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# Genetic, biochemical and clinical spectrum of patients with mitochondrial trifunctional protein deficiency identified after introduction of newborn screening in the Netherlands

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**Abstract**

*Introduction* Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) is included in many newborn screening (NBS) programs. Acylcarnitine-based NBS for LCHADD not only identifies LCHADD, but all different deficiencies of the mitochondrial trifunctional protein (MTP), a multi-enzyme complex involved in long-chain fatty acid β-oxidation. Besides LCHAD, MTP harbors two additional enzyme activities: long-chain enoyl-CoA hydratase (LCEH), and long-chain ketoacyl-CoA thiolase (LCKAT). Deficiency of one or more MTP activities causes generalized MTP deficiency (MTPD), LCHADD, LCEH deficiency (not yet reported), or LCKAT deficiency (LCKATD).

*Aim* To gain insight in the outcomes of MTP-deficient patients diagnosed after introduction of NBS for LCHADD in the Netherlands.

*Methods* Retrospective evaluation of genetic, biochemical and clinical characteristics of MTP-deficient patients, identified since 2007.

*Results* Thirteen patients were identified: seven with LCHADD, five with MTPD and one with LCKATD. All LCHADD patients (one missed by NBS, clinical diagnosis) and one MTPD patient (clinical diagnosis) were alive. Four MTPD patients and one LCKATD patient developed cardiomyopathy and died within one month and 13 months of life, respectively. Surviving patients did not develop symptomatic hypoglycemia, but experienced reversible cardiomyopathy and rhabdomyolysis. Five LCHADD patients developed subclinical neuropathy and/or retinopathy.

*Conclusion* Patient outcomes were highly variable, stressing the need for accurate classification of and discrimination between the MTP deficiencies to improve insight in the yield of NBS for LCHADD. NBS allowed prevention of symptomatic hypoglycemia, but current treatment

options failed to treat cardiomyopathy and prevent long-term complications. Moreover, milder patients, who might benefit from NBS, were missed due to normal acylcarnitine profiles.

**Synopsis**

Accurate classification of and discrimination between the different MTP deficiencies are required to improve insight in the true yield of NBS for LCHADD.

**Author Contributions**

M.S. and G.V. were involved in acquisition of data, interpretation of data and drafting of the manuscript. All authors were involved in the interpretation of data and reviewing and editing the manuscript. L.B., A.M.B, T.G.J.D, M.C.V., M.W. G.V., S.A.F., I.C., W.L.P. and M.S. were involved in the long-term care of patients. S.F. and L.IJ. were involved in the biochemical analysis for all patients. E.D., R.M. and R.K.V.P. are involved in the implementation in and evaluation of the Dutch NBS program. M.S. and G.V. take responsibility for the collection of data, the interpretation and publication. All authors have given final approval of the version to be published.

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**Conflicts of interest**

Marit Schwantje, Sabine Fuchs, Lonneke de Boer, Annet Bosch, Inge Cuppen, Eugenie Dekkers, Terry Derks, Sacha Ferdinandusse, Lodewijk IJlst, Riekelt Houtkooper, Rose Maase, Ludo van der Pol, Maaike de Vries, Rendelien Verschoof-Puite, Ronald Wanders, Monique Williams, Frits Wijburg and Gepke Visser declare to have no potential conflicts of interests. None of the authors have accepted reimbursements, fees, funds, or salaries from an organization that may in any way

gain or lose financially from the results reported in this manuscript. None of the authors have any competing interests regarding relevant financial activities outside the submitted work, intellectual property or any other relationships.



**Ethics approval**

The study has been approved by the medical ethics committee of the University Medical Center Utrecht (METC10-430/C; METC19-234/M).

**Patient consent statement**

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. All living patients/patients' parents gave their written consent to participate in this study.

**An availability of data and material statement**

Additional clinical data, not described in this article, will be shared upon (reasonable) request to the corresponding author.

**Key words**

Newborn screening; long-chain fatty acid oxidation; mitochondrial trifunctional protein complex; LCHAD deficiency; MTP deficiency; LCKAT deficiency

**Introduction**

Mitochondrial trifunctional protein (MTP) is a multi-enzyme complex, which catalyzes the last three steps of mitochondrial long-chain fatty acid β-oxidation (lcFAO) following the first step catalyzed by very-long-chain acyl-CoA dehydrogenase.[1,2] The MTP-complex harbors three enzyme activities: long-chain enoyl-CoA hydratase (LCEH), long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), and long-chain ketoacyl-CoA thiolase (LCKAT).[1,2] Deficient function of one or more of these enzyme activities leads to either generalized MTP deficiency (MTPD, OMIM #609015), isolated LCHAD deficiency (LCHADD, OMIM #609016), or isolated LCKAT deficiency (LCKATD, no OMIM entry).[3,4] Until now, isolated LCEH deficiency has not been reported.

In many countries LCHADD is included in population newborn screening (NBS) programs. NBS for LCHADD is generally based on abnormal acylcarnitine profiles measured by tandem mass spectrometry (MS/MS).[2] In the Netherlands, positive NBS results are followed by genetic and enzymatic analysis to confirm or rule out the diagnosis, as required to reduce the risk of misdiagnosis.[5] Acylcarnitine profiles however do not fully discriminate between LCHADD, MTPD or LCKATD. Thus, screening for LCHADD results in screening for MTPD and LCKATD as well.

