated with a high incidence of autosomal recessive diseases (Zlotogora 2014).

As in previous reports, the mutations identified in our subjects in both *MCCC1* and *MCCC2* were mostly missense mutations and were widely distributed in all encoding exons (Grunert et al 2012; Yang et al 2014; Dantas et al 2005; Jung et al 2012). Four mutations occurred in more than one family, suggesting a founder effect in the Bukharian Jews (two mutations) and in the Tunisian Jews (one mutation) and among the Moslem Arabs (one mutation). Future studies are needed to estimate precisely the prevalence of these mutations in each relevant population. Notably, the c.994 C>T mutation in the *MCCC2* gene occurred in one Arab Moslem family and in one Druze family which are considered two separate ethnic groups. This mutation was previously reported in a Swiss individual (Dantas et al 2005) suggesting it is a hot spot in *MCCC2* gene rather than a founder.

Since the American College of Medical Genetics and Genomics recommendation to include 3MCCD in the core panel of newborn screening (Pediatrics, 2006 Supp.), data based on these screens has begun to accumulate. The reporting of 3MCCD is somewhat controversial mainly because of its benign course (Arnold et al 2012; Lam et al 2013; Yang et al 2014). Our study supports the benign nature of 3MCCD. The relatively high number of maternal MCCD cases in the current study that were diagnosed coincidentally provides an unexpected view of the natural history of the disease. It appears that 19 of the 20 affected mothers were completely asymptomatic and their history was also unremarkable for metabolic crises, neurological impairment, intellectual disability, or other features. Moreover, although first degree siblings of those mothers were not tested for 3MCCD, their history when available was unremarkable. In addition, 17 of the 20 individuals with primary 3MCCD were completely asymptomatic. Of the three symptomatic individuals one had a medical condition clearly not related to 3MCCD, and the other two were born prematurely and their symptoms could be partially attributed to complications of prematurity. Thus, we hypothesize that 3MCCD, basically a benign metabolic condition, may serve as a predisposing factor in certain individuals with acquired situations such as prematurity or severe infection. In conclusion, given the lack of reliable biochemical and molecular parameters that predict the long term outcome and the expected asymptomatic course in the majority of 3MCCD individuals we suggest this disorder should be excluded from the extended NBS, with follow-up and treatment, when necessary, confined to only those subjects identified when clinical presentation prompted metabolic work-up.

Finally, our study confirms that acylcarnitine bloodspot NBS is useful and effective in identifying primary and maternal 3MCCD. However, based on the above data and considering the balance of benefit to risk, the Israeli Ministry of Health has now decided to exclude this condition from its national extended NBS program. The Israeli NBS program still monitors C5OH as a secondary marker for beta ketothiolase deficiency but not for biotinidase deficiency, a disorder not on our screening panel.

Compliance with Ethical Standards

Conflict of interest None.

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