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Multiple acyl-CoA dehydrogenase deficiency and population newborn screening

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Multiple acyl-CoA dehydrogenase deficiency and population newborn screening

Connecting the dots

Willemijn van Rijt

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Multiple acyl-CoA dehydrogenase deficiency and population newborn screening

Connecting the dots

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The origin of this thesis came from two patients, born in the summers of 2012 and 2013, who have been diagnosed with multiple acyl-CoA dehydrogenase deficiency (MADD; also known as glutaric aciduria type II). MADD is an ultra-rare (defined as <1 : 50,000 according to European Union; regulation No 536/2014) disorder of mitochondrial fatty acid oxidation and amino acid metabolism.

Although MADD is not included in the Dutch population newborn blood spot screening (NBS) program, both patients were identified after false-positive NBS test results for other inborn errors of metabolism (IEM). Despite being diagnosed with the same disease, the difference in the clinical course between the two patients is striking. The first patient was clinically ascertained already on the first day of life with deep hypoglycemia and severe ventricular tachyarrhythmias. This combined clinical presentation is suspect for an IEM related to long-chain mitochondrial fatty acid oxidation, which turned out to be MADD. During the first year of life, she developed leukodystrophy and cardiomyopathy that did not respond to the standard treatment options. Contrarily, the second patient has remained asymptomatic under regular treatment.

Both patients inspired the scientific exploration of several topics on the clinical presentation, treatment and monitoring of MADD, as well as the considerations regarding the national NBS program. Furthermore, the acute, life-threatening clinical presentation in the first patient urged us to contemplate the relationship between IEMs and unexpected death in early childhood.

1

General introduction and thesis outline

Inborn errors of metabolism and population newborn screening

Inborn errors of metabolism (IEM) are (ultra-)rare complex and heterogeneous disorders. They can be defined as conditions that lead to a disruption of a metabolic pathway, including a deficient transporter or enzyme, or a cofactor involved.[1] This can result in the accumulation of (toxic) metabolites prior, and deficiencies of metabolites after the metabolic block.[2] More than 1000 well-characterized IEMs have been described in literature.[1] The global, cumulative birth prevalence of IEMs is estimated to be at least 1 : 2,000 live births.[3] Population newborn blood spot screening (NBS) programs can identify patients suffering from IEMs, mostly via tandem mass spectrometry (MS/MS) analysis of metabolites in dried blood spots (DBS), including acylcarnitines and amino acids.[4-6] Due to differences in decision-making criteria, NBS programs vary worldwide, and sometimes even within countries, for example with respect to the screening panel, sampling window, analytical techniques, storage conditions of DBS.[7,8]

Already in 1968, Wilson and Jungner stated that, although "*in theory, … screening is an admirable method of combating disease … In practice, there are snags.*"[9(p7)] "*The central idea of early disease detection and treatment is essentially simple. However, the path to its successful achievement (on the one hand, bringing to treatment those with previously undetected disease, and, on the other, avoiding harm to those persons not in need of treatment) is far from simple though sometimes it may appear deceptively easy.*"[9(p26)] Hence, proper and careful evaluation of population screening programs is essential, particularly when it concerns incapacitated persons and minors. For decades, Wilson and Jungner's principles of early disease detection[9], as presented in **Table 1**, have provided a starting point in the decision-making process about which disorders qualify for population NBS. These criteria were supplemented by the World Health Organization in 2008.[10] In the Netherlands, based on these principles, disorders are classified with respect to the eligibility for population NBS, as shown in **Table 2**.[11,12] Currently, 17 IEMs are implemented in the Dutch NBS program and another eight IEMs are planned to be included.[12] In the most recent recommendation of the Health Council of the Netherlands, multiple acyl-CoA dehydrogenase deficiency (MADD; also known as glutaric aciduria type II; online mendelian inheritance in man (OMIM) #231680) was classified as 'category 3 condition' due to the lack of evidence for sufficient health gain through detection by NBS.[11,13]

Table 1. The principles of early disease detection.

Principles based on the disease
The condition sought should be an important health problem
There should be a recognizable latent or early symptomatic stage
The natural history of the condition, including development from latent to declared disease, should be adequately understood
Principles based on the treatment of the disease
There should be an accepted treatment for patients with recognized disease
Facilities for diagnosis and treatment should be available
There should be an agreed policy on whom to treat as patients
Principles based on the screening test
There should be a suitable test or examination
The test should be acceptable to the population
Case-finding should be a continuing process and not a ‘once and for all’ project
Cost considerations
The costs of case finding (including diagnosis and treatment) should be economically balanced in relation to expenditure on medical care as a whole

Reproduced from [9].

Table 2. Classification for the assessment whether disorders are eligible for inclusion in the Dutch newborn screening program.

Category 1: conditions that qualify for inclusion
Neonatal screening prevents significant, irreversible damage and/or yields substantial health gains for the child
A test of proven quality is available
Category 2A: conditions that require further study
Neonatal screening prevents significant, irreversible damage and/or yields substantial health gains for the child
A test of proven quality is not (yet) available
Category 2B: conditions that may be considered for inclusion after weighing the advantages and disadvantages, including cost-effectiveness
Neonatal screening yields health gains
A test of proven quality is available
Category 3: conditions that do not qualify for inclusion
Neonatal screening yields no health gains
There may be other advantages for quality of life, such as shortening the diagnostic process (without prevention or limitation of damage to health).

Reproduced from [11].

Inborn errors of metabolism that can escape identification

Population NBS programs have dramatically improved outcomes of many patients with IEMs.[14] However, some newborns escape early identification, diminishing the potential health gain. IEMs can be overlooked due to the limited number of disorders included in NBS programs; because of clinical ascertainment or (unexpected) death before the NBS test results become available or even before the NBS sample has been drawn[14-17]; or due to false-negative NBS test results[18-20]. The risk of under-detected IEMs is reinforced by the nonspecific clinical presentations, making timely awareness extremely difficult.[21] For example, confirmed sepsis does not exclude an IEM as cause of death, and can even form a provoking factor.[22]

Several retrospective studies identified IEMs as causative factor in 0.9-6% of the unexpected deaths in early childhood, including sudden infant death.[23-28] In fact, a mitochondrial fatty acid oxidation (FAO) disorder should a priori be considered in any unexpected death in the first week of life, or even preverbal children, until proven otherwise.[2] Hepatic dysfunction (e.g. hypoketotic hypoglycemia, hyperammonemia), cardiac symptoms (e.g. arrhythmias, cardiomyopathy) and severe rhabdomyolysis can cause multi-organ failure and result in sudden death.[29] The risk of neonatal symptoms and signs is possibly aggravated by the physiologic adaptations during the perinatal transition. The evolution from intrauterine to extrauterine life is characterized by important changes from fetal (i.e. *passive*) towards neonatal (i.e. *active*) physiologic key-processes, such as glucose homeostasis and thermoregulation. Most neonates undergo a period of (prolonged) fasting when the continuous feeding through the placenta switches to intermittent feeding, particularly those that are breastfed, because the breast milk is often not available at full rate from the first day on. Additionally, there is a shift in cardiac energy metabolism from pre-dominantly glucose- and lactate-driven towards mitochondrial fatty acid-driven.[30] These changes leave individuals with a compromised energy metabolism, especially patients suffering from mitochondrial FAO disorders such as MADD, vulnerable to life-threatening metabolic derangements and sudden infant death.

Multiple acyl-CoA dehydrogenase deficiency

Primary MADD results from a genetic defect in the electron transfer flavoproteins (*ETFA*, OMIM #608053; or *ETFB*, OMIM #130410) or ETF dehydrogenase (*ETFDH*, OMIM #231675). The impaired electron transfer towards the mitochondrial respiratory chain causes a decrease in oxidized flavin adenine dinucleotide (FAD), which becomes unavailable for FAD-dependent dehydrogenases in mitochondrial FAO and amino acid metabolism. This results in energy deficiency and the accumulation of toxic metabolites.[31] MADD can also be secondary, resulting from genetic defects

of riboflavin transport or flavin adenine dinucleotide synthesis (i.e. MADD-like diseases).[32]

Figure 1 presents a timeline of milestones in medical literature on MADD. The first MADD patient was reported in 1976.[33] A Turkish boy presented clinically with neonatal hypoglycemia, acidosis and a 'sweaty feet' odor. Urinary organic acid analysis revealed massive elevations of glutaric- and lactic acid, combined with increased excretion of other urinary organic acids including isobutyric-, isovaleric-, and alpha-methylbutyric acid. Since then, several case reports and small cohort studies have led to the identification of the involved gene defects.

The clinical presentation of MADD is heterogeneous. Historically, patients are classified into three subtypes: neonatal onset patients with or without congenital anomalies (type I and II, respectively) and patients with a later, relatively milder onset (type III).[34] Congenital anomalies include polycystic kidneys, hypospadias, and neuronal migration defects. Neonatal onset patients suffer from acute, overwhelming symptoms early in life including life-threatening metabolic derangements, cardiomyopathy, leukodystrophy, and hypotonia.[31] The clinical course in MADD patients with a later disease onset ranges from hypoglycemia to lipid storage myopathy, exercise intolerance, fatigue and chronic vomiting.[31] In all MADD subtypes, periods of increased metabolic stress and catabolism can provoke acute, life-threatening events, even in adulthood.[35-38]

Several laboratory abnormalities can be indicative of the diagnosis MADD:

- metabolite studies in blood (acylcarnitines): typically increased acylcarnitines include C4-, C5-, C5-DC-, C6-, C8-, C10-, C12:1-, C12-, C14:2-, C14:1-, C14-, C16-, and C18:1-carnitine, and several molar ratios.[31,32,39]
- metabolite studies in urine (organic acids, acylglycines): typically increased excretions include ethylmalonic-, glutaric-, 3-hydroxyisovaleric-, 2-hydroxyglutaric-, 5-hydroxyhexanoic-, adipic-, suberic-, sebacic-, and dodecanedioic acid, and isobutyrylglycine, isovalerylglycine, hexanoylglycine, and suberylglycine.[31,32]
- acylcarnitine assays in cultured skin fibroblasts.[40]
- FAO flux assays in cultured skin fibroblasts.[41-43]
- immunoblot analyses of cultured skin fibroblast extracts.[44,45]
- enzymatic studies in cultured skin fibroblasts: the activity of ETF[46] and ETFDH[34].
- molecular studies: sequencing of *ETFA*[47], *ETFB*[48] and *ETFDH*[49].

The application of next generation sequencing identified novel genes of which the variants can cause MADD-like clinical and/or biochemical phenotypes, next to MADD caused by variants in the classic three genes. These disorders can be

categorized as MADD-like diseases[32]:

- riboflavin transport disorders (i.e. genetic variants in SLC52A1, SLC52A2, SLC52A3; OMIM #615026, #614707, and #211530/211500, respectively).[50-54]
- FAD metabolism disorders (i.e. genetic variants in SLC25A32, FLAD1; OMIM #616839 and #255100, respectively).[55-60]

Patients with MADD are mostly identified through clinical ascertainment, but in some geographical areas, the disorder is included in NBS programs using MS/MS analysis of acylcarnitines in DBS.[35,61-63] In 2011, MADD was categorized as 'disorder to consider for NBS' by the EU Network of Experts on NBS[64], whereas in the United States of America, the disorder is listed as 'secondary condition' on the Recommended Uniform Screening Panel.[65] To date, several (regions of) European countries screen for MADD, as depicted in **Figure 2**.[66,67] MADD is not included in the Dutch NBS program, although patients can be identified after false-positive acylcarnitine test results for other IEMs, so-called 'bycatch' (n = 4 since 2003).[13,62]

The exact prevalence of MADD is unknown. In the European Union (EU), the estimated total number of patients was 120, as surveyed by the European Reference Network for Hereditary Metabolic Disorders in 2018 (unpublished data). This would result in a minimal prevalence of 0.01 : 50,000 (estimated population of the EU by Eurostat: 512.6 million as of 1 January 2018). In reports on the experiences with expanded NBS, the frequency of MADD per 50,000 screened newborns ranged from 0.11 (i.e. Australia)[14] to 0.23 (i.e. New England, USA)[63] to 0.48 (i.e. Portugal)[68] in Western countries, whereas this concerned 0.74 and 0.85 in the United Arab Emirates[69] and Singapore[70], respectively.

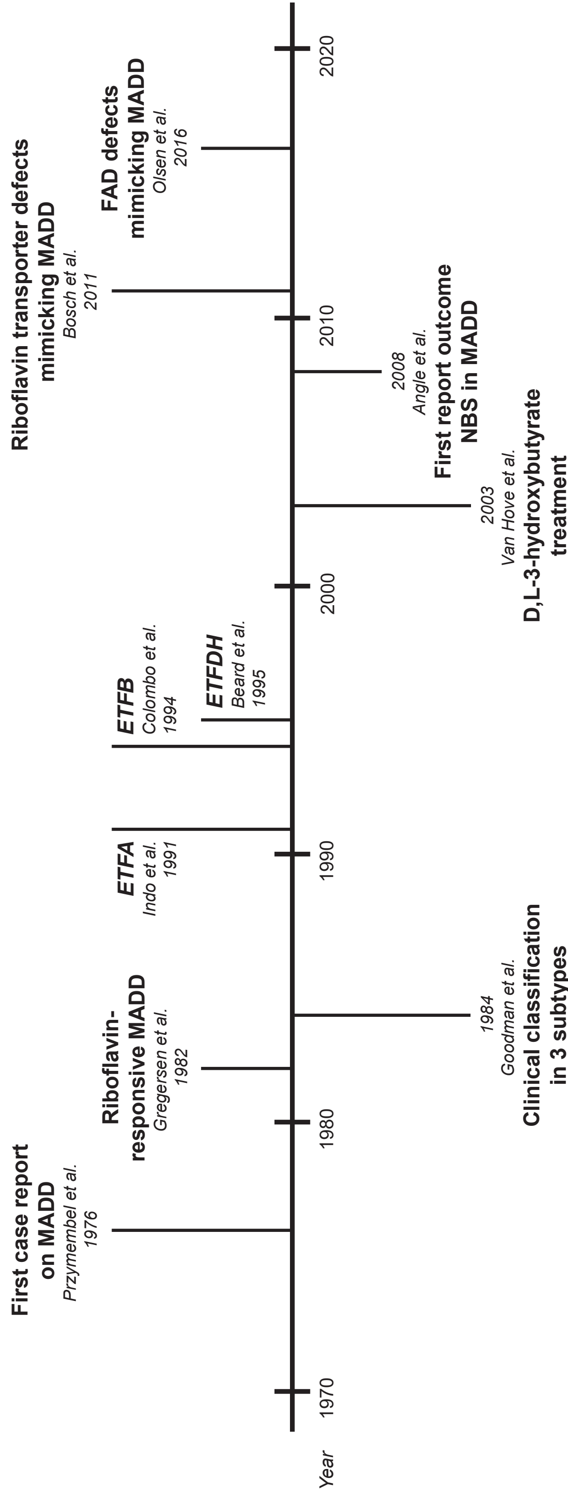

Figure 1. The scientific history of multiple acyl-CoA dehydrogenase deficiency. A timeline on the major advancements in the history of MADD.