Structurally, the MTP-complex is a hetero-octamer consisting of four α- and four β-subunits. LCHAD and LCEH activities are catalyzed by the α-subunit and both are encoded by the *HADHA* gene. LCKAT activity is catalyzed by the β-subunit, which is encoded by the *HADHB* gene.[4,6] Both *HADHA* and *HADHB* are located on chromosome 2p23. Variants in *HADHA* may

cause a reduced LCHAD activity. The most common cause of LCHADD is the c.1528G>C (p.Glu510Gln) variant, which has been identified in at least one allele in most patients.[7] Homozygosity for this variant causes LCHADD, whereas heterozygosity for this variant in combination with another *HADHA* variant may result in either LCHADD or MTPD, depending on the effect of the second variant on the stability of the MTP-complex.[7,8] Similarly, some *HADHB* variants leave the MTP-complex intact and only affect the enzymatic activity of the β-unit, resulting in LCKATD, whereas other variants affect proper formation or the stability of the MTP-complex, causing loss of all three enzyme activities resulting in MTPD. The fact that *HADHA* and *HADHB* variants can cause LCHADD and LCKATD, respectively, but also MTPD, complicates the diagnostic process. Hence, both genetic diagnostics and analysis of the enzyme activities of the MTP-complex are essential for accurate discrimination between these related but distinct diseases.[9]

Patients with MTPD and LCHADD display heterogeneous clinical phenotypes varying from early-onset life-threatening cardiomyopathy, hypoketotic hypoglycemia and liver failure, to a later-onset form with myopathy, episodic rhabdomyolysis, peripheral neuropathy and/or pigmentary retinopathy.[3] LCKATD has only been reported in three patients: two with cardiomyopathy and all with fatal outcome within two months of life.[10,11]

In the Netherlands, LCHADD has been included in the NBS program since 2007. Although LCHADD is the intended target disorder of NBS, also patients with MTPD and LCKATD can be identified by NBS. To increase insight in patient outcomes, we evaluated the genetic,

biochemical and clinical characteristics of patients with MTP deficiency identified since introduction of LCHADD in the Dutch NBS program.

## Methods

*Study design and setting*

We conducted a retrospective evaluation of genetic, biochemical and clinical characteristics of patients with MTP deficiency (comprising LCHADD, MTPD and LCKATD), diagnosed since the introduction of LCHADD in the Dutch NBS program (2007) until May 2021. We included both patients identified by NBS and patients diagnosed after clinical presentation. The study was approved by the medical ethics committee of the University Medical Center Utrecht (METC10-430/C; METC19-234/M). Informed consent was obtained from living patients.

*Patients and diagnosis*

Patients were identified via the Dutch Diagnosis Registration Metabolic Diseases (DDRMD, www.ddrmd.nl). In the Netherlands, NBS is performed within 72–168 hours after birth by measuring hydroxy-C16-carnitine levels with MS/MS in dried blood spots (DBS) (initial cut-off point (January 2007-October 2010): ≥0.20 μmol/L, adjusted cut-off point since October 2010: ≥0.08 μmol/L). NBS results were available within ten days after birth. Diagnosis was confirmed with biochemical and genetic analysis. Patients were classified based on enzyme activities in LCHADD, MTPD and LCKATD patients.

*Biochemical studies*

Primary patient skin fibroblasts were cultured in Ham's F-10 medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), 25 mmol/L HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin and 250 µg/mL amphotericin in a humidified atmosphere of 5% CO2 at 37°C. To investigate thermo-sensitivity in patient #5, cells were cultured at 40°C for two weeks followed by enzyme and long-chain fatty acid β-oxidation (lcFAO)-flux analysis. LcFAO-flux analysis was performed in fibroblasts, as described earlier by measuring the production of radiolabeled $H_2O$ from [9,10-$^3$H(N)]-oleic acid.[12-14] The palmitate loading test was performed in fibroblasts by adding 120 µmol/L [U-13C]palmitate and 0.4 mmol/L L-carnitine to the medium, essentially as described by Diekman et al.[15] After 96 hours, acylcarnitines in the medium were measured by MS/MS.

LCHAD and LCKAT activities were measured in lymphocyte and/or fibroblast homogenates using 3-keto-palmitoyl-CoA as substrate. Since approximately 75% of 3-hydroxyacyl-CoA dehydrogenase activity as measured with 3-keto-palmitoyl-CoA is catalyzed by LCHAD and the remaining 25% by short-chain hydroxyacyl-CoA dehydrogenase (SCHAD), the 3-ketopalmitoyl-CoA dehydrogenase activity is measured after a pre-incubation (30 min) with and without N-ethylmaleimide (30 mmol/L), which inactivates LCHAD but not SCHAD. LCHAD incubations were performed in 0.2 mol/L potassium phosphate, 0.1 mol/L 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.2, 1 g/LTriton X-100 and 0.4 mmol/L NADH with 0.066 mg/mL protein at 37°C. The LCKAT incubations were performed in a solution of 0.1 mol/LTRIS pH 8.6, 25 mmol/LMgCl2, 0.2 mg BSA/mL, 3 mmol/L Coenzyme A, and 1 g/L Triton X-100 with 0.066 mg/ml protein at 37°C. Reactions were started with 0.18 mmol/L 3-keto-palmitoyl-CoA and

stopped after 5 minutes for LCHAD and after 10 minutes for LCKAT with 0.33 mol/L HCl and cooling on ice. After 5 minutes, the samples were neutralized with 2 mol/L KOH/0.6 M MES, and 30% (v/v) acetonitrile was added. After a further 5 minutes on ice the samples were centrifuged for 5 minutes at 20,000 *x* g at 4°C. The supernatant was subjected to ultra-high performance liquid chromatography on a C18 column (Waters Acquity HSS C18 1.8 µm 2.1x100mm). Resolution of the different CoA-esters (substrate and products) was achieved by a linear gradient of acetonitrile (from 33% to 37% (v/v)) in 16.9 mmol/L sodium phosphate buffer (pH 6.9) at a flow rate of 0.5 mL/min under continuous monitoring at 260 nm.

*Genetic analysis*

Genetic analysis was performed by Sanger sequencing of all exons and flanking intronic sequences of the *HADHA* and *HADHB* genes. Sequence data were compared to the reference sequences NM_000182.4 (*HADHA*) and NM_000183.2 (*HADHB*) with nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

*Clinical characteristics*

Historical data were collected from the treating metabolic centers and the Dutch Expertise Center for lcFAO-disorders. At the expertise center, patients were seen by a pediatric multidisciplinary team comprising of metabolic specialists, dieticians, neurologists, and cardiologists. Ophthalmologic investigations were performed in the treating metabolic center.

**Results**

From January 2007 until May 2021, 2,463,575 children were born in the Netherlands.[16] NBS was performed in over 99% of the newborns, leading to referral of 41 newborns (January 2007-October 2010): 3 newborns, October 2010-May 2021: 38 newborns).With thirty false positives, eleven of the referred newborns were diagnosed with MTP deficiency. Seven were hospitalized preceding the abnormal NBS results (Table 1). Two additional patients were diagnosed after clinical presentation (patients #5 and #8). Of the 13 patients, five had MTPD, seven LCHADD, and one LCKATD. Hydroxy-C16-carnitine levels in NBS samples and blood plasma after referral or clinical presentation are shown in Table 2.

*Biochemical characteristics*

The results of genetic and enzymatic analyses are summarized in Table 3. MTPD patients had both reduced LCHAD and LCKAT activities, LCHADD patients had only reduced LCHAD activity and the LCKATD patient only reduced LCKAT activity. Although LCKAT activity in fibroblasts was also slightly reduced in patients #10 and #12, LCKAT activity was normal in lymphocytes, resulting in the final diagnosis of LCHADD.

Patient #5 had relatively high residual LCHAD and LCKAT activities in fibroblasts cultured at the standard temperature of 37°C. After culturing the fibroblasts at 40°C, LCHAD and LCKAT activities decreased 3 and 5 times compared to 37°C, respectively. Additionally, the lcFAO-flux decreased from 99% of controls at 37°C to 34% at 40°C. These results show a thermo-sensitive MTP deficiency (Supplementary Material 1).

In the LCHADD patients, lcFAO-flux was below 28% except for patient #8, who was missed by NBS and showed an lcFAO-flux of 83%. Interestingly, the palmitate loading test was completely normal in patient #8, while all other LCHADD patients showed the characteristic accumulation of hydroxy-C16-carnitine (Table 2).

*Genetic characteristics*

All MTPD patients were homozygous or compound heterozygous for *HADHB* variants (Table 3). All LCHADD patients carried *HADHA* variants, with at least one allele carrying the common c.1528G>C (p.Glu510Gln) variant. The LCKATD patient was compound heterozygous for the following *HADHB* variants: c.182G>A (p.Arg61His) and c.1289T>C (p.Phe430Ser).

*Clinical characteristics*

**Generalized MTPD**

Two male and three female patients had MTPD. In all patients, pregnancies were complicated with hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, acute fatty liver of pregnancy (AFLP) or pre-eclampsia (Table 1). Four MTPD patients died due to cardiomyopathy within the first month of life (median age of death: 8 days, range: 3-31 days); one MTPD patient is currently eleven years old and experiences illness- and exercise-induced muscle symptoms.

The deceased patients were hospitalized before NBS could be performed or before the results became available. All had hypertrophic or dilated cardiomyopathy with a severely reduced left ventricle (LV) function, before the 8$^{th}$ day of life. Lactic acidosis was reported for three of them. At time of death, MTP deficiency was suspected in all, and treatment by means of glucose

infusion, inotropic and diuretic drugs had been initiated. In addition to multiple hypoglycemic events and cardiomyopathy, patient #3 had infantile respiratory distress syndrome without improvement despite treatment with surfactant and high frequency ventilation. Patient #4 also developed necrotizing enterocolitis. Creatine kinase (CK) levels were elevated up to 5,876 U/L. Patients #3 and #4 were previously described by Diekman et al.[17]

MTPD patient #5 is a now eleven-year-old girl. NBS showed normal levels of hydroxy-C16-carnitine. From the age of three years she experienced fever-associated episodes of muscle weakness and exercise intolerance. Episodes lasted two to four weeks and began when body temperature normalized. Between episodes she experienced exercise intolerance with a maximum walking time of 15 minutes and exercise-induced muscle weakness. On clinical examinations, muscle tendon reflexes were normal. Extensive biochemical, metabolic and genetic diagnostics did not reveal abnormalities. Acylcarnitine profiles and CK levels, measured at seven different occasions, were always normal. At ten years of age, whole exome sequencing showed homozygosity for the following *HADHB* variant*:* c.397A>G (p.Thr133Ala). Subsequent enzyme analyses showed a thermo-sensitive MTP deficiency.

Cardiac screening with echocardiography showed normal cardiac function. Sensory nerve action potential (SNAP) amplitudes and conduction velocities (NCVs) were normal, but electromyography showed sporadic and small motor unit potentials.

***Development and growth***

Patient #5 had normal motor and cognitive development. She had a normal height corrected for target height, but was overweight (Table 4).

***Dietary treatment***

Patients #1 to #4 received intravenous glucose infusion. Patient #3 was additionally treated with parenteral feeding, carnitine supplementation (100 mg/kg/day), long-chain triglyceride (LCT-)restriction and medium-chain triglyceride (MCT-)enrichment. Since diagnosis, patient #5 was treated with, but poorly complied to an LCT-restricted, MCT-supplemented and carbohydrate-enriched diet during illness.

**Isolated LCHADD**

Four male and three female patients had LCHADD. All are alive with a current median age of ten years (range: 3.9–13.3y) and a similar follow-up duration. In two patients, pregnancies were complicated with HELLP syndrome (Table 1). Clinical characteristics are summarized in Table 4.

Five LCHADD patients were identified by NBS. Patient #10, the younger sibling of patient #11, was diagnosed prenatally. Despite initiation of dietary measures immediately after birth, NBS was still positive (Table 2). Patient #8 was missed by NBS (false negative NBS results) and presented with increased CK and ALAT during a bout of gastroenteritis at the age of nine months. Mild muscular hypotonia and microcephaly (-2.7SD) were noticed. An abnormal acylcarnitine profile suggestive of LCHADD led to confirmatory enzymatic and genetic testing. The identification of this patient resulted in a lowering of the NBS cut-off point for hydroxy-C16-carnitine in DBS from ≥0.20 µmol/L to ≥0.08 µmol/L. A retrospective study was conducted of all newborns born in the period 01-01-2007 to 01-10-2010 (n=500.000). In the 23 DBS of newborns with hydroxy-C16-carnitine >0.08 µmol/L and <0.22 µmol/L, homozygosity or

heterozygosity for the common c.1528G>C (p.Glu510Gln) variant was not found, suggesting that no cases were missed in this period.