Figure 2. Population newborn screening for multiple acyl-CoA dehydrogenase deficiency in Europe. Summarized from [66] and [67]. European countries (with regions) that screen for MADD are depicted in blue.

Upon diagnosis, it remains difficult to predict disease severity. This particularly concerns patients identified through NBS, since the aim of screening is to start early treatment in order to prevent clinical symptoms. Treatment options include 1) dietary fat- and protein restrictions to avoid the accumulation of toxic metabolites; 2) fasting avoidance to prevent energy deficiency; and 3) supplementation with carnitine (dose: 50 - 100 mg/kg/day) and glycine (dose: 150 - 300 mg/kg/day) for conjugation of toxic metabolites; riboflavin (dose: 100 - 300 mg/day), which is a precursor of FAD, in riboflavin-responsive MADD, possibly in conjunction with coenzyme Q10 (dose: 60 - 240 mg/day) in case of secondary coenzyme Q10 deficiency.[31,71-74] Additionally, an emergency protocol can offer assistance in the home management by caregivers and first hour in-hospital management to prevent catabolism and subsequent metabolic derangements.[75] Whereas neonatal onset MADD patients often do not respond to riboflavin therapy, the majority of patients with later onset MADD, particularly caused by a genetic defect in ETFDH, are riboflavin-responsive.[72,76,77] Although individual management strategies based on the regular treatments options are generally sufficient in type III MADD, morbidity and mortality remain high in neonatal onset MADD patients, despite early identification.[31,42,63]

In healthy individuals, once glycogen storages become depleted, mitochondrial FAO is essential to maintain energy homeostasis and in turn fuels hepatic ketogenesis.[78,79] Ketone bodies (KB) form an important alternative substrate for the brain, heart and skeletal muscle.[80-82] In MADD patients, the impaired mitochondrial FAO can disturb ketogenesis, leading to insufficient endogenous KB concentrations. Hence, KB supplementation may provide an effective therapeutic approach. Up to 2012, successful treatment with sodium-D,L-3-hydroxybutyrate (D,L-3-HB, a KB) has been described in four severely affected MADD patients, adding new perspectives to the management of MADD.[83,84] The D,L-3-HB salt was dosed up 900 mg/kg/day (salt-free dose: 738 mg/kg/day) and resulted in significant improvement of cardiomyopathy, leukodystrophy and hypotonia.

In our type II MADD patient, the clinical symptoms worsened despite D,L-3-HB supplementation at the previously reported dose. It was hypothesized that -in this patient- this dose could not entirely correct for the presumed severe metabolic block. In a case report, we described how a physiological dose of 2600 mg/kg/day of D,L-3-HB salt (salt-free dose: 2132 mg/kg/day) compensated for the deficient KB synthesis and resulted in substantial improvement of the clinical course.[85]

Thesis outline

The chapters of this thesis can be categorized into two parts that are closely intertwined, as demonstrated by the conceptual framework in Figure 3.

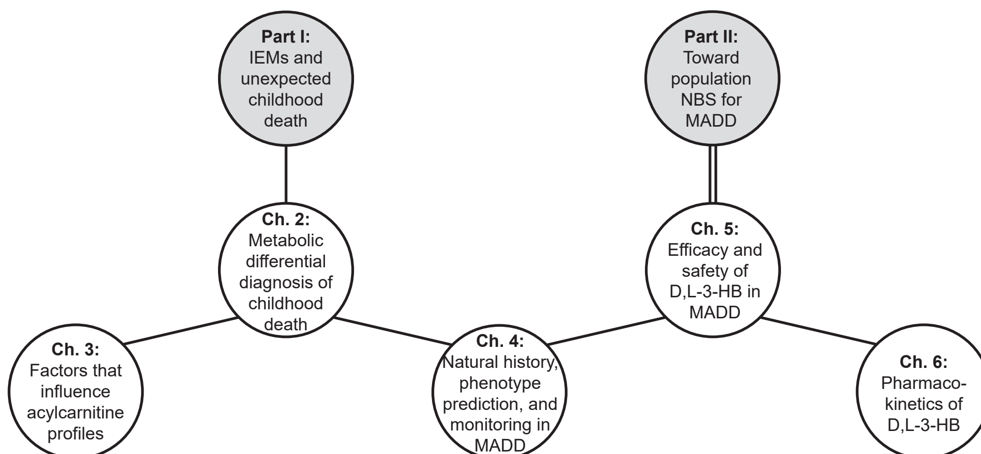

Figure 3. The conceptual framework of this thesis.

Part I – Inborn errors of metabolism and unexpected childhood death

MADD is an exemplary mitochondrial FAO disorder that can cause severe neonatal symptoms and is associated with unexpected death in infancy and childhood. Presumably, many other IEMs are associated with a similar risk. Increased knowledge is important, because it improves timely recognition, and by that may lead to prompt initiation of treatment in order to minimize the risk of life-threatening events and death.

The first major objective of this thesis is to study which IEMs are associated with unexpected death in early childhood, and how their detection through acylcarnitine profile analysis can be improved. To this aim, the following questions will be addressed:

- Which IEMs should be included in the differential diagnosis of unexpected death in early childhood, and to what extent are these disorders treatable, and detectable by MS/MS analysis in DBS? (chapter 2)
- To what extent do metabolic stress and long-term storage affect acylcarnitine profiles? (chapter 3a and 3b)

Part II – Toward population newborn screening for multiple acyl-CoA dehydrogenase deficiency

MADD is not included in the Dutch NBS program. Complicating factors concern the limited knowledge on the natural history, disease severity prediction and monitoring of the spectrum of MADD patients, and the absence of (sufficient, systematic evidence of) an effective treatment for severely affected patients.

The second major objective of this thesis is to provide evidence to re-evaluate the possibility of NBS for MADD in the Netherlands, based on the principles of early disease detection by Wilson and Jungner.9 The following questions will be addressed:

- What is the natural history of MADD? (chapter 4)
- Which (bio)markers can be used for phenotype prediction and monitoring in MADD patients? (chapter 4)
- What is the efficacy and safety of D,L-3-HB treatment in MADD? (chapter 5)
- What are the pharmacokinetic parameters in terms of absorption and distribution after D,L-3-HB administration? (chapter 6)

Finally, the results and future perspectives of this thesis are discussed in chapter 7.

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PART I

Inborn errors of metabolism and unexpected childhood death

2

Inborn errors of metabolism that cause sudden infant death: a systematic review with implications for population neonatal screening programmes

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ABSTRACT

Background:
Many inborn errors of metabolism (IEM) may present as sudden infant death (SID). Nowadays increasing numbers of patients with IEMs are identified pre-symptomatically by population newborn bloodspot screening (NBS) programmes. However, some patients escape early detection because their symptoms and signs start before NBS test results become available; they die even before the sample for NBS has been drawn; or because there are IEMs which are not included in the NBS programmes.

Objectives and methods:
This was a comprehensive systematic literature review to identify all IEMs associated with SID, including their treatability and detectability by NBS technologies. Reye syndrome (RS) was included in the search strategy because this condition can be considered as a possible pre-stage of SID in a continuum of aggravating symptoms.

Results:
43 IEMs were identified that were associated with SID and/or RS. Of these 1) 26 can already present during the neonatal period, 2) treatment is available for at least 32, and 3) 26 can currently be identified by the analysis of acylcarnitines and amino acids in dried bloodspots.

Conclusion:
We advocate an extensive analysis of amino acids and acylcarnitines in blood/plasma/dried bloodspots and urine for all children who died suddenly and/or unexpectedly, including neonates in whom blood has not yet been drawn for the routine NBS test. The application of combined metabolite screening and DNA-sequencing techniques would facilitate fast identification and maximal diagnostic yield. This is important information for both clinicians who need to maintain clinical awareness, and for decision-makers to improve population NBS programmes.

INTRODUCTION

Many inborn errors of metabolism (IEM) that cause cellular energy deficiency and/or intoxication are associated with sudden infant death (SID). Based on retrospective studies, approximately 0.9 to 6% of all SID cases involve IEMs.[1-3] Although these studies were subject to several forms of selection bias, they form the rationale behind metabolic autopsy protocols for young children, which include analyses of amino acid and acylcarnitine profiles in plasma/urine.[4]

Since the 1990s, tandem mass spectrometry (MS/MS) of dried blood spots (DBS) has been developed to perform high-throughput simultaneous quantitative analysis of different diagnostic metabolites in small amounts in biological samples.[5] As a consequence, in the last two decades population newborn bloodspot screening (NBS) programmes have expanded to include many IEMs. Patients with treatable IEMs can remain undetected by population NBS programmes for several reasons. In some IEMs, symptoms and signs including death may already occur prior before the NBS test results becoming available or even before blood for testing has been drawn, annulling the benefits of NBS.[6-10] This is especially relevant in areas where neonatal blood is collected relatively late, for instance in the Netherlands (i.e. 72-168 hours after birth).[11,12] Worldwide, across different areas, population NBS programmes differ with respect to the methodological aspects and the disorders screened.

Systematic studies on the percentage of IEMs in SID cases are required because, although rare, SID that would have been preventable due to the IEM concerned being treatable does still occur. Therefore, we performed this comprehensive systematic literature review to identify IEMs that 1) are associated with SID, 2) have clinical ascertainment during the neonatal period, 3) are treatable and 4) are detectable by MS/MS.

METHODS

Search strategy

A literature search for relevant references was performed according to the Cochrane Collaboration methodology. The CINAHL, Cochrane, PubMed and Embase public databases were searched using both Medical Subject Heading (MeSH) terms and free text. A detailed presentation and assessment of the search strategy, including the Preferred Reporting Items for Systematic review and Meta-Analysis Protocols (PRISMA-P) 2015 checklist, is presented in **Supplemental Data 1** (accessible online). **Figure 1** presents the flow chart of the detailed search strategy together

with the steps of the systematic review.

The following search terms were included to further optimize our search. The term 'mitochondrial fatty acid oxidation' was included because, based on previous studies and our personal expertise, this disease group has the highest incidence among IEMs associated with both SID[1-3] and NBS programmes.[6,7,10] SID is historically defined as occurring in the first year of life. We therefore expanded our search strategy, adding the term 'sudden unexpected death of infant'. Originally, Reye syndrome (RS) was described as a non-inflammatory encephalopathy in childhood, associated with hepatic dysfunction.[13] Since the 1980s, it has been recognized as a presenting symptom of IEMs rather than an etiologic diagnosis.[14] We considered RS as a potential pre-stage of SID in a continuum of aggravating symptoms. We therefore also included the term 'Reye syndrome' in our search.

All reports published since 1990 were included, corresponding with the first publications about the availability of MS/MS and the general progressions made with molecular and enzymatic confirmatory testing in the field of IEMs. References published before 1990 were only included when available upon request. Two independent reviewers (GK and TD) performed title and abstract screenings. Consensus on inclusion was reached during regular meetings. Subsequently, three independent (WvR, GK and TD) screened the full-text articles of all selected references. The inclusion of a diagnosis as a cause of SID and/or RS was based on the presence of detailed patient data and a confirmed diagnosis in the full-text articles. Specific exclusion criteria were: 1) no detailed patient data reported; 2) a lack of accessibility of the articles; 3) confirmatory metabolite, molecular or enzymatic studies were inconclusive; 4) there had been a (possible) additional contributing cause of death; 5) patients were above 18 years old; and 6) the abstract and/or article was not available in either English or Dutch.

Data analysis

All IEMs were classified according to the Society for the Study of Inborn Errors of Metabolism classification of IEMs.[15] Based on the included references, associations between confirmed diagnoses and SID and/or RS were documented (e.g. **Table 1**: '+' in the SID column indicates that the particular IEM has been associated with SID, with the corresponding references presented in **Supplemental Table 1** (accessible online)). Neonatal clinical presentation was reported based on detailed patient data from the included references. Based on recent textbooks and literature, the treatability[16] and detectability by MS/MS of a DBS[17-19] were documented, respectively.

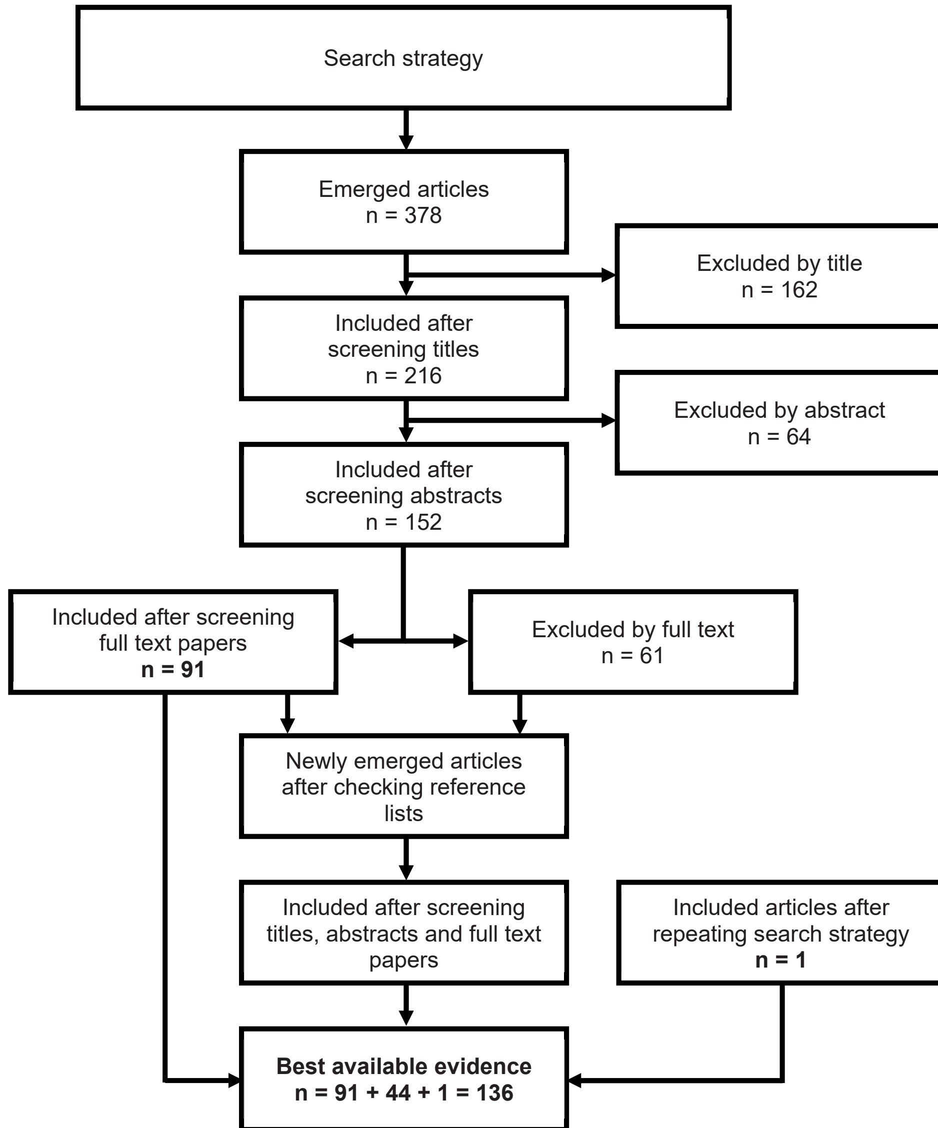

Figure 1. Flowchart of the detailed search strategy. CINAHL, Cochrane, PubMed and Embase were searched using both MeSH terms and free text: (“Metabolism, Inborn Errors”[Mesh] OR “inborn errors of metabolism” OR “mitochondrial fatty acid oxidation”) AND (“Sudden Infant Death”[Mesh] OR “sudden infant of death” OR “sudden infant death syndrome” OR “unexpected death” OR “sudden unexpected death of infant” OR “Reye Syndrome”[Mesh]) AND (Humans[Mesh]) AND (“Infant, Newborn, Child, Adolescent”[Mesh] OR newborn OR infant OR child). The search was conducted on 15 February 2013. Due to the time that elapsed between the execution of the search and the completion of the paper, the search was repeated on 28 August 2015 to screen for possible extra IEMs. This lead to the inclusion of only one more IEM associated with either SID and/or RS: dihydrolipoamide dehydrogenase deficiency (DLD deficiency; OMIM #246900).