Three patients had mild reversible abnormalities on echocardiography. Patient #6 only needed dietary measures for reversibility, whereas patient #11 and #7 were also temporarily treated with diuretics and ACE-inhibitors. At the time of diagnosis, patients #11 and #7 had feeding problems. Patient #7 had asymptomatic hypoglycemia, low carnitine plasma concentration (free carnitine: 6.2 µmol/L, ref: 20-55) and a slightly delayed motor development, normalizing after treatment initiation.

In patient #9, glucose concentrations were undetectable after birth ("low"). In the first days of life, the total glucose intake could not be decreased below 9.6 mg/kg/min. Besides patient #7, no other patient experienced a hypoglycemic event after diagnosis. ALAT was normal in all, except during rhabdomyolysis.

Illness-induced rhabdomyolysis was reported in three patients (patients #9, #10 and #11). Two patients experienced one (patient #6) or two (patient #7) episodes of severe muscle pain preceded by physical activity from the age of eight and eleven years, respectively. Patient #11 reported muscle pain after minimal exercise from the age of four years onwards, and experienced several episodes of muscle pain after physical activity with increased CK levels from eight years onwards.

On neurologic examination, muscle strength was normal in all patients. Four showed decreased or absent tendon reflexes from the age of eight to eleven years onwards (Table 4). Clinical sensory examination was normal in most patients, with the exception of patient #8, who had abnormal vibration sense at the age of twelve years. Only patient #8 showed abnormal nerve conduction studies (NCS). NCS revealed a decreased SNAP amplitudes in the n. suralis and

decreased compound muscle action potential amplitudes in the n. peroneus at the age of nine years, which remained stable during follow-up.

Pigmentary retinopathy without visual impairment or night blindness was diagnosed by ophthalmoscopy in three patients at the age of two, five and seven years in patients #12, #11 and #6, respectively.

***Dietary treatment***

After diagnosis, all patients were advised a maximal fasting time according to age. All but patient #7 used an LCT-restricted and MCT-enriched diet from diagnosis, in two (patients #11 and #12) combined with breastfeeding. Patient #7 started an LCT-restricted and MCT-enriched diet at four months of age, and used carnitine supplementation temporarily. For patient #12, the diet was stopped gradually upon initiation of solid foods, but restarted at the age of 2.5 years. All patients on dietary treatment used supplementation of Omega-3 and Omega-6 fatty acids. Four patients (patient #6, #7, #10, #11) needed a percutaneous endoscopic gastrostomy because of feeding problems (behavioral problems, frequent vomiting).

***Development and growth***

Most patients had a normal height and weight (Table 4). All had normal early cognitive development and all patients older than four years attended a regular primary school. Patient #7 was slightly delayed in motor development at four months, which recovered after treatment of his cardiomyopathy. Patient #9 had cerebral palsy due to birth asphyxia and showed a delayed motor development, improving with physiotherapy. Patient #12 was a bottom scooter and started walking at 22 months.

**Isolated LCKATD**

The LCKATD patient was born after an uncomplicated pregnancy and delivery. Because of low birth weight (<2.3SD) she was admitted and treated for hypoglycemia. She gradually deteriorated and developed cardiorespiratory insufficiency with lactic acidosis. At day four, echocardiography showed reduced LV function (LVFS: 15%) with initially normal cardiac dimensions, later biventricular hypertrophy. She developed rhabdomyolysis. Upon treatment with intravenous glucose, LCT-restriction, MCT- and ketone-supplementation, inotropic drugs, diuretics and ACE-inhibitors, she improved and cardiac function recovered.

After discharge at the age of one month, she had a relatively stable period. At four months, she developed epilepsy, which was treated with Levetiracetam and later Zonisamide. From nine months onwards, she experienced several metabolic exacerbations with deterioration of cardiac function and increased CK levels. During the fourth exacerbation, cardiac function did not recover despite intravenous glucose and cardiac medication. She died at the age of 13 months.

***Development and growth***

She had a motor developmental delay. At the age of twelve months, her length was +0.9SD and her weight for height: +0.1SD.

***Dietary treatment***

She was treated with an LCT-restricted and MCT- and ketone-supplemented diet through tube feeding. Ketones were supplemented as sodium-D,L-3-hydroxybutyrate (300mg/kg/day increasing to 500mg/kg/day). Extensive details of this patient have been described in a separate report (under submission).

**Discussion**

Since the introduction of acylcarnitine-based NBS for LCHADD in the Netherlands, seven LCHADD patients (resulting birth prevalence: approximately 1 in 350,000) were diagnosed, of whom one was missed by NBS. Thereafter, the cut-off point for referral was adjusted. Since acylcarnitine profiles in LCHADD, LCKATD and MTPD are similar, not only patients with LCHADD, but also four with MTPD and one with LCKATD (birth prevalence: 1 in 2,500,000) were diagnosed by NBS. During the study period, an additional patient with MTPD (birth prevalence: 1 in 500,000) was diagnosed based on clinical symptoms. All LCHADD patients and this latter patient are still alive. The four patients with MTPD referred by NBS died within their first month of life and the LCKATD patient at the age of 13 months, all because of cardiac failure.

None of the LCHADD patients developed symptomatic hypoglycemia or neurological symptoms whereas in an international pre-NBS cohort study, hypoglycemia was reported as one of the presenting symptoms in 78% of the LCHADD patients. Moreover, a quarter of the pre-NBS patients had psychomotor retardation before or at the time of diagnosis.[18] These differences point to a significant beneficial effect of NBS. However, LCHADD patients detected by NBS still developed cardiomyopathy (three out of six, excluding patient #8, who was missed by NBS) and episodes of rhabdomyolysis (five out of six), despite treatment with a maximum fasting time according to age and an LCT-restricted and MCT-enriched diet since diagnosis by NBS for most.