RESULTS

This systematic review included a total of 136 references. Table 1 presents the 43 IEMs associated with either SID and/or RS, concerning mostly disorders of mitochondrial fatty acid oxidation, the urea cycle and organic acidurias. References of all included articles are presented in Supplemental Table 1 (accessible online). Out of these 43 IEMs, 26 had presented already during the neonatal period of which 15 are treatable and also detectable by MS/MS methodologies. In at least 32 of the 43 IEMs, a specific dietary and/or pharmacological treatment is available in order to prevent clinical presentation. Identification by population NBS programmes by MS/MS analysis of amino acids and/or acylcarnitines in DBS is possible in 26 of the 43 IEMs.

Table 1. Inborn errors of metabolism associated with sudden infant death and/or Reye syndrome.

Disorder	Neonatal presentation	RS	SID	Treatable	DBS	Phenotype OMIM #
Amino acid and peptide metabolism						
Urea cycle disorders						
Carbamoylphosphate synthetase I deficiency	+	+	+	+	+ [f]	237300
Ornithine transcarbamylase deficiency [c]	+	+	+	+	+ [f]	311250
Citrullinaemia type I [c]	+	+	+	+	+ [f]	215700
Argininosuccinic aciduria	-	-	+	+	+ [f]	207900
Organic acidurias						
Glutaric aciduria type I [c d]	-	-	+	+	+ [f]	231670
Propionic aciduria [c e]	+	+	-	+	+ [f]	232000
Methylmalonic aciduria [c e]	+	+	+	+	+ [f]	251000
Isovaleric aciduria [c d]	-	-	+	+	+ [f]	243500
Methylglutaconic aciduria type I	-	+	-	+	+ [f]	250950
Methylglutaconic aciduria type II (Barth syndrome)	+	+	+	+	-	302060
3-Hydroxy-3-methylglutaric aciduria [d]	+	+	-	+	+ [f]	246450
Alpha-methylacetoacetic aciduria [e]	-	+	-	+	+ [f]	203750
L-2-hydroxyglutaric aciduria	-	-	+	-	-	236792
Disorders of the metabolism of branched-chain amino acids not classified as organic acidurias						
[a]Dihydrolipoamide dehydrogenase deficiency	+	+	-	-	-	246900
Disorders of phenylalanine or tyrosine metabolism						
Tyrosinaemia type I [c d]	+	+	+	+	+ [f]	276700
Disorders of serine, glycine glycerate metabolism						
Nonketotic hyperglycinaemia [c]	+	-	+	+/-	+ [f]	238300

Continues on the next page

Disorder	Neonatal presentation	RS	SID	Treatable	DBS	Phenotype OMIM #
Disorders of amino acid transport						
Lysinuric protein intolerance	-	-	+	+	-	222700
Carbohydrate metabolism						
Disorders of fructose metabolism						
Hereditary fructose intolerance	-	+	-	+	-	229600
Disorders of glycerol metabolism						
Glycerol kinase deficiency	+	+	-	+	-	307030
Disorders of gluconeogenesis						
Fructose-1,6-biphosphatase deficiency	+	+	-	+	-	229700
Phosphoenolpyruvate carboxykinase deficiency	-	+	+	-	-	261650
Glycogen storage disorders						
Glycogen storage disease type Ia (von Gierke disease)	+	+	+	+	-	232200
Glycogen storage disease type Ib	-	-	+	+	-	232220
Glycogen storage disease type II (Pompe disease)	-	-	+	+	+ [g]	232300
Fatty acid and ketone body metabolism						
Disorders of carnitine transport and the carnitine cycle						
Carnitine transporter deficiency [e]	+	+	+	+	+ [f]	212140
Carnitine palmitoyltransferase I deficiency [e]	+	+	+	+	+ [f]	255120
Carnitine acylcarnitine translocase deficiency [e]	+	+	+	+	+ [f]	212138
Carnitine palmitoyltransferase II deficiency [e]	+	+	+	+	+ [f]	255110
Disorders of mitochondrial fatty acid oxidation						
Very long-chain acyl-CoA dehydrogenase deficiency [c d]	+	+	+	+	+ [f]	201475
Mitochondrial trifunctional protein deficiency	+	+	+	+/-	+ [f]	143450

Isolated deficiency of long-chain 3-hydroxylacyl-CoA dehydrogenase [d]	+	+	+	+	+ [f]	143450
Medium-chain acyl-CoA dehydrogenase deficiency [c d]	+	+	+	+	+ [f]	201450
[b]Medium-chain 3-ketoacyl-CoA thiolase deficiency	+	-	+	+	-	602199
3-alpha-hydroxyacyl-CoA dehydrogenase deficiency	+	+	+	+	+ [f]	231530
Multiple acyl-CoA dehydrogenase deficiency [c]	+	+	+	+/-	+ [f]	231680
Energy metabolism						
Disorders of pyruvate metabolism						
Pyruvate dehydrogenase complex deficiency	-	+	-	+/-	-	NS
Mitochondrial respiratory chain disorders						
Point mutations of mtDNA	+	+	+	-	-	NS
Ubiquinone (CoQ10) deficiency (Non-LS)	-	+	+	+	-	607426
Complex I deficiency; riboflavin responsive (ACAD9)	-	+	+	-	-	611126
Complex I deficiency	-	-	+	-	-	252010
Complex IV deficiency	+	+	+	-	-	220110
Metabolism of vitamins and (non-protein) cofactors						
Disorders of biotin metabolism						
Biotinidase deficiency [d]	-	-	+	+	+ [h]	253260
Holocarboxylase synthetase deficiency [d]	-	-	+	+	+ [f]	253270

In each column, ‘+’ indicates that the particular IEM has been associated with the respective parameter, with the corresponding references presented in Supplemental Table 1 (accessible online). [a]Dihydrolipoamide dehydrogenase deficiency (DLD deficiency; OMIM #246900); [b]medium chain 3-ketoacyl-CoA thiolase deficiency (MCKAT deficiency; OMIM #602199); these IEMs were not included in the list of the Society for the Study of Inborn Errors of Metabolism, but were found via the search strategy and were therefore included as IEMs associated with either SID and/or RS. [c]Reported to have caused clinical ascertainment and/or neonatal death before NBS test results were available.[6,7,10] [d]Included in the expanded Dutch population NBS programme since 2007.[25] [e]Recommended in 2015 for expansion of the Dutch population NBS programme.[25] [f]According to McHugh et al.[17]; [g]according to Kishnani et al.[18]; [h]according to Gonzalez et al.[19]. Abbreviations (in alphabetical order): ACAD9, acyl-CoA dehydrogenase family member 9; CoQ10, coenzyme Q10; LS, Leigh syndrome; NS, not specified.

DISCUSSION

This unique systematic literature review identified at least 43 IEMs associated with SID and/or RS, 26 of which can already present during the neonatal period. At least 32 are considered as treatable disorders and 26 are currently detectable by MS/MS analysis of amino acids and/or acylcarnitines in DBS. The remaining 17 cannot be detected by current metabolite screening methods, but require additional testing either by expanding the metabolic testing options or by means of genetic and/or enzymatic laboratory methods. Out of the 26 IEMs in which feasibility of clinical ascertainment within the neonatal period has been reported, at least 15 are treatable and also detectable by MS/MS analysis. This is important information for the improvement of population NBS programmes because early detection and subsequent appropriate treatment can prevent clinical presentation and even death (Table 1). Moreover, considering the results of our study, we propose that diagnostic (laboratory) protocols can be improved for children (including neonates) presenting with sudden/unexpected death.

There is no doubt that expanded population NBS programmes have significantly improved the outcomes of many patients, but there is still a subset of patients that unfortunately escapes early identification.[20] In one group, this is because limited numbers of IEMs are included in the NBS programmes. It is important to realize that population NBS programmes vary worldwide, and may even within countries. In another group, it is because the symptoms and signs present before the NBS test results become available or even before blood has been drawn. This is aggravated by the relative late drawing of blood and/or follow-up after positive test results in some areas/countries. In the Netherlands, neonatal blood for the NBS test is collected between 72 and 168 hours after birth.[11,12] In 2013, the response rate for the NBS programme was 99.35%. Referral to a metabolic physician was initiated before day 8 in 62% of the positive neonates, but 441 out of 173,118 newborns died (etiology was not specified) before blood could be drawn.[11] The reports on population NBS programmes from Australia, the USA, and Germany present patients with clinical ascertainment and sometimes even neonatal death before NBS test results have become available (see: c in Table 1).[6,7,10] In line with these reports, since the expansion of the NBS programme in our country (Table 1), clinical symptoms and signs have often preceded the NBS test results, sometimes even leading to early death, in cases of very long-chain acyl-CoA dehydrogenase deficiency, long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency/mitochondrial trifunctional protein deficiency, medium-chain acyl-CoA dehydrogenase deficiency, maple syrup urine disease and galactosemia (unpublished data). Patients may

also escape early identification due to false-negative NBS results (e.g. patients with carnitine transporter deficiency or very long-chain acyl-CoA dehydrogenase deficiency[21,22]) or for analytical reasons, which is of concern for patients with carnitine palmitoyltransferase 2 deficiency[23]. These examples stir up the debate on whether the NBS test should be performed earlier in life and/or at two different time points.

The general view on 'the metabolic autopsy' originated from case studies and small retrospective cohort studies that introduced bias.[4] It is generally recognized that low incidences and aspecific symptoms and signs cause an under-diagnosis of IEMs.[9] Our study strengthens the rationale that, despite a low incidence of individual IEMs, the neonates who died deserve at least an MS/MS analysis of amino acids and acylcarnitines in a DBS, when feasible. For most of the disorders listed in Table 1, the associated recurrence rate for affected families is at least 25%.

Several methodological issues in this study should be mentioned. First, the retrospective design of many of the cohort studies and case studies included might have introduced both a publication bias and a data availability bias as 1) the reports do not always describe detailed patient data and 2) obviously not all SID cases due to IEMs get reported in the literature. Second, there are many factors including aspecific symptoms that lead to under-diagnosis of IEMs in neonates.[9] Third, despite our extensive and detailed search strategy, we cannot exclude the possibility that a few references were missed. This was emphasized by the fact that, after including the full-text articles in the first round (n = 91), new references still emerged via the reference lists of excluded and included full-text articles. In order to optimize the search strategy, we conducted a second (n = 44) and third (n = 1) screening round. Fourth, in the medical literature, the definition of SID is not always consistently applied with regard to age range and clinical symptoms and signs. In an attempt to overcome this, we added the term 'sudden unexpected death of infant' to our search strategy. Last, some included IEMs exemplify only one protein deficiency in a large metabolic pathway involving many enzymes and transporters that could potentially create a similar clinical picture. Therefore, we believe, based on our systematic review, that the IEMs included in Table 1 should be considered as the minimal number of IEMs associated with SID and/or RS. Despite expanding NBS programmes, clinical awareness needs to remain high amongst neonatologists and paediatricians because many IEMs have not yet been implemented in NBS programmes. It is possible that early recognition of clinical presentations and subsequent diagnostic testing could prevent fatal outcomes.[24]

In summary, our systematic review identified the IEMs that are associated with RS and SID, a significant proportion of which are treatable disorders. In our opinion, the analysis of amino acids and acylcarnitines in blood/plasma/DBS and

urine should be part of post-mortem diagnostic protocols, next to isolation of DNA and preferentially, material for functional tests such as the analysis of cultured skin fibroblasts. The combination of metabolite screening and DNA-sequencing techniques would harbor the best of both methods, i.e. fast identification and a high diagnostic yield.

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3a

Changes in pediatric plasma acylcarnitines upon fasting for refined interpretation of metabolic stress

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ABSTRACT

Background:

Childhood fasting intolerance is a life-threatening problem associated with various inborn errors of metabolism. Plasma acylcarnitines reflect fatty acid oxidation and help determine fasting intolerance etiology. Pediatric reference values of plasma acylcarnitines upon fasting are not available, complicating interpretation of stress samples.

Methods:

Retrospective analysis of supervised clinical fasting studies between 01/2005 and 09/2012. Exclusion criteria involved patients with (suspected) disorders, repeated tests or incomplete results. Remaining children were grouped according to age: group A (≤ 24 months), B (25 - 84 months) and C (≥ 85 months). Median and 2.5th to 97.5th percentiles of basic metabolic parameters and acylcarnitines were determined at start and end of testing on the ward and analyzed for significant differences ($p < 0.05$).

Results:

Out of 127 fasting studies, 48 were included: group A (n = 13), B (n = 23) and C (n = 12). Hypoglycemia occurred in 21%. Children from group C demonstrated significantly higher end glucose concentrations while end ketone body concentrations were significantly lower compared to younger children. In all groups, free carnitine and C3-carnitine significantly decreased upon fasting, while C2-, C6-, C12:1-, C12-, C14:1-, C14-, C16:1- and C16-carnitine significantly increased. End concentrations of C6-, C12:1-, C12-, C14:1-, C14-, C16:1-, C16- and C18:1-carnitine were significantly lower in children ≥85 months compared to younger children.

Conclusions:

Fasting-induced counter-regulatory mechanisms to maintain energy homeostasis are age-dependent. This influences the changes in basic metabolic parameters and acylcarnitine profiles. Our data enable improved interpretation of the individual fasting response and may support assessment of minimal safe fasting times or treatment responses in patients.

INTRODUCTION

During childhood, fasting intolerance is a common, possibly life-threatening problem associated with hypoglycemia, metabolic acidosis and/or hyperammonemia. Hypoglycemia occurs when there is an imbalance between glucose production, utilization and (re)absorption.[1,2] Adequate fasting adaptation depends on three main pillars which provide counter-regulatory mechanisms to maintain energy homeostasis: a functioning liver and endocrine system, enzymes for production and utilization of metabolic fuels and sufficient energy substrate stores.[1,2] In the initial postprandial fasting state, hepatic glycogenolysis provides a sufficient energy balance. As fasting duration progresses, glycogen stores get exhausted and hepatic gluconeogenesis from alternative substrates as lactate and alanine becomes the main energy source. Lipolysis is activated and fatty acid oxidation in mitochondria and peroxisomes increases. This in turn provides energy required for gluconeogenesis and triggers ketogenesis.[1,2] After prolonged fasting, ketone bodies (KB) finally replace glucose as the brain's major energy source.[3,4] Defects in these pillars can cause hypoglycemia.

Hypoglycemia can result in irreversible neurological impairment or even death.[1,5,6] In order to prevent possible future metabolic derangements, the knowledge on fasting tolerance etiology is of large importance and therefore needs to be assessed. Possible underlying diagnoses as endocrine disorders, liver failure, and various inborn errors of metabolism (IEM) should be excluded.[1,2,6] The diagnostic work up of fasting intolerance consists of a combination of medical history (i.e. clinical symptoms, age at onset, time of last meal, family history), physical examination and analysis of metabolic parameters in blood samples (e.g. glucose, lactate, insulin, cortisol, growth hormone, 3-hydroxybutyrate (3-HB), free fatty acids (FFA), acylcarnitines, amino acids) and urine (e.g. organic acids).[7-11] Molecular analysis, and if necessary, confirmation by analysis of enzyme activity, effectively complements metabolite screening for diagnosis of several IEMs.[6,11-14]

Plasma acylcarnitine profiles represent an estimation of the mitochondrial fatty acid oxidation (FAO) status and can be measured by tandem mass spectrometry analysis (MS/MS). Deviations in plasma acylcarnitine profiles are used to identify IEMs, mainly disorders of mitochondrial FAO and several organic acidurias.[8,10,11] Reference values for plasma acylcarnitine profiles in a normal, fed state are available.[9,15,16] Contrarily, little is known on changes in acylcarnitine concentrations due to metabolic stress as prolonged fasting.[7,17,18] This complicates the interpretation of plasma acylcarnitines in stress samples. Therefore, the aim of this study is to describe fasting-induced changes in plasma acylcarnitine concentrations in children at various ages.

MATERIAL AND METHODS

This study involved a retrospective analysis of fasting test results, including basic metabolic parameters and acylcarnitine profiles. The Medical Ethical Committee of the University Medical Center Groningen confirmed that the Medical Research Involving Human Subjects Act did not apply, rendering official study approval unnecessary (METc code 2011/173). The study protocol was performed in accordance with the Declaration of Helsinki and approved for waived consent as it concerned retrospective, anonymous data.

Subject selection

Children who experienced a prior episode of (suspected) hypoglycemia underwent a supervised clinical fasting study in the metabolic unit of the Beatrix Children's Hospital, University Medical Center Groningen. Since MS/MS analysis of plasma acylcarnitine profiles has been performed routinely in our hospital since 2005, children who underwent a fasting study between January 2005 and September 2012 were included. Children were excluded from data analysis in case of 1) a proven metabolic or endocrine disorder; 2) suspicion for a causative IEM which could not be excluded by further diagnostic studies (e.g. riboflavin transporter deficiencies, glucose transporter type I deficiency, multiple acyl-CoA dehydrogenase deficiency, mitochondrial respiratory chain disorders); 3) a known disease such as a genetic disorder, epilepsy, cardiac anomalies or chronic disorder; 4) mental retardation or developmental delay; 5) repeated fasting tests or 6) only one acylcarnitine profile available from either the start or end of testing on the clinical ward.

Procedure supervised clinical fasting studies

Depending on the child's age and the clinician's appraisal, children started fasting on the day or night (mostly between 18.00 and 20.00 o'clock) before the supervised clinical fasting test, directly after the last regular meal. The next morning at 9.00 AM, testing started on the clinical ward. During the fasting period, children were only allowed to drink water or tea without additions. Throughout the entire test, children were observed for symptoms related to hypoglycemia, such as lethargy, paleness, clamminess, tachycardia, irritability and sweating. Blood glucose concentrations were measured at least hourly, but frequently more often, to guarantee safety. The intended duration of testing was based on age, as young children are known to have a shorter fasting capacity than older children.[7,19,20] Premature termination of the test occurred in case of hypoglycemia, defined as plasma glucose < 2.6 mmol/l[21], and/or symptoms suggestive of hypoglycemia.

At multiple intervals during testing on the ward, blood samples were collected for analysis of plasma whole blood glucose, pyruvate, lactate, acetoacetate, 3-HB and FFA. Plasma acylcarnitine profiles were measured at the start and end of testing on the ward using flow-injection MS/MS analysis, as described previously.[22] No blood samples were obtained before fasting to minimize the inconvenience of blood sampling. Moreover, the response of plasma acylcarnitines concentrations upon fasting is most informative after prolonged fasting, i.e. when fatty acids are mobilized.

Data analysis

Based on previous studies which demonstrated age-dependent fasting adaptation[7,9,18,20,23] and acylcarnitine concentrations[15], three age groups were defined: group A (≤ 24 months); group B (25 - 84 months); group C (≥ 85 months). Median (range) and 2.5th to 97.5th percentiles of basic metabolic fasting parameters and plasma acylcarnitine profiles were determined using the percentile function in Microsoft Excel. The relative change in concentrations was calculated by dividing the absolute change by the concentration at the start of the fasting test, multiplied by 100. Data was further analyzed using IBM SPSS Statistics for Windows, version 25.0 (IBM Corporation, Armonk, New York, USA), GraphPad Prism, version 5.0 (GraphPad Software, La Jolla, California, USA) and SIMCA Software, version 14.0 (Umetrics, Umea, Sweden). Due to small sample sizes, only non-parametric tests were performed. Differences in parameters at the start and end of testing on the ward within each group were analyzed using Wilcoxon Signed Ranks Test. Differences in parameters between groups were first detected via Kruskal-Wallis analysis and subsequently objectified using Mann-Whitney U Test. A p-value of < 0.05 was considered statistically significant. Bonferroni correction was performed if required. A principal component analysis on acylcarnitine profiles and basic metabolic parameters in plasma at the start and end of the fasting test was used to test for population distribution. This multivariate analysis technique is often used to visualize complex data and explore components that determine variation in a dataset. It is also useful to identify outliers in complex datasets.

RESULTS

Out of 127 children who underwent a supervised clinical fasting study between January 2005 and September 2012, 79 were excluded based on former mentioned criteria. Fasting test results of 48 children remained for further analysis: group A (n

= 13; median age: 17 months (range 1 - 23 months)); group B (n = 23; median age 45 months (25 - 81 months)); group C (n = 12; median age 126 months (89 - 194 months)). 52% of included subjects were male (n = 25) and 48% female (n = 23). Median fasting duration at the end of testing in group A, B and C, respectively, was 18 hours (10 - 22 hours), 20 hours (17 - 22 hours) and 21.5 hours (17.5 - 24 hours).

Out of 48 children, ten (21%) reached hypoglycemia of which most were found in group A (n = 4, 31%) and group B (n = 5, 22%) compared to group C (n = 1, 8%). Fasting duration until hypoglycemia increased with age, as demonstrated in **Figure 1**. Concentrations of basic metabolic parameters at the start and end of testing on the ward are presented in **Table 1A**. Glucose concentrations decreased significantly in all age groups upon fasting, with a smaller decline in children above seven years of age. In all groups, KB concentrations and the product of glucose and KB significantly increased upon fasting, while FFA/3-HB and FFA/KB significantly decreased. FFA significantly increased in group B and C, while group A only demonstrated a slight FFA mobilization from 0.69 to 0.91 mmol/L. End KB concentrations were significantly lower in older children. The fasting response in relation to age is presented in **Figure 2**.

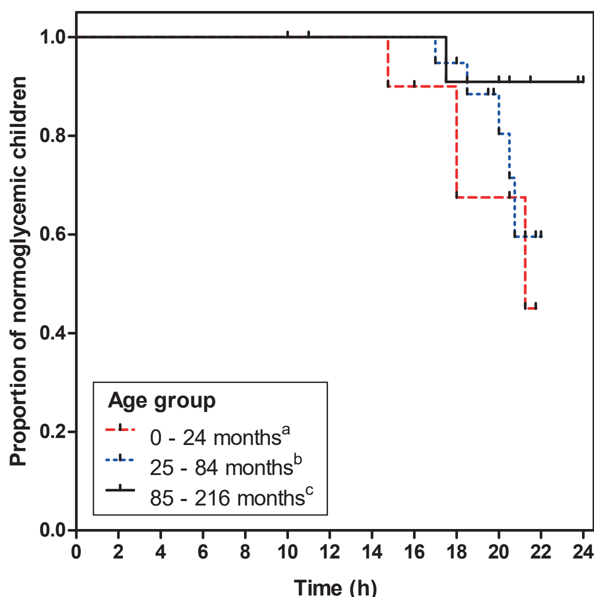


Figure 1. Proportion of children that maintained normoglycemia till the end of fasting as a function of time. Kaplan-Meier plot that visualizes the fasting duration until hypoglycemia, categorized according to age. [a]Calculated from n = 12; [b]calculated from n = 19; [c]calculated from n = 11, due to missing data; │ = censored subject.

Table 1A. Basic metabolic parameters and acylcarnitine profiles in plasma during fasting.

Parameters (mmol/L or ratio)	Group A ≤ 24 months, n = 13		Group B 25 - 84 months, n = 23		Group C ≥ 85 months, n = 12	
	Start *t = 14 h* *(5 h - 15 h)*[a]	End *t = 18 h* *(10 h - 22 h)*[a]	Start *t = 15 h* *(13 h - 15.5 h)*[b]	End *t = 20 h* *(17 h - 22 h)*[b]	Start *t = 15 h* *(11.5 h - 17.5 h)*[c]	End *t = 21.5 h* *(17.5 h - 24 h)*[c]
Glucose	4.1 (3.1 - 6.4)	3.2 (2.1 - 4.7)[d]	4.3 (2.9 - 5.4)	3.1 (2.5 - 4.5)[d]	4.5 (3.3 - 5.6)	4.2 (2.6 - 5.1)[def]
Lowest glucose	3.0 (2.0 - 3.9)		2.9 (1.7 - 3.8)		3.9 (2.6 - 4.7)[de]	
Pyruvate	0.12 (0.06 - 0.16)	0.08 (0.05 - 0.14)[d]	0.08 (0.03 - 0.13)	0.08 (0.05 - 0.13)	0.08 (0.05 - 0.12)[e]	0.06 (0.03 - 0.09)[def]
Lactate	1.15 (0.66 - 2.07)	0.90 (0.70 - 1.81)	0.80 (0.46 – 2.00)	1.30 (0.60 - 2.05)[d]	0.70 (0.53 - 1.05)[e]	0.70 (0.50 - 1.15)[f]
AcAc	0.25 (0.04 - 0.74)	0.72 (0.12 - 1.51)[d]	0.13 (0.03 - 0.98)	0.70 (0.16 - 1.43)[d]	0.02 (0.01 - 0.26)[ef]	0.21 (0.03 - 0.61)[def]
3-HB	0.71 (0.07 - 1.89)	2.19 (0.30 - 4.20)[d]	0.60 (0.04 - 2.18)	2.49 (0.56 - 4.66)[d]	0.05 (0.03 - 0.92)[ef]	0.66 (0.16 - 2.73)[def]
3-HB/AcAc	2.97 (1.62 - 4.54)	2.92 (1.71 - 4.96)[d]	2.85 (1.44 - 4.96)	3.45 (2.45 - 4.72)[d]	2.32 (1.14 - 11.93)	3.66 (2.83 - 6.73)[de]
KB	0.95 (0.11 - 2.63)	2.81 (0.43 - 5.68)[d]	0.72 (0.07 - 3.14)	3.19 (0.73 - 6.04)[d]	0.08 (0.04 - 1.18)[ef]	0.85 (0.19 - 3.26)[def]
FFA	0.69 (0.40 - 1.44)	0.91 (0.49 - 2.03)	0.92 (0.19 - 1.51)	1.72 (0.74 - 2.87)[de]	0.42 (0.24 - 1.51)[ef]	0.79 (0.39 - 2.17)[df]
FFA/3-HB	1.25 (0.47 - 8.27)	0.55 (0.33 - 1.69)[d]	1.47 (0.55 - 7.95)	0.63 (0.28 - 2.42)[d]	6.10 (1.36 - 15.35)[ef]	0.99 (0.51 - 3.14)[de]
FFA/KB	0.94 (0.35 - 4.92)	0.45 (0.25 - 1.21)[d]	1.18 (0.40 - 4.60)	0.50 (0.22 - 1.84)[d]	3.69 (0.97 - 9.73)[ef]	0.84 (0.39 - 2.43)[de]
Glucose*KB	4.41 (0.44 – 8.69)	7.60 (1.75 - 18.69)[d]	3.10 (0.27 - 13.23)	8.98 (2.55 - 20.67)[d]	0.32 (0.19 - 3.91)[ef]	4.20 (0.84 - 9.43)[df]

Table 1B. Basic metabolic parameters and acylcarnitine profiles in plasma during fasting.

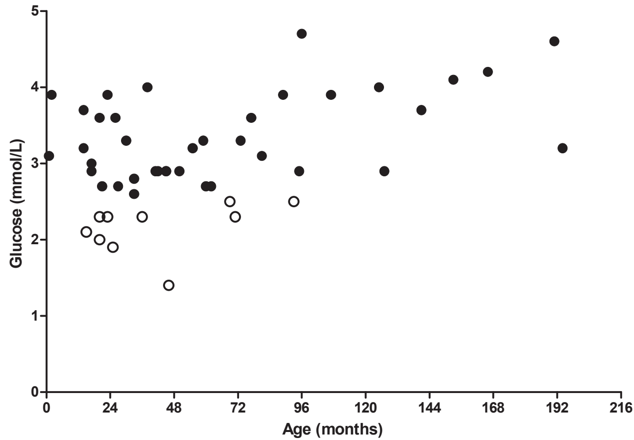
AC (µmol/L)	Reference value	Group A ≤ 24 months, n = 13		Group B 25 - 84 months, n = 23		Group C ≥ 85 months, n = 12	
		Start *t = 14 h (5 h - 15 h)*[a]	End *t = 18 h (10 h - 22 h)*[a]	Start *t = 15 h (13 h - 15.5 h)*[b]	End *t = 20 h (17 h - 22 h)*[b]	Start *t = 15 h (11.5 h - 17.5 h)*[c]	End *t = 21.5 h (17.5 h - 24 h)*[c]
Total	20 - 70	39.79 (27.93 - 51.68)	33.87 (27.50 - 47.53)[d]	36.46 (24.88 - 49.72)	35.82 (23.29 - 44.13)[d]	36.36 (21.84 - 50.19)	33.70 (17.59 - 49.14)[d]
C0	16 - 55	25.61 (18.99 - 37.68)	16.79 (10.51 - 31.24)[d]	25.25 (16.98 - 38.58)	16.62 (8.65 - 29.95)[d]	25.14 (16.18 - 41.78)	17.87 (10.31 - 37.38)[d]
C2	1.2 - 25.0	10.44 (6.24 - 22.20)	13.78 (8.28 - 25.46)[d]	9.40 (3.78 - 17.95)	12.27 (6.35 - 23.40)[d]	6.63 (3.83 - 13.84)	9.21 (5.55 - 20.73)[d]
C3	0.08 - 1.10	0.30 (0.17 - 0.52)	0.22 (0.13 - 0.43)[d]	0.25 (0.14 - 0.38)	0.20 (0.12 - 0.33)[d]	0.27 (0.17 - 0.48)	0.20 (0.09 - 0.35)[d]
C4	0.02 - 0.30	0.08 (0.05 - 0.16)	0.08 (0.05 - 0.16)	0.07 (0.04 - 0.19)	0.07 (0.04 - 0.20)	0.07 (0.03 - 0.12)	0.07 (0.03 - 0.12)
C5	0.01 - 0.25	0.05 (0.03 - 0.12)	0.06 (0.03 - 0.11)	0.06 (0.03 - 0.11)	0.06 (0.03 - 0.08)	0.07 (0.04 - 0.12)	0.06 (0.04 - 0.10)
C6	0.01 - 0.15	0.05 (0.03 - 0.10)	0.08 (0.05 - 0.11)[d]	0.05 (0.03 - 0.13)	0.07 (0.04 - 0.14)[d]	0.03 (0.03 - 0.07)[ef]	0.04 (0.03 - 0.09)[def]
C8	0.01 - 0.35	0.11 (0.06 - 0.17)	0.12 (0.08 - 0.18)	0.10 (0.04 - 0.20)	0.11 (0.06 - 0.22)[d]	0.09 (0.04 - 0.20)	0.09 (0.05 - 0.20)
C10:1	0.01 - 0.30	0.09 (0.04 - 0.14)	0.09 (0.05 - 0.13)	0.08 (0.03 - 0.14)	0.09 (0.05 - 0.17)[d]	0.07 (0.03 - 0.14)	0.07 (0.04 - 0.16)
C10	0.01 - 0.45	0.17 (0.09 - 0.22)	0.18 (0.09 - 0.25)	0.12 (0.05 - 0.28)	0.14 (0.07 - 0.30)[d]	0.10 (0.05 - 0.23)	0.11 (0.05 - 0.25)
C12:1	0.00 - 0.45	0.20 (0.07 - 0.47)	0.42 (0.11 - 0.68)[d]	0.09 (0.04 - 0.49)	0.26 (0.07 - 0.92)[d]	0.07 (0.03 - 0.25)	0.12 (0.05 - 0.48)[def]