Furthermore, peripheral neuropathy and pigmentary retinopathy were detected preclinically in four of the LCHADD patients detected by NBS during routine evaluation. The course of these

complications is often progressive and longer follow-up is needed to investigate the clinical consequences in this population. It has been reported that the long-term complications are less severe in LCHADD patients under optimal and/or earlier initiated dietary therapy.[19,20] However, the early development of subclinical long-term complications in our patients and patients previously reported in literature show that NBS diagnosis and early initiation of current treatment strategies cannot fully prevent these complications.

During 14 years of NBS for LCHADD in the Netherlands, thirty referred newborns had false positive NBS results. The percentage of false positives was calculated to be approximately 0.0003% before adjustment of the cut-off point for NBS, and 0.002% after adjustment. The increase of false positive NBS results was a logical consequence of lowering the cut-off point of NBS for LCHADD. Although the amount of false positives was comparable to numbers previously reported by Sander et al. (0.001%)[10], comparison to other NBS programs is difficult because of variability between used screening markers, cut-off points and classification of MTP deficiencies. One LCHADD patient had false negative NBS results (14%). Other LCHADD patients missed by NBS have been reported in literature[21], but the true amount of false negatives worldwide is thus far unclear.[22]

The LCHADD patient missed by NBS and detected by increased CK and ALAT during a bout of gastroenteritis at the age of nine months had no metabolic derangements during the twelve years of follow-up, but developed asymptomatic peripheral neuropathy. Grünert et al.[23] described a similar presentation in four patients with MTP deficiency due to biallelic *HADHA* variants (enzyme activities not reported) who were also missed by NBS. These four patients showed

isolated peripheral neuropathy as the presenting symptom and in three of them this was the only symptom. Since MTP-related peripheral neuropathy is hypothesized to be caused by toxicity of long-chain 3-hydroxyacyl fatty acid metabolites, it is surprising that this was the presenting symptom in patients without a characteristic NBS acylcarnitine profile. Although plasma acylcarnitines may not fully represent accumulation of acylcarnitines in nerves[24], this may suggest a different pathophysiological mechanism causing neuropathy in MTP deficiencies.

The target disorder for NBS in the Netherlands is LCHADD and therefore, the MTPD patient clinically diagnosed at the age of nine years can officially not be classified as a false negative NBS result. The diagnosis was established by whole exome sequencing in combination with enzyme diagnostics because of unexplained muscle symptoms. Interestingly, her acylcarnitine profile was repeatedly normal. Normal acylcarnitine profiles under healthy conditions, and in one patient even during metabolic decompensation, have been described in literature for other MTPD patients with a (neuro)myopathic phenotype.[25,26] Dietary treatment combined with avoidance of provoking factors proved beneficial for most of them. Consequently, our patient might have had a more favorable outcome with less metabolic decompensations and a shorter diagnostic trajectory, had she been identified by NBS.

MTPD patients identified by NBS died early in life despite early diagnosis and treatment initiation. Although MTPD is known for its high mortality rates[27], the broad clinical spectrum also comprises milder phenotypes with peripheral neuropathy and/or adult-onset myopathy.[28,29] Knowledge regarding the precise distribution of mild and severe phenotypes among the whole MTPD population is insufficient, but an overrepresentation of the severe phenotype in our cohort

is possible. This might be the result of the genotypic spectrum with (expected) frameshift variants and deletions, which are associated with severe disease phenotypes.[28] However, given the fact that the deceased MTPD patients were already admitted before NBS results became available, it is also plausible that this severe MTPD phenotype is underreported in other patient cohorts.

It seems unlikely that an earlier diagnosis either by prenatal diagnosis or cord blood analysis would have prevented the fatal outcome of the severe MTPD patients. This implies that currently available treatment options are insufficient and improved treatment strategies are needed. Recent studies suggest a beneficial effect of treatment with betahydroxybutyrate and triheptanoin in patients with (long-chain) fatty acid oxidation disorders.[30-33] The reported LCKATD patient, who was treated with sodium-D,L-3-hydroxybutyrate, had a longer survival time than LCKATD patients previously reported in literature and potentially benefited from this treatment. However, both treatment strategies require tolerance for enteral feeding, which is a sometimes unavoidable obstacle in the treatment of critically ill patients. Intravenous betahydroxybutyrate has been investigated in adults with cardiac disease and might be a promising future treatment option.[34]

NBS might be more beneficial for patients with a milder, myopathic phenotype of MTPD, but for these patients acylcarnitine-based NBS might be inadequate due to their often normal acylcarnitine profiles. An alternative approach is NBS by next generation sequencing (NGS). NGS has already identified patients with homozygous or compound heterozygous variants in *HADHA* and *HADHB* within cohorts of patients with myopathic symptoms or peripheral neuropathy, not identified by acylcarnitine profiling.[35] To further classify the patients and

prevent identification of variants without functional consequences, genetic-based NBS should always be combined with enzyme analyses.

Prognostication after referral by NBS by acylcarnitine profiling for a possible MTP deficiency is difficult, because of the broad phenotypic spectrum. Spiekerkoetter et al.[28] reported a genotype-phenotype correlation for MTPD, where *HADHB* missense variants were expected to present with milder phenotypes than premature termination or frameshift variants. Although functional studies were also expected to allow prognostication, a clear correlation between MTP enzyme activities and clinical phenotype has not been established.[28,36] Despite the detection of a correlation between the results of metabolic flux studies in cultured skin fibroblasts and phenotypic severity[28,36], the current knowledge on a relation between enzymatic or genotypic characteristics and clinical outcomes is still too limited for accurate prognostication. Even LCHADD patients with identical genotypes (homozygosity for the common c.1528G>C (p.Glu510Gln) variant) show heterogeneous clinical phenotypes, suggesting influence of environmental and other genetic factors.[18]

**Conclusion**

We report the outcome of 13 patients diagnosed since introduction of LCHADD in the Dutch NBS program. Since NBS for LCHADD by acylcarnitine profiling identifies all three MTP deficiencies, the identified patients comprised seven LCHADD, five MTPD and one LCKATD. Their clinical presentation and outcomes were highly variable. Additionally, milder patients with normal acylcarnitine profiles, for whom dietary treatment might be most beneficial, were missed. This could be solved with NBS by NGS followed by enzyme analysis.