C12	0.01 - 0.35	0.17 (0.09 - 0.32)	0.32 (0.12 - 0.49)[d]	0.10 (0.05 - 0.35)	0.24 (0.06 - 0.58)[d]	0.08 (0.05 - 0.21)[e]	0.10 (0.07 - 0.38)[def]
C14:1	0.00 - 0.50	0.22 (0.07 - 0.54)	0.40 (0.11 - 0.78)[d]	0.12 (0.04 - 0.66)	0.42 (0.08 - 1.05)[d]	0.07 (0.04 - 0.29)[e]	0.18 (0.07 - 0.51)[def]
C14	0.00 - 0.15	0.07 (0.04 - 0.15)	0.13 (0.06 - 0.26)[d]	0.04 (0.02 - 0.17)	0.12 (0.03 - 0.31)[d]	0.03 (0.02 - 0.07)[e]	0.06 (0.03 - 0.17)[def]
C16:1	0.00 - 0.15	0.05 (0.03 - 0.14)	0.10 (0.04 - 0.19)[d]	0.04 (0.01 - 0.14)	0.11 (0.03 - 0.22)[d]	0.02 (0.01 - 0.12)[e]	0.05 (0.03 - 0.17)[df]
C16	0.05 - 0.30	0.14 (0.09 - 0.21)	0.19 (0.11 - 0.28)[d]	0.10 (0.07 - 0.19)[e]	0.18 (0.07 - 0.27)[d]	0.09 (0.07 - 0.15)[e]	0.12 (0.10 - 0.21)[de]
C18:2	0.01 - 0.25	0.08 (0.04 - 0.14)	0.08 (0.04 - 0.14)	0.08 (0.03 - 0.15)	0.10 (0.05 - 0.16)[d]	0.06 (0.02 - 0.12)	0.07 (0.02 - 0.15)[d]
C18:1	0.04 - 0.55	0.25 (0.13 - 0.37)	0.23 (0.17 - 0.42)	0.21 (0.09 - 0.35)	0.28 (0.13 - 0.44)[d]	0.14 (0.05 - 0.27)[e]	0.19 (0.07 - 0.31)[df]
C18	0.01 - 0.15	0.06 (0.04 - 0.07)	0.06 (0.04 - 0.08)	0.05 (0.03 - 0.08)	0.06 (0.04 - 0.09)[d]	0.04 (0.03 - 0.06)	0.05 (0.04 - 0.07)[d]

Basic metabolic parameters (A) and acylcarnitine concentrations (B) in plasma during fasting. Data are presented as median (2.5^{th} - 97.5^{th} percentile). The presented reference values are used by our center for children > 1 month of age. [a]Median (range) fasting duration calculated from n = 12; [b]calculated from n = 19; [c]calculated from n = 11 due to missing data. A p-value of $p < 0.05$ was considered statistically significant, [d]between start and end of test; [e]compared to group A; [f]compared to group B ($p < 0.01$ after Bonferroni correction for analyses between age groups). Abbreviations (in alphabetical order): 3-HB, 3-hydroxybutyrate; AC, acylcarnitine; AcAc, acetoacetate; FFA, free fatty acids; KB, ketone bodies.

3a

A)



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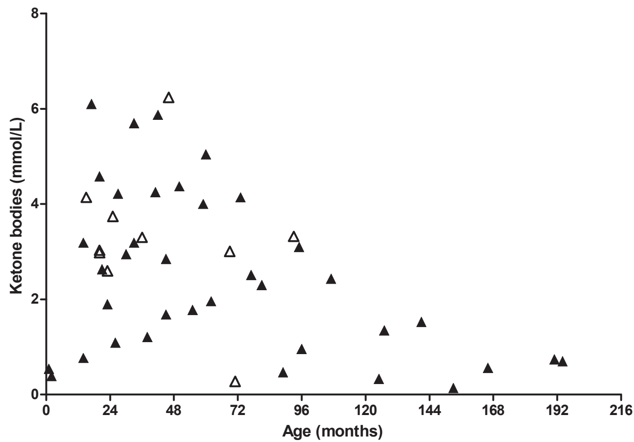

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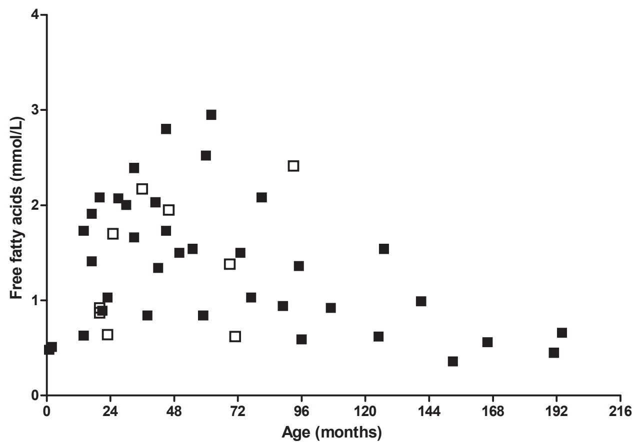

Figure 2. Basic metabolic parameters in plasma at the end of fasting in relation to age. Glucose levels (A) from n = 48; KB concentrations (B) from n = 47 due to missing data; FFA concentrations (C) from n = 46 due to missing data. Filled symbols represent children who maintained normoglycemia during the fasting test, open symbols represent children who reached hypoglycemia.

Plasma acylcarnitine profiles at the start and end of testing on the ward are presented in **Table 1B**. Since only C5 was significantly different between male and female subjects at the end of fasting, no subgroups were formed based on gender. The corresponding acylcarnitine ratios used for diagnostic differentiation and follow-up of IEM-patients[16,24] are demonstrated in **Table 2**. Upon fasting, all groups showed a significant decrease in free carnitine and C3-, while C2-, C6-, C12:1-, C12-, C14:1-, C14-, C16:1- and C16-carnitine significantly increased. In addition, end concentrations of C18:2-, C18:1- and C18-carnitine significantly increased in group B and C while C8-, C10:1- and C10-carnitine increased significantly only in group B. Significantly higher end concentrations of C6-, C12:1-, C12-, C14:1-, C14-, C16:1-, C16- and C18:1-carnitine were demonstrated in younger children compared to group C. Almost all acylcarnitine ratios changed significantly upon fasting, except for C4/C8 in groups A and C, C14:1/C12:1 in group A, (C16+C18:1)/C2 in groups B and C, C5/C3 and C8/C10 in group C, respectively. Only C14:1/C2, C14:1/C10 and C14:1/C16 were significantly lower in group C compared to younger children. The relative changes in plasma acylcarnitine concentrations and their corresponding ratios upon fasting are presented in **Figure 3**.

A principal component analysis model on the basic metabolic parameters and acylcarnitine profiles in plasma at the start and end of the fasting test demonstrated evenly distributed normo- and hypoglycemic subjects as presented in **Supplementary Figure 1**.

Table 2. Plasma acylcarnitine ratios during fasting.

AC ratio	Disorder	Group A ≤ 24 months, n = 13		Group B 25 - 84 months, n = 23		Group C ≥ 85 months, n = 12	
		Start *t = 14 h (5 h - 15 h)*[a]	**End** *t = 18 h (10 h - 22 h)*[a]	**Start** *t = 15 h (13 h - 15.5 h)*[b]	**End** *t = 20 h (17 h - 22 h)*[b]	**Start** *t = 15 h (11.5h - 17.5h)*[c]	**End** *t = 21.5 h (17.5 h - 24 h)*[c]
C0/ (C16+C18)	CPT-I, CPT-II, CACT	133.83 (77.39 - 204.40)	62.56 (33.15 - 167.73)[d]	178.59 (79.99 - 255.51)	71.09 (31.79 - 206.83)[d]	164.83 (130.53 - 341.86)[e]	102.62 (53.27 - 226.70)[d]
C3/C2 *<u>100</u>	PROP, MUT, Cbl A-D, B12 def, MCD	2.57 (1.50 - 6.28)	1.54 (0.76 - 3.27)[d]	2.80 (1.15 - 8.27)	1.53 (0.73 - 5.31)[d]	4.38 (2.24 - 9.61)	2.09 (1.08 - 3.88)[d]
C3/C16	PROP, MUT, Cbl A-D, CPT-I, B12 def, MCD	1.79 (0.96 - 3.86)	1.20 (0.71 - 3.11)[d]	2.57 (1.06 - 4.77)	1.14 (0.53 - 4.49)[d]	2.74 (1.90 - 6.67)	1.58 (0.67 - 2.85)[d]
C4/C2 *<u>100</u>	SCAD, MADD, IBG, EE, FIGLU	0.81 (0.51 - 1.17)	0.56 (0.25 - 0.96)[d]	0.82 (0.33 - 2.21)	0.57 (0.25 - 1.48)[d]	0.85 (0.56 - 1.78)	0.61 (0.40 - 1.05)[d]
C4/C3	MCD, B12 def, Cbl A-D, PROP, EE, IBG, FIGLU, MADD, SCAD	0.29 (0.19 - 0.47)	0.35 (0.26 - 0.61)[d]	0.25 (0.13 - 0.63)	0.39 (0.17 - 0.80)[d]	0.24 (0.13 - 0.35)	0.32 (0.20 - 0.47)[d]
C4/C8	IBG, SCAD, EE, FIGLU	0.80 (0.37 - 1.21)	0.83 (0.32 - 1.10)	0.76 (0.35 - 2.18)	0.69 (0.32 - 1.87)[d]	0.71 (0.31 - 1.68)	0.71 (0.38 - 1.41)
C5/C0 *<u>100</u>	IVA, 2MBG, MADD, EE	0.23 (0.10 - 0.45)	0.36 (0.14 - 0.61)[d]	0.23 (0.12 - 0.44)	0.36 (0.20 - 0.73)[d]	0.25 (0.16 - 0.44)	0.36 (0.14 - 0.43)[d]
C5/C2 *<u>100</u>	IVA, MADD, 2MBG, EE	0.52 (0.23 - 1.89)	0.30 (0.17 - 0.94)[d]	0.66 (0.27 - 1.47)	0.43 (0.20 - 1.08)[d]	0.96 (0.46 - 2.66)	0.58 (0.35 - 1.60)[d]

C5/C3	MCD, B12 def, MUT, Cbl A-B, PROP, IVA, EE, MADD, 2MBG	0.17 (0.10 - 0.45)	0.20 (0.14 - 0.47)[d]	0.22 (0.12 - 0.44)	0.28 (0.17 - 0.51)[d]	0.23 (0.14 - 0.40)	0.32 (0.16 - 0.55)
C8/C2 *100	MCAD, MADD	1.14 (0.66 - 1.88)	0.78 (0.50 - 1.50)[d]	1.01 (0.70 - 2.25)	0.86 (0.55 - 1.98)[d]	1.10 (0.79 - 3.58)	0.92 (0.46 - 1.94)[d]
C8/C10	MCAD	0.84 (0.64 - 0.98)	0.73 (0.55 - 0.97)[d]	0.84 (0.61 - 1.22)	0.75 (0.61 - 1.19)[d]	0.82 (0.68 - 1.10)	0.79 (0.60 - 1.08)
C14:1/C2 *100	VLCAD, MADD, LCHAD/TFP	2.14 (0.86 – 4.00)	3.37 (1.09 - 5.63)[d]	1.47 (0.93 - 3.65)	3.49 (1.40 - 6.55)[d]	0.99 (0.79 - 2.69)	1.71 (0.73 - 4.65)[df]
C14:1/C10	VLCAD	1.62 (0.72 - 2.47)	2.96 (1.02 - 4.02)[d]	1.16 (0.69 - 2.36)	2.89 (1.12 - 5.18)[d]	0.74 (0.57 - 1.34)[ef]	1.74 (0.79 - 3.20)[def]
C14:1/C12:1	VLCAD	1.24 (1.00 - 1.80)	1.29 (0.93 - 2.00)	1.34 (0.89 - 1.76)	1.45 (1.10 - 2.06)[d]	1.11 (0.94 - 1.56)	1.30 (0.92 - 2.02)[d]
C14:1/C16	VLCAD, MADD, LCHAD/TFP	1.23 (0.48 - 2.87)	2.47 (0.64 - 4.30)[d]	1.25 (0.50 - 3.61)	2.62 (1.16 - 4.70)[d]	0.83 (0.49 - 1.85)	1.60 (0.61 - 2.46)[df]
(C16+C18:1)/ C2 *100	CPT-I, CPT-II, CACT	3.36 (2.32 - 6.96)	3.08 (1.84 - 5.78)[d]	3.78 (2.56 - 5.30)	3.21 (2.18 - 6.29)	3.18 (2.00 - 6.69)	2.99 (1.92 - 6.71)
GA-II index*	MADD	0.04 (0.01 - 0.27)	0.26 (0.03 - 0.72)[d]	0.02 (0.00 - 0.31)	0.17 (0.01 - 1.61)[d]	0.02 (0.00 - 0.16)	0.03 (0.01 - 0.49)[d]

Data are presented as median (2.5^{th} - 97.5^{th} percentile). Some ratios are multiplied by 100 or 10000 to clarify the differences between start and end of test. [*]GA-II index: [(C4*C5*C8*C14)/(C0*C3)*10000]. [a]Median (range) fasting duration calculated from n = 12; [b]calculated from n = 19; [c]calculated from n = 11 due to missing data. A p-value of $p < 0.05$ was considered statistically significant, [d]between start and end of test; [e]compared to group A; [f]compared to group B ($p < 0.01$ after Bonferroni correction for analyses between age groups). Abbreviations (in alphabetical order): 2MBG, 2-short/branched chain acyl-CoA dehydrogenase deficiency (OMIM #610006); AC, acylcarnitine; B12 def, vitamin B12 deficiency; CACT, carnitine-acylcarnitine translocase deficiency (212138); Cbl, cobalamin (complementation group); CPT-I, carnitine palmitoyltransferase Ia deficiency (255120); CPT-II, carnitine palmitoyltransferase II deficiency (255110); EE, ethylmalonic encephalopathy (602473); FIGLU, formiminoglutamic acidemia (229100); IBG, isobutyryl-CoA dehydrogenase deficiency (611283); IVA, isovaleryl-CoA dehydrogenase deficiency (243500); LCHAD, long-chain L-3-Hydroxy dehydrogenase deficiency (609016); MADD, multiple acyl-CoA dehydrogenase deficiency (231680); MCAD, medium-chain acyl-CoA dehydrogenase deficiency (607008); MCD, holocarboxylase synthetase deficiency (253270); MUT, methylmalonic acidemia (251000, 251100, 251110); PROP, propionic acidemia (606054); SCAD, short-chain acyl-CoA dehydrogenase deficiency (201470); TFP, trifunctional protein deficiency (609015); VLCAD, very long-chain acyl-CoA dehydrogenase deficiency (201475).