The most apparent benefit of NBS for LCHADD was the prevention of symptomatic hypoglycemia. However, currently available treatment options could not prevent all symptoms and long-term complications. To enhance the benefits of NBS, more effective treatment strategies are needed. Moreover, accurate classification of and discrimination between the different MTP deficiencies utilizing both enzymatic and genetic analysis is required to improve insight in the yield of NBS, prognosis prediction and patient outcomes.

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| Patient number | MTP deficiency | Sex | Current age | Birth weight (in gr) | Pregnancy duration (wk+days) | Apgar scores (1 min, 5 min) | Pregnancy complications | Consan-guinity | Ethnicity[#] | Phenotype at time of diagnosis | | Other disease? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Diagnosed by NBS? | Signs and symptoms | |
| #1 | *MPTD* | F | † day 3 | 1580 | 33+2 | 1, 7 | AFLP | Unknown | Other African | Yes* | Prenatal cardiomyopathy, lactic acidosis (33 mmol/L), hypoglycemia (*hosp*) | Trombopenia |
| #2 | | M | † day 5 | 2455 | 36+2 | 8, 10 | HELLP | No | Other African | Yes* | Cardiomyopathy, lactic acidosis (10.3 mmol/L) (*hosp*) | No |
| #3 | | M | † day 31 | 1275 | 30+0 | 7, 8 | Pre-eclampsia, IUGR | Yes | Unknown | Yes | Cardiomyopathy, lactic acidosis (>10 mmol/L), hypoglycemia (*hosp*) | IRDS |
| #4 | | F | † day 10 | 2110 | 35+1 | 4, 8 | Pre-eclampsia | Yes | Northern African | Yes | Cardiomyopathy (*hosp*) | NEC |
| #5 | | F | 11.9y | 3700 | 38 | 'good start' | Pre-eclampsia, maternal diabetes | Yes | Northern African | No | Fever-induced muscle weakness, exercise intolerance, leg pain | No |
| #6 | *LCHADD* | M | 10.4y | 3240 | 38+0 | >9, >9 | No | No | Caucasian | Yes | Weight loss, jaundice, fever, desaturation (*hosp*) | No |
| #7 | | M | 12.5y | 1130 | 31+2 | 7, 8 | HELLP, IUGR | No | Caucasian | Yes | No | No |
| #8 | | M | 13.3y | 3030 | 41 | 5, 9 | No | No | Caucasian | Missed by NBS | Gastroenteritis with increased CK (1,796 U/L) and ALAT (470 U/L) | No |
| #9 | | M | 7.2y | 2560 | 36+5 | 2, 7 | ICP, HELLP | No | Caucasian | Yes | Fetal distress during delivery, postpartum hypoglycemia, increased NT-proBNP (3,100 pg/mL) (*hosp*) | CP due to asphyxia |
| #10 | | F | 5.9y | 2990 | 39+0 | 9, 10 | No | No | Caucasian | Prenatal** | n.a. | No |
| #11 | | F | 10.3y | 2970 | 38+6 | 'good start' | No | No | Caucasian | Yes | Weight loss (*hosp*) | No |
| #12 | | F | 3.9y | 3120 | 37+6 | 'good start' | No | No | Caucasian | Yes | No | No |
| #13 | *LCKATD* | F | † 13mo | 2194 | 38+5 | 9, 10 | No | No | Caucasian | Yes* | Hypoglycemia, rhabdomyolysis (CK: 10,004 U/L), cardiomyopathy (NT-proBNP >35,000 pg/mL), lactic acidosis (8.3 mmol/L) | Epilepsy |

Table 1: Baseline and birth characteristics of all patients with MTP deficiency. Y = years, gr = grams, wk = weeks, min = minutes, AFLP: acute fatty liver of pregnancy, HELLP: Hemolysis, elevated liver enzymes and low platelets syndrome, IUGR: intrauterine growth retardation, ICP: intrahepatic cholestasis of

pregnancy, IRDS: infantile respiratory distress syndrome, NEC: necrotizing enterocolitis, CP: cerebral palsy, CK: creatine kinase, ALAT: alanine aminotransferase, NT-proBNP: N-terminal pro-brain natriuretic peptide, † = deceased. Hosp: the patient was hospitalized preceding the abnormal NBS results. #According to the Dutch Perinatal Dictionary and Data (PWD). *An lcFAO-disorder was already suspected based on clinical presentation, before NBS results became available. **Prenatal diagnosis through family screening (sibling of patient #11). Reference values: lactate < 2.0 mmol/L, CK <145-200 U/L, ALAT < [illegible] U/L, NT-proBNP <125-320 pg/mL.