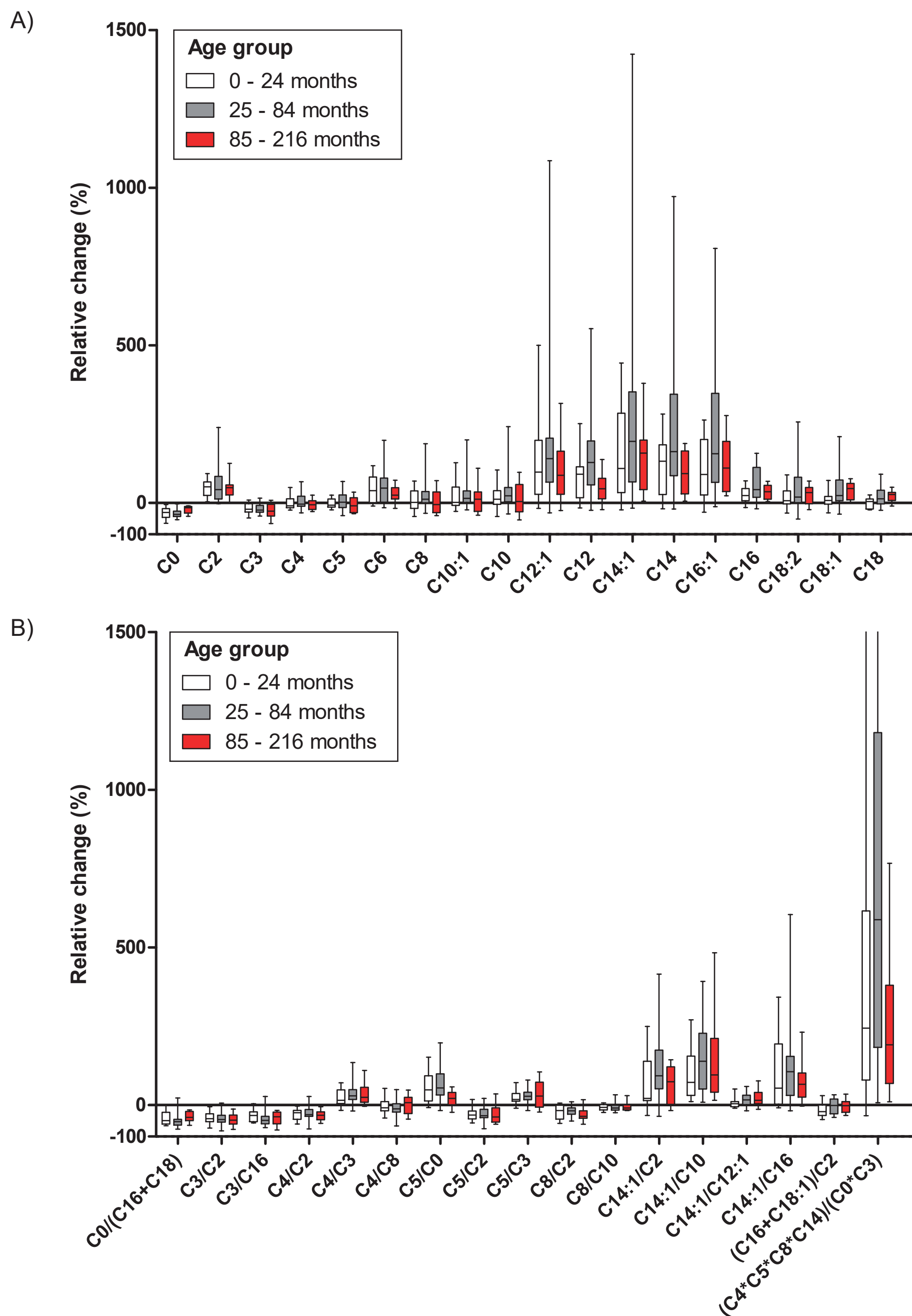

Figure 3. The relative changes in plasma acylcarnitine concentrations upon fasting. Each boxplot represents the 2.5th - 97.5th percentile of the relative changes in acylcarnitine concentration (A) and the corresponding molar ratios (B) upon fasting, categorized according to age. Corresponding significance is presented in Table 1.

DISCUSSION

Until now, the influence of metabolic stress on plasma acylcarnitine concentrations has only been studied to a limited extent.[7,17,18] This study demonstrates that prolonged fasting causes significant changes in various long-, medium-, and short-chain plasma acylcarnitine concentrations of and their corresponding ratios, together with changes in basic metabolic fasting parameters. Interestingly, the course of fasting-induced counter-regulatory mechanisms to maintain energy homeostasis, and thus the extent of acylcarnitine accumulation was age dependent. In children above seven years of age, concentrations of various plasma acylcarnitines were significantly lower compared to younger children. Although acylcarnitine ratios also changed significantly upon fasting, they seemed more robust for age influence, emphasizing their usefulness for diagnostic purposes. There is a potential risk of false-positive results when reference values for acylcarnitine profiles in a normal, fed state are used for the interpretation of stress samples. Our data facilitate improved, age-dependent interpretation of stress blood samples in order to discriminate normal from abnormal fasting responses. Particularly for the diagnosis of IEMs that may (only) result in biochemical abnormalities during periods of metabolic stress this might be of importance. Furthermore, these data can also refine the assessment of the minimal safe fasting time and evaluation of treatment response in patients.

It is known that younger children demonstrate earlier FFA mobilization and subsequent ketogenesis compared to children above seven years of age. This is probably caused by more rapid depletion of glycogen and gluconeogenic substrate storages, and a relatively higher total energy requirement.[7,10,23] In our study, end KB concentrations, the product of end glucose and KB, and end FFA levels were significantly lower in children above seven years of age compared to children aged two to seven years. This indicates less or later activity of mitochondrial FAO and ketogenesis in older children, as reflected in Figure 2, which results in a lower degree of plasma acylcarnitine accumulation. Interestingly, FFA mobilization upon fasting was not significant in children below two years of age. Although this might be due to the small sample size, other causes can perhaps include less available substrate or an increased flux. The relatively high energy requirement in infancy and childhood appears largely determined by brain development.[25,26] The estimated endogenous glucose production rate is highest in early childhood.[27] While energy required for growth declines from ~40% of the total energy requirement in the first month to <2% in the second year of life,[25] the brain's glucose demand maximizes during childhood.[28] Moreover, alanine concentrations have been reported lowest from approximately two to six years of life, reflecting low substrate availability for gluconeogenesis.[29] At

these ages, fasting can thus result in increased FFA mobilization and ketogenesis in order to preserve brain metabolism. This corresponds to our data, where most notable fasting-induced changes in metabolic parameters were found in children between two and seven years of age.

Prolonged fasting causes a significant increase in various long-, medium-, and short-chain plasma acylcarnitine concentrations, with the most significant changes in C12:1- to C16:1-carnitines and C14:1/C2, C14:1/C10, C14:1/C16 and (C4*C5*C8*C14)/(C0*C3) ratios. The relative change in the molar ratios was generally smaller and seemed less affected by age. Therefore, they enable enhanced interpretation of acylcarnitine profiles with improved sensitivity and specificity.[8] The (C4*C5*C8*C14)/(C0*C3) ratio that was proposed by Sahai et al. for improved diagnosis of MADD[24], was highly influenced by fasting, which may invalidate its application. Its diagnostic value remains to be investigated. Interestingly, there was a significant decrease in total carnitine, free carnitine and C3-carnitine in all age groups. The decrease in total carnitine concentrations is largely determined by the absolute change in free carnitine, even though acylcarnitine concentrations of several chain lengths increase upon fasting. Possible explanations for the decrease in free carnitine upon fasting include interference of the exogenous supply, increased uptake by tissues, or increased utilization for acylcarnitine formation.[30] C3- or propionylcarnitine is mainly derived from valine and isoleucine metabolism, and to some extent also from other sources including threonine, methionine, and odd chain fatty acids.[31] Its decrease may suggest increased utilization upon fasting, but this remains to be elucidated.

A study limitation concerns the relatively small sample size. Methodologically, it would have also been preferred to use a cohort without medical history instead of children who underwent a supervised clinical fasting study to evaluate the cause of previous (suspected) hypoglycemia. Ethically however, it would not be justifiable to perform clinical fasting studies in children without any suspicion of a disease. As strict exclusion criteria were adhered, we considered the remaining children as children who suffered (suspected) hypoglycemia, but who could otherwise be defined as apparently healthy subjects. All fasting test results included in our study were considered normal. Each child with a glucose concentration of <3.0 mmol/L at the end of the test demonstrated a KB concentration of > 1.8 mmol/L, which differentiates them from FAO disorder patients.[7] Increased concentrations of C14:1- and C14:2-carnitines appear to be a result of fasting-induced lipolysis. The C14:1/C12:1 ratio may help distinguish C14:1-carnitine elevations due to a physiologic fasting response from elevations due to VLCAD deficiency, avoiding a false-positive test result.[32] In our data, the individual end C14:1/C12:1 ratios did not exceed the

range described for physiologic fasting responses.

The ten subjects who developed hypoglycemia during the fasting test were not excluded. Based on fasting parameters in plasma, an underlying metabolic disorder was not considered to be likely. Principal component analyses revealed no differences between normo- (n = 38) and hypoglycemic subjects (n = 10), as shown in Supplementary Figure 1. Moreover, end FFA and KB concentrations did not significantly differ between both groups. Although some statistical differences were detected in end plasma acylcarnitine concentrations, the age-dependent fasting response might have been a confounding factor since nine out of ten subjects who reached hypoglycemia (90%) were below seven years of age. After children above seven years of age were excluded from statistical analysis, only C10-carnitine was significantly higher in hypoglycemic subjects with a median value of 0.20 versus 0.13 µmol/L ($p=0.003$). However, these values are within the C10-carnitine ranges as presented in Table 1B. One of the most commonly used explanations for hypoglycemia in children involves idiopathic ketotic hypoglycemia (IKH). These otherwise healthy children present with hyperketotic hypoglycemia, usually combined with a period of metabolic stress as intercurrent illness or prolonged fasting.[6,33] It remains debatable whether IKH is a pathophysiological condition or rather a physiologic state. Contrarily to previous beliefs, it has been suggested that these children represent the "lower tail of the Gaussian distribution of fasting tolerance in children" in whom there is a discrepancy between glucose production and utilization.[34] Relatively larger peripheral energy demands in young children as a result of higher brain to body proportions and impaired gluconeogenesis due to limited supplies of gluconeogenic substrates, particularly alanine, have both been mentioned as possible key players.[34,35] Therefore, IKH was not defined as an exclusion criterion in this study. However, a recent retrospective cohort study identified pathogenic variations in 20 out of 164 children with IKH concerning five genes associated with milder types of glycogen storage diseases including type 0, VI and IX.[36] It cannot be ruled out that our study cohort contained patients suffering from mild ketotic IEMs or undiscovered disorders associated with hypoglycemia. Furthermore, a positive carrier status for one of these diseases or synergistic heterozygosity[37,38], which theoretically could result in (slight) biochemical abnormalities upon fasting, even in the absence of clinical symptoms, has not been excluded. However, a principal component analysis model on individual acylcarnitine profiles and basic metabolic parameters in plasma at the start and end of fasting demonstrated an even population distribution without outliers, as presented in Supplementary Figure 1.

Supervised clinical fasting studies have been performed historically for diagnostic and treatment purposes in patients with fasting intolerance. Possible

complications, although rare, include severe hypoglycemia, metabolic acidosis, cardiac arrhytmias and organ failure.[6,11] Since the late 1990s, alternative diagnostic techniques have become available, with higher sensitivity and specificity. While diagnostic supervised clinical fasting studies have mostly been abandoned and are considered obsolete, fasting studies after diagnosis can still provide useful information regarding minimal safe fasting time or treatment response, presumed that they are carried out under strict observation.[6,11,18]

CONCLUSIONS

The described age-dependent changes in plasma acylcarnitine concentrations in children upon (prolonged) fasting can, in conjunction with basic metabolic parameters, be used for refined interpretation of individual response to fasting. In patients with a confirmed diagnosis, these parameters can also contribute to the determination of a minimal safe fasting time and evaluation of treatment responses.

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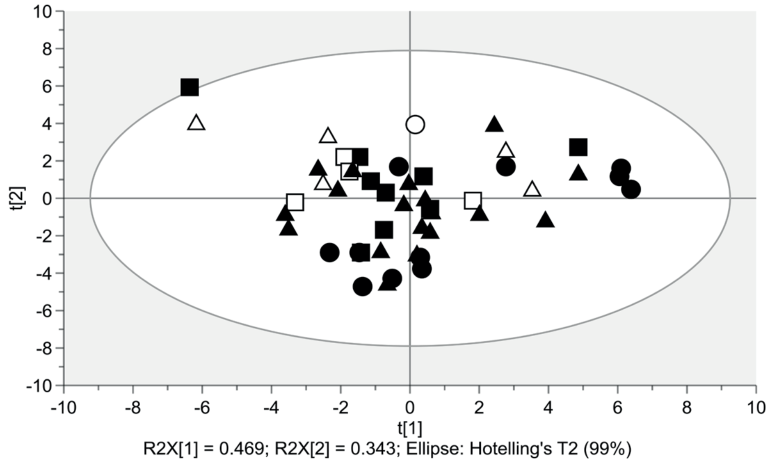
SUPPLEMENTARY APPENDIX

Supplementary Figure 1.