| Patient number | MTP deficiency | NBS results (in µmol/L, in DBS) | Measurement of acylcarnitines in plasma after positive NBS or during diagnostic presentation (in µmol /L, measured in blood plasma) | | | | | Palmitate loading test (in nmol/4days·mg protein) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C16-OH | C0 (ref) | C14:1 (ref) | C14-OH (ref) | C16-OH (ref) | C18-OH (ref) | C12 (0.0-0.8) | C14 (0.0-0.4) | C16 (0.0-4.3) | C16-OH (0-0.07) |
| #1 | *MTPD* | 1.67* | 23.53 (22.3-54.8) | 1.70 (0.02-0.18) | 0.34 (0-0.04) | 1.78 (0-0.02) | 0.48 (0-0.4) | 0.8 | 2.5 | 26.4 | 2.5 |
| #2 | | 1.40 | 25.17 (22.3-54.8) | 2.17 (0.02-0.18) | 0.35 (0-0.04) | 1.50 (0-0.02) | 0.53 (0-0.4) | - | - | - | - |
| #3 | | 0.63 | 9.82 (22.35–54.8) | 0.41 (0.02-0.18) | - | 0.44 (0) | - | 0.2 | 1.6 | 34.2 | 1.6 |
| #4 | | 1.44 | 6.9 (22.35–54.8) | 0.1 (0.02–0.18) | 0.04 (0) | 0.59 (0) | - | - | - | - | - |
| #5 | | 0.03 | 43 (22.3-54.8) | 0.06 (0.02-0.18) | 0.01 (0-0.04) | 0.00 (0-0.02) | 0.00 (0-0.02) | 0.6 | 0.2 | 2.4 | 0.0 |
| #6 | *LCHADD* | 1.47 | 12.55 (22.3-54.8) | 0.02 (0-0.04) | 0.04 (0 -0.04) | 0.14 (0 -0.02) | 0.14 (0-0.04) | 1.2 | 1.1 | 7.6 | 1.1 |
| #7 | | 0.32 | 17.99 (20-55) | 0.56 (0-0.17) | 0.12 (0-0.04) | 0.23 (0-0.02) | 0.00 (0-0.05) | 1.0 | 0.8 | 6.9 | 1.0 |
| #8 | | 0.11 | 30.85 (22.30-54.8) | 1.12 (0.02-.18) | 0.18 (0-0.04) | 0.14 (0-0.02) | 0.03 (0-0.04) | 0.1 | 0.2 | 2.0 | 0.0 |
| #9 | | 0.19 | 12 (5-35) | 0.53 (0.01-0.34) | 0.07 (0-0.01) | 0.15 (0-0.01) | 0.12 (0-0.01) | - | - | - | - |
| #10 | | 0.11 | 11.41 (20-55)** | 0.10 (0-0.17)** | 0.04 (0-0.04)** | 0.15 (0-0.02)** | 0.12 (0-0.05)** | 1.5 | 1.4 | 13.7 | 1.8 |
| #11 | | 2.19 | 30.36 (20-55) | 0.92 (0-0.17) | 0.25 (0-0.04) | 0.62 (0-0.02) | 0.00 (0-0.05) | - | - | - | - |
| #12 | | 1.02 | 26.0 (12-46) | 2.37 (0-0.15) | 0.52 (0-0.01) | 1.00 (0-0.02) | - | 1.3 | 1.1 | 9.9 | 1.0 |
| #13 | *LCKATD* | 4.49 | 75.62 (25-65) | 0.32 (0-0.04) | 0.42 (0-0.04) | 2.81 (0-0.02) | 0.77 (0-0.05) | 0.2 | 0.5 | 14.0 | 0.9 |

Table 2: Acylcarnitine levels measured in 1) newborn screening bloodspots, 2) blood plasma directly after referral by NBS or during diagnostic trajectory (for patients #5 and #8), 3) the palmitate loading test performed in patient fibroblasts. *NBS was performed too early (at day 1 of life) **For patient #10, acylcarnitines were measured in umbilical cord blood, after prenatal diagnosis through family screening. DBS: dried blood spots. Reference values and the number of decimals are shown as measured and reported by the performing metabolic laboratory.

| Patient number | MTP deficiency | Affected gene | Genetic variants | | Enzyme activities in lymphocytes | | Enzyme activities in skin fibroblasts | | lcFAO-flux in skin fibroblasts |
|---|---|---|---|---|---|---|---|---|---|
| | | | Allele 1 | Allele 2 | LCHAD | LCKAT | LCHAD | LCKAT | |
| [illegible] | *MTPD* | *HADHB* | c.181C>T (p.Arg61Cys) | c.254+5 G>A (suspected splicing defect) | 20% | 5% | 20% | 5% | - |
| #2 | | *HADHB* | c.18C>A (p.Tyr6X) | c.631-1 G>A (splicing defect) | 29% | 3% | - | - | - |
| #3 | | *HADHB* | c.354+5delG (skipping exon 6) | c.354+5delG (skipping exon 6) | - | - | 14%* | 18%* | - |
| #4 | | *HADHB* | c.209+1G>C (suspected splicing defect) | c.209+1G>C (suspected splicing defect) | 50%* | 8%* | - | - | - |
| #5 | | *HADHB* | c.397A>G (p.Thr133Ala) | c.397A>G (p.Thr133Ala) | - | - | 40% | 33% | 99% |
| | | | | | | | At 40°C: 10% | At 40°C: 7% | At 40°C: 34% |
| [illegible] | *LCHADD* | *HADHA* | c.1528G>C (p.Glu510Gln) | c.1528G>C (p.Glu510Gln) | 23% | 161% | 10% | 138% | 24% |
| #7 | | *HADHA* | c.1528G>C (p.Glu510Gln) | c.1528G>C (p.Glu510Gln) | 41% | 89% | 7% | 103% | 28% |
| #8 | | *HADHA* | c.1528G>C (p.Glu510Gln) | c.982G>A (p.Gly328Arg) + c.1072C>A (p.Gln358Lys) | 26% | 88% | 10% | 92% | 83% |
| [illegible] | | *HADHA* | c.1528G>C (p.Glu510Gln) | c.1528G>C (p.Glu510Gln) | 19% | 133% | - | - | - |
| #10 | | *HADHA* | c.1528G>C (p.Glu510Gln) | c.2099delG (p.Gly700GlufsX30) | 19% | 82% | 10% | 43% | 27% |
| #11 | | *HADHA* | c.1528G>C (p.Glu510Gln) | c.2099delG (p.Gly700GlufsX30) | 25% | 89% | - | - | - |
| #12 | | *HADHA* | c.1528G>C (p.Glu510Gln) | c.1432delG (p.Ala478Leufs*17) | 41% | 89% | 14% | 56% | 22% |
| #13 | *LCKATD* | *HADHB* | c.182G>A (p.Arg61His) | c.1289T>C (p.Phe430Ser) | 146% | 9% | 74% | 4% | 17% |