A)



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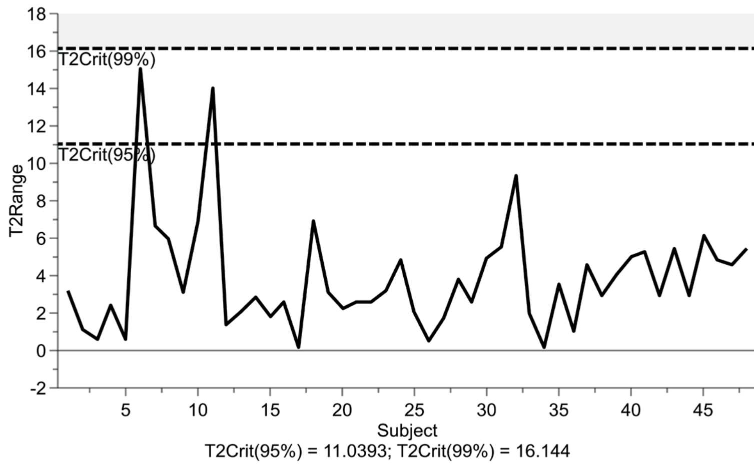

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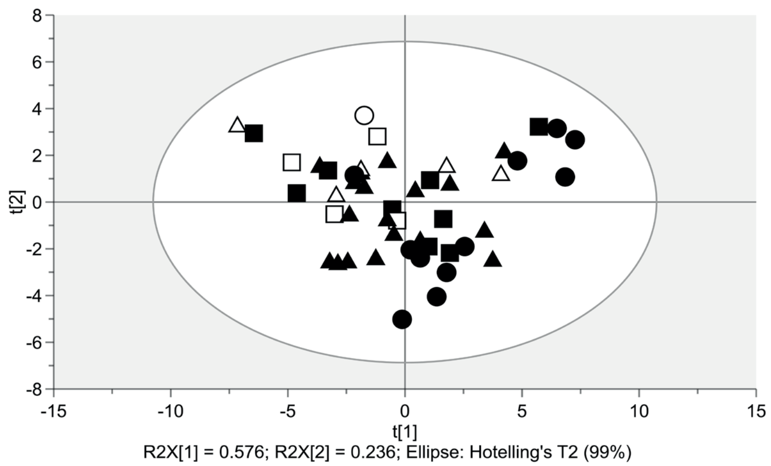

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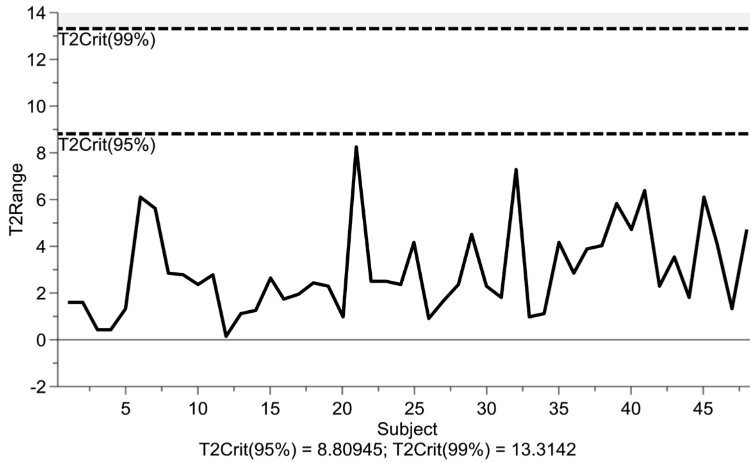

Supplementary Figure 1. Principal component analysis models on acylcarnitine profiles and basic metabolic parameters in plasma at the start and end of the fasting test. Scores scatter plots at the start and end of fasting (A and C, respectively) presenting children aged 0 - 24 months (■), 25 - 84 months (▲) and 85 - 216 months (●); normoglycemic children (filled symbols), hypoglycemic children (open symbols). The plot shows the scores of the second principal component versus the scores of the first principal component, including the 99% Hotelling's T2 ellipse to check for outliers. The principal components consist of combinations of metabolites and explain most of the variance in the data. The data points are randomly distributed around zero, indicating that the data follow a normal distribution and no outliers are present. It also shows that the normo- and hypoglycemic children overlap. Absence of outliers above the 95% and 99% T2 range is also demonstrated by the corresponding Hotellings'T2 range plot (B and D, respectively). The Hotelling's T2 range defines the area corresponding to the 95% or 99% confidence. Values above the 95% limit are suspect for outliers, and values above the 99% limit can be regarded as strong outliers.

3b

Instability of acylcarnitines in stored dried blood spots: the impact on retrospective analysis of biomarkers for inborn errors of metabolism

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ABSTRACT

Stored dried blood spots (DBS) can provide valuable samples for retrospective diagnosis of inborn errors of metabolism, and for validation studies for newborn blood spot screening programs. Acylcarnitine species are subject to degradation upon long-term storage at room temperature, but limited data is available on the stability in original samples and the impact on acylcarnitine ratios. We analysed complete acylcarnitine profiles by flow-injection tandem mass spectrometry in 598 anonymous DBS stored from 2013 to 2017, at +4 °C during the first year and thereafter at room temperature. The concentrations of C2-, C3-, C4-, C5-, C6-, C8-, C10:1-, C10-, C12:1-, C12-, C14:1-, C14-, C16:1-, C16-, C18:2-, C18:1-, C18-, C5OH+C4DC-, C18:1OH-, and C16DC-carnitine decreased significantly, whereas a positive trend was found for free carnitine. Only the C4/C8-, C8/C10-, C14:1/C10- and C14:1/C16-ratio appeared robust for the metabolite instability. The metabolite instability may provoke wrong interpretation of test results in case of retrospective studies, and risk inaccurate estimation of cut-off targets in validation studies when only stored control DBS are used. We recommend to include control DBS in diagnostic, retrospective cohort studies; and for validation studies, to use fresh samples and repeatedly re-evaluate cut-off targets.

INTRODUCTION

The development of tandem mass spectrometry (MS/MS) enabled rapid determination of acylcarnitine and amino acid profiles.[1,2] This technique is commonly used in population newborn bloodspot screening (NBS) to screen for disorders of mitochondrial fatty acid oxidation, and organic acid- and amino acid metabolism.[3] The primary process of NBS programs (e.g. screening panel, sampling window, analytical techniques, storage time of filter paper cards and storage conditions) varies worldwide, and even within countries.[4,5] In The Netherlands, 17 inborn errors of metabolism (IEM) are currently included in the NBS program.[6] The dried blood spots (DBS) on filter paper cards are generally stored for a maximum period of five years after laboratory analysis.[6]

Many IEMs are associated with unexpected death in early childhood, resulting from energy deficiency or intoxication.[7] The often non-specific clinical presentations imply a risk of IEMs to remain unrecognized as cause of death. Stored DBS can provide a valuable sample for post-mortem investigations after unexpected death in early childhood.[8-10] This especially holds for countries where only a limited selection of IEMs is included in the NBS program and/or diagnostic resources are scarce.[4] Moreover, stored DBS can be used for the evaluation of biomarkers of IEMs considered to be included in the NBS program.[11]

A study we recently performed on the prevalence of IEMs in children who died in early childhood, using metabolite analysis in stored DBS, was complicated by the impact of long-term storage on the acylcarnitine profiles. While the stability of C3DC-, C5DC-carnitine and saturated acylcarnitine species has been analysed in stored, spiked DBS[12,13], the available data on acylcarnitine concentrations in stored, original DBS is limited and only concerns the stability of C3DC-, C0-, C2-, C3, C4, and C5-carnitine.[14-16] Besides, the impact of metabolite stability on acylcarnitine ratios, which can be used for diagnostic differentiation and characterization of IEMs[3], has never been assessed. Hence, we systematically analysed the complete acylcarnitine profiles in original DBS stored from 2013 to 2017. Here, we report how metabolite instability can complicate the interpretation of retrospectively analysed acylcarnitine biomarkers for IEMs in stored DBS, and reflect on the potential risks of using control DBS stored at room temperature for validation studies for NBS programs.

MATERIALS AND METHODS

The Medical Ethical Committee of the University Medical Center Groningen confirmed that the Medical Research Involving Human Subjects Act did not apply and that official approval of this study by the Medical Ethical Committee was not required (METc code 2016/694). The Dutch NBS program is regulated by the National Institute for Public Health and the Environment (‘Rijksinstituut voor Volksgezondheid en Milieu’ (RIVM) in Dutch). Its Research Committee on Neonatal Screening (‘Werkgroep Onderzoek Neonatale Hielprikscreening’ (WONHS) in Dutch) authorized the study. A waiver of consent was granted by both the METc and WONHS, since the study concerned anonymized samples.

In The Netherlands, neonatal blood, sampled from the heel, is spotted on filter paper cards between 72 and 168 hours after birth and subsequently analysed in one of the five regional screening laboratories, according to national protocols.[6] All filter paper cards were stored at +4 °C for one year at the respective regional screening laboratory for quality assurance purposes. If authorized by the parents, the filter paper cards were stored at room temperature for another four years at the central archive of the RIVM reference laboratory, for quality assurance purposes and anonymized retrospective biomarker studies. There was no conditioning of humidity. The cards were stored in sealed bags (200-300 per bag) in cardboard boxes (20-30 bags per box). Once a year, in January of calendar year “X”, all filter paper cards of calendar year “X-6” are destroyed.[6] If the parents object to the use of residual anonymized blood samples for scientific research, the filter paper card is destroyed after one year.

This study is part of a larger research project on the prevalence of IEMs in children who died in early childhood. DBS included in the current study were from children with normal NBS results (i.e. control samples in the aforementioned study). In October 2018 (start of study), the available stored filter paper cards dated back to 2013. A total of 120 anonymized, DBS on filter paper cards per complete storage year, i.e. 2013 to 2017, were retrieved from the storage facilities of the RIVM, resulting in five storage year cohorts (i.e. 2013, 2014, 2015, 2016 and 2017). Till analyses, the DBS were kept in sealed bags with freshly added silica sachets, stored in a refrigerator at the laboratory of Metabolic Diseases in Groningen, The Netherlands. The number of spots per year cohort, i.e. 120, permits nonparametric determination of reference intervals.[17,18] Filter paper cards of children whose parents did not authorize the use for anonymous research purposes were excluded.

Flow-injection MS/MS analysis was used to quantitatively determine the acylcarnitine profiles in the DBS. Three 3.2 mm Ø discs were punched out of the

DBS. Acylcarnitines were extracted by vortexing for 30 minutes at 600 rpm after addition of 6 deuterium-labeled internal standards in methanol/MilliQ H2O (80/20 v/v). Supernatant was transferred to a vial and analysed by flow-injection tandem-mass spectrometry (Sciex API4500, Framingham, USA). Concentrations of C0 and the individual acylcarnitines were quantified by multiple reaction monitoring. C0 by an m/z transition of 162->103 and acylcarnitines by selected precursor ions with m/z 85 as the common product ion, as described previously.[19] Data evaluation was performed with Analyst® MD 1.6.2 Instrument Control and Data Processing Software (AB Sciex, Framingham, USA). The measurements took place between November 2018 and July 2019. Quality control specimens were analysed parallel with the study samples to monitor the precision during the study period. Concentrations were expressed in micromole per liter, with two decimal places.

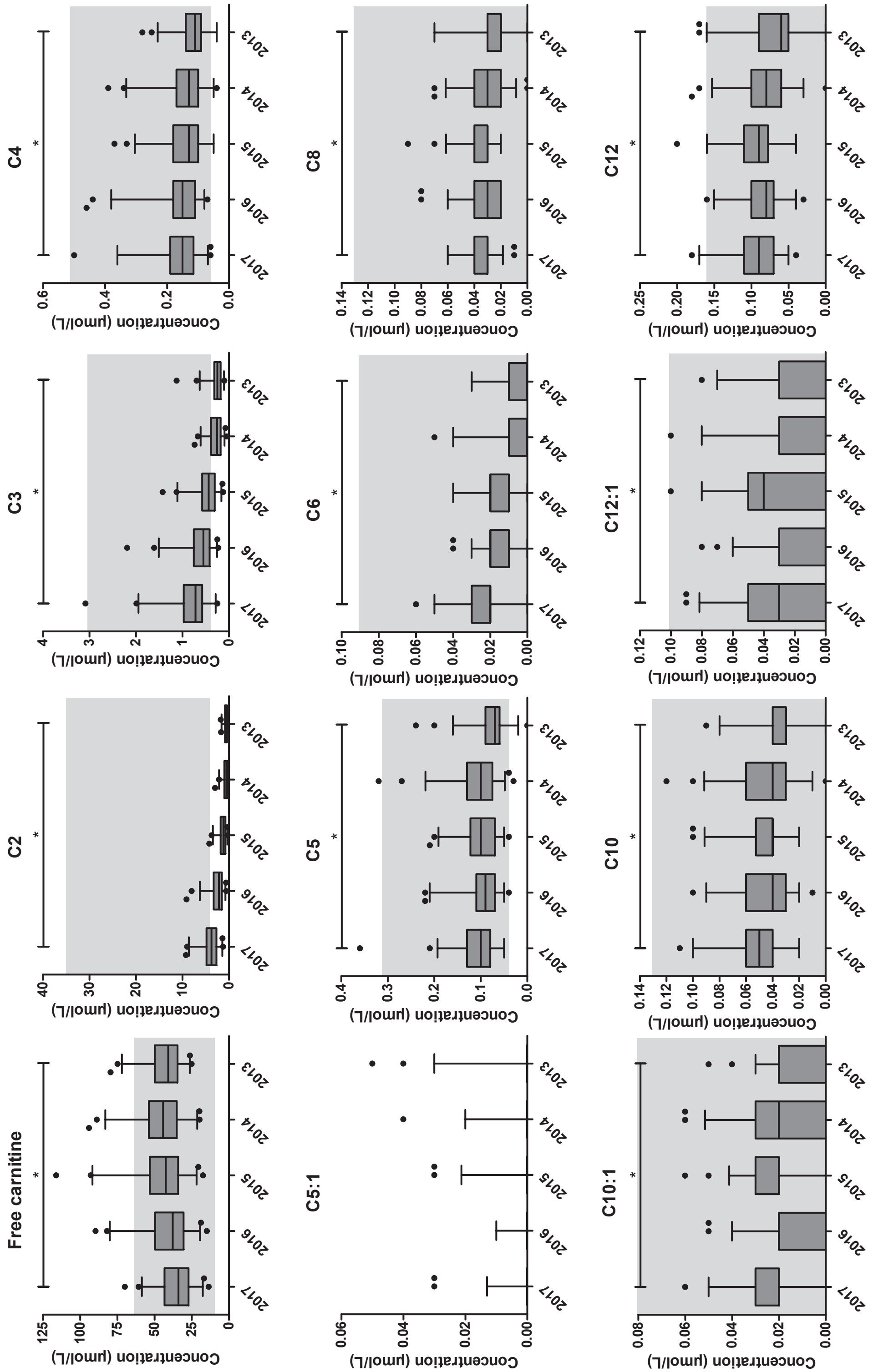
Data analysis was performed using SIMCA Software, version 15.0.2 (Umetrics, Umea, Sweden), Microsoft Excel with the Analyse-it add-in, version 4.81.6, and IBM SPSS Statistics for Windows, version 23 (IBM Corporation, Armonk, New York, USA). GraphPad Prism, version 5.0 (GraphPad Software, La Jolla, California, USA) was used to create graphs. First, a principal component analysis was used to visualize and explore the complete dataset. Cases outside the 99% Hotelling's T2 range were identified as strong outliers and excluded from further data analysis. Next, the distributions of the acylcarnitine concentrations and their molar ratios per storage year cohort were assessed. Jonckheere's trend test was used to test for significant trends upon storage duration, a p-value of < 0.05 was considered statistically significant. The test was not applied when $> 50\%$ of the cases had concentrations at or below the detection limit (i.e.< 0.01 - 0.05, depending on acylcarnitine species investigated), when $> 50\%$ of the cases concerned zero or infinite values for molar ratios, or when a visual trend was absent. For the acylcarnitine species with a significant change in the concentration upon long-term storage, regression analysis was used to define the trend. For linear trends, the annual decay rates were estimated from the slope of the trend line equations. The four-year percent decays were calculated from the estimated decay rates and the median acylcarnitine concentrations in 2017. For other trend types, we calculated the four-year percent decays from the difference in the median concentrations in 2017 and 2013. Finally, we evaluated the possible consequences of metabolite instability for adequate interpretation of acylcarnitine biomarkers for IEMs upon long-term storage room temperature. To this aim, we investigated the potential impact on 1) retrospective MS/MS analysis in stored DBS for the detection of IEMs, and 2) validation studies in stored control DBS to determine cut-off targets for NBS programs.