Table 3: Enzymatic and genetic characteristics of all patients with MTP deficiency. LCHAD and LCKAT activities in the patient samples are expressed as % of the mean of the reference values. Shown long-chain fatty acid β-oxidation (lcFAO)-flux is the mean of two independent experiments and is expressed as % of the mean lcFAO-flux in two or three control cell lines measured in the same experiment. *Measurements were performed with the formerly used spectrophotometric assay, as described by Wanders et al.[9]

| Patient | Cardiac | | | | Muscular | | Hepatic | Neuropathic | | Ocular | Growth | | | Diet |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Abnormalities on ECG (age detected) | Abnormalities on echocardiography | | | Muscle pain and/or rhabdomyolysis | | Hypoglycemia, lowest known glucose level (age) | Abnormal reflexes (age first detected) | Peripheral neuropathy on NCS (if yes: age detected, if no: age last NCS) | Pigmentary retinopathy on ophthalmoscopy (if yes: age detected, if no: age last ophthalmoscopy) | Height (SD) | Height corrected for TH (SD) | Weight for height (SD) | Total fat, MCT and LCT (in energy%) |
| | | Abnormalities (age detected) | Symptoms | Reversibility | Illness-induced (max CK) | Exercise-induced (max CK, age 1st episode) | | | | | | | | |
| #1 | n.r. | Hypertrophic CMP, lowest LVFS 2.7% (prenatal) | Cardiac failure, death | No | Yes, 628 U/L | n.a. | Yes, 1.6 mmol/L (postpartum) | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| #2 | n.r. | Dilated CMP, lowest LV FS 9% (day 4) | Cardiac failure, death | No | Yes, 5,876 U/L | n.a. | No | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| #3 | n.r. | Hypertrophic, dilated CMP, low cardiac output (day 6) | Cardiac and respiratory failure, death | No | Not measured | n.a. | Yes, multiple events (postpartum glucose: 1.8 mmol/L) | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| #4 | n.r. | Dilated CMP, low cardiac output (1 wk) | Cardiac failure, death | No | Yes, 478 U/L | n.a. | No | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| #5 | No | No | No | - | Yes, muscle weakness (CK always normal) | Yes, muscle weakness (CK not measured, 9y) | No | No | No (8y)# | Not performed | -0.06 | + 0.7 | +3.5 | Not calculated |
| #6 | No | Mild LV hypertrophy, normal LV function (1wk) | No | Yes, after start MCT-diet | No | 1 episode with myoglobinuria (42,900 U/L, 8y) | No | Decreased ankle jerk reflexes (10y) | No (9y) | Yes (7y) | +1.76 | +0.95 | -0.04 | Total: 25<br>LCT: 32<br>MCT: 68 |
| #7 | No | Dilated CMP Lowest LVFS: 16% (4mo) | Delay in motor development, feeding problems | Yes, with spironolactone, furosemide, captopril | No | 2 episodes (CK not measured, 11y) | Yes, 2.9 mmol/L (4mo) | Absent triceps and radialis reflexes, decreased knee tendon reflexes (11y) | No (11y) | No (11y) | -0.32 | +0.11 | + 1.0 | Total: 23<br>LCT: 34<br>MCT: 66 |
| #8 | No | No | - | - | 1 episode before diagnosis (1,796U/L) | No | No | Absent upper and lower limb reflexes (9y) | Yes, decreased SNAP and CMAP (9y) | No (12y) | +1.69 | +1.19 | -2.18 | Total: 38<br>LCT: 39<br>MCT: 61 |
| #9 | No | No | - | - | 1 episode (63,238U/L) | No | Yes, 'low'** (neonatal) | No | Not performed | No (6y) | +0.38 | -0.5 | -0.57 | Total: 35<br>LCT: 23<br>MCT: 77 |
| #10 | Aspecific abnormal | No | - | - | 4 episodes (76,937U/L) | No | No | No | No (9y) | No (5y) | -0.82 | -0.02 | +0.92 | Total: 18<br>LCT: 45<br>MCT: 55 |

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| | repolarization (4y) | | | | | | | | | | | | | |
| #11 | No | Hypertrophic CMP Lowest LVFS: 20% (5mo) | Feeding problems | Yes, with hydrochloro-thiazide | 1 episode (1,943U/L) | 3 episodes* (6,038U/L, 8y) | No | Absent upper and lower limb reflexes (8y) | No (5y) | Yes (5y) | -2.39 | -1.55 | + 1.14 | Total: 22 LCT: 40 MCT: 60 |
| #12 | Aspecific abnormal repolarization (2y) | No | - | - | No | No | No | No | No (2y)[##] | Yes (2y) | -2.38 | -2.55 | -1.08 | Total: 34 LCT: 100 MCT: 0 |
| #15 | n.r. | CMP, lowest LVFS 14% (day 4) | Cardiac failure, death | | 5 episodes (33,715 U/L) | n.a. | Yes, levels unknown (postpartum) | No | Not performed | Not performed | +0.9 | n.r. | +0.1 | Not calculated |

Table 4: Clinical characteristics of all surviving patients. Most recent growth and dietary characteristics of the expertise center for lcFAO-disorders are shown. For patient #5, who did not visit the expertise center, most recent characteristics of the treating metabolic center are shown. Y = years, mo = months, wk = week, ECG: electrocardiogram, n.r.: not reported, n.a.: not applicable, LV: left ventricle, CMP: cardiomyopathy, LVFS: left ventricle fractional shortening, CK: creatine kinase (reference value: <145-200), NCS: nerve conduction studies, SNAP: sensory nerve action potential, CMAP: compound muscle action potential, SD: standard deviation, MCT: medium-chain triglycerides, LCT: long-chain triglycerides, energy%: energy percentage of total calorie intake. *Patient #11 already experienced exercise induced muscle pain from infancy, but increased CK levels were not measured until the age of eight years. **Glucose concentrations were undetectable after birth ('low'). [#]Electromyography was also performed. Results were nonspecific, showing sporadic and small motor unit potential, not specific for a myopathy. [##]NCS showed decreased CMAP in the n. peroneus and decreased sensory nerve conduction velocity in the n. suralis. These abnormalities were most likely caused by a low temperature of the lower extremities.