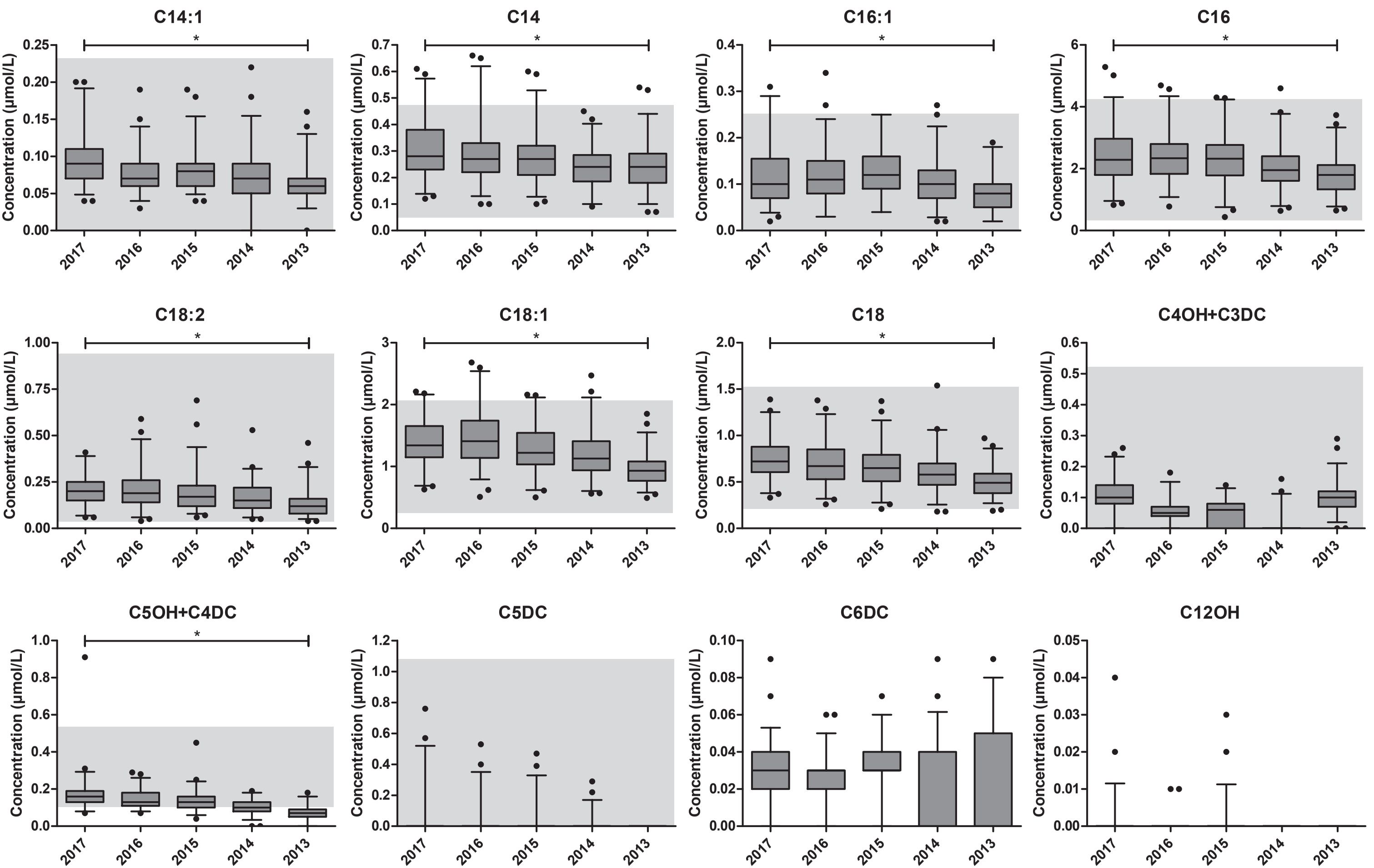
RESULTS

A total of 598 DBS were analysed; 119 DBS from storage years 2013 and 2014 and 120 DBS from storage years 2015, 2016 and 2017. The data of the low and high quality control samples per acylcarnitine species are depicted in **Table S1**. The mean inter-assay coefficient of variation was < 20% for all acylcarnitine species, except for some acylcarnitines (i.e. unsaturated-, hydroxy-, dicarboxylic, and very long-chain acylcarnitine species) with concentrations close to the detection limit. Upon outlier detection via principal component analysis (n = 598, four components, UV scaling, cumulative R2X = 0.536; cumulative Q2 = 0.373), 20 cases were excluded from the dataset (i.e. 2014: n = 6/119; 2015: n = 6/120; 2016: n = 1/120; 2017: n = 7/120).

The changes in carnitine and acylcarnitine concentrations per storage year cohort are presented in **Figure 1**. Jonckheere's trend test revealed a significant negative trend upon long-term storage for C2-, C3-, C4-, C5-, C6-, C8-, C10:1-, C10-, C12:1-, C12-, C14:1-, C14-, C16:1-, C16-, C18:2-, C18:1-, C18-, C5OH+C4DC-, C18:1OH-, and C16DC-carnitine concentrations. A positive trend was found for free carnitine. The total carnitine concentration (i.e. the sum of acylcarnitines plus free carnitine) did not change upon long-term storage.

Using regression analysis, the trend type for free carnitine was defined as polynomial, whereas for C2- and C3-carnitine the trend appeared exponential. For the remaining acylcarnitine species, the trend was defined as linear. The estimated decays of the acylcarnitine species are presented in **Table S2**. The observed decreases in absolute concentrations between 2017 and 2013 were greatest for C2-, C3-, C16-, C18:1, C18-carnitine. The most substantial percentual drop in concentrations was found for C2-, C3-, C6-, C5OH+C4DC-, and C18:1OH-carnitine, with estimated four-year decays above 60%. The estimated four-year decay ranged between 22% to 34% for the remaining saturated acylcarnitines, and between 30% to 45% for the unsaturated acylcarnitines and C16DC-carnitine.


Free carnitine
C2
C3
C4
C5:1
C5
C6
C8
C10:1
C10
C12:1
C12
Concentration (µmol/L)
2017
2016
2015
2014
2013
*


C14:1
C14
C16:1
C16
C18:2
C18:1
C18
C4OH+C3DC
C5OH+C4DC
C5DC
C6DC
C12OH
Concentration (µmol/L)
2017
2016
2015
2014
2013
*

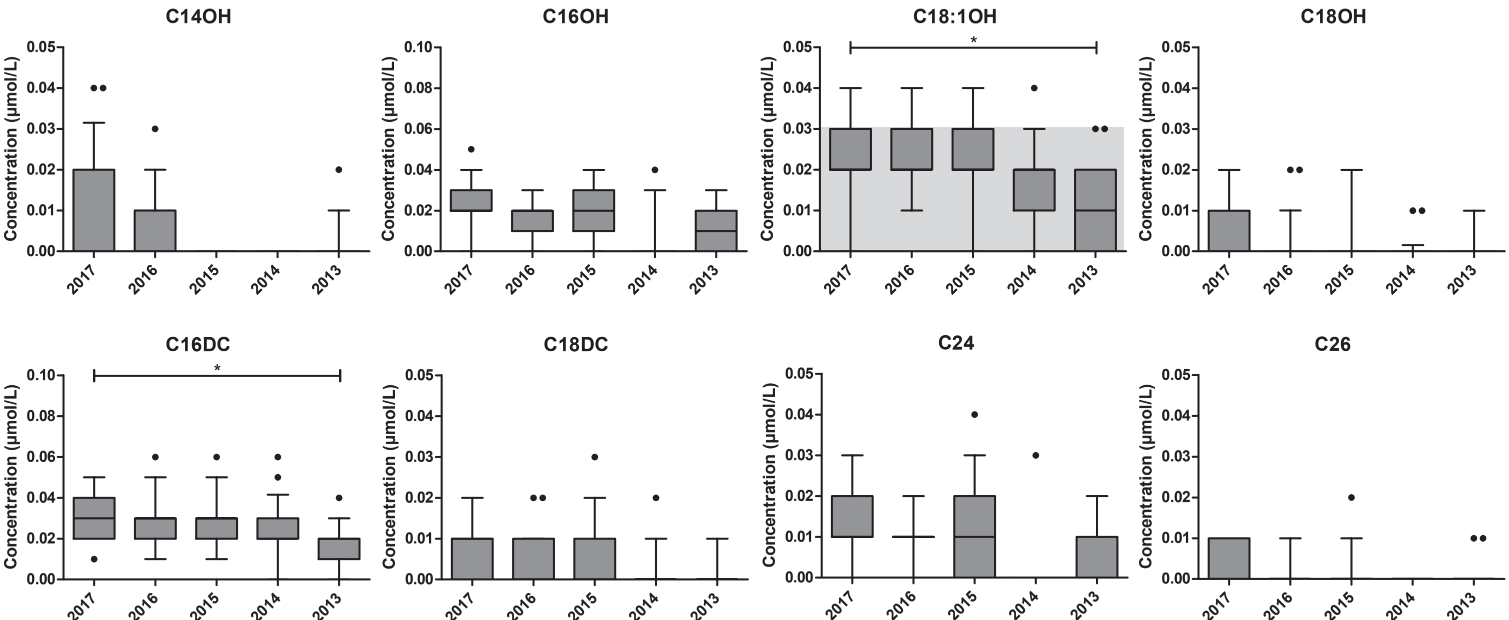
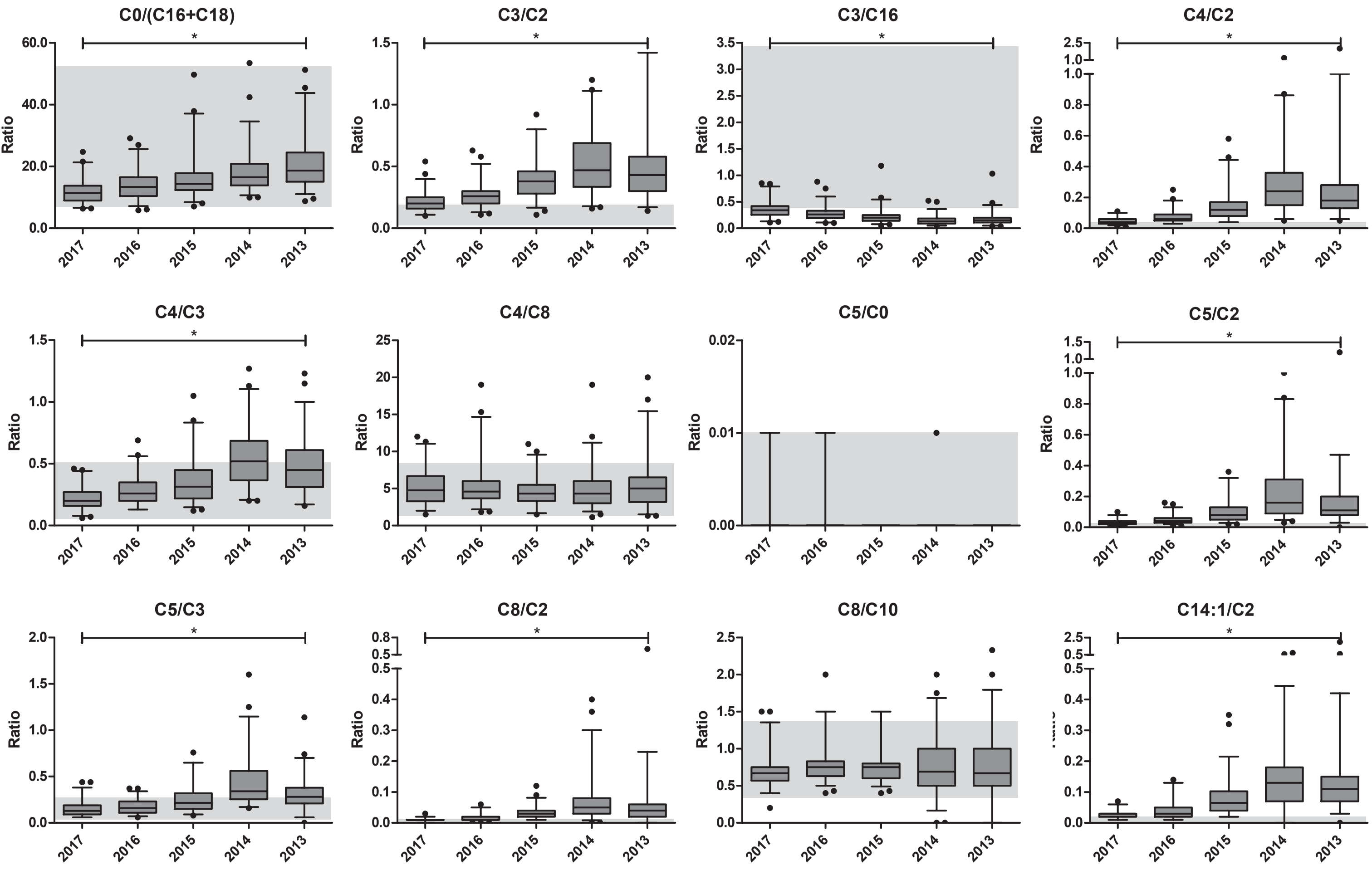


Figure 1. The changes in carnitine and acylcarnitine concentrations. The impact of metabolite instability upon long-term storage at room temperature. The boxplots represent the first quartile, median and third quartile. The whiskers extend to the 2.5th and 97.5th percentiles. Shaded areas represent the reference ranges as determined by our centre for the neonatal population. For C5:1- C12OH-, C14OH-, C16OH-, and C18OH-, C6DC-, C16DC-, C18DC-, C24-, and C26-carnitine, the upper reference limit is below the detection limit (i.e. not detectable). *Statistically significant trend in the concentration upon storage duration, as determined by Jonckheere's trend test. The following parameters were excluded from statistical analysis: C5:1-, C4OH+C3DC-, C5DC-, C6DC-, C12OH-, C14OH-, C16OH-, C18OH-, C18DC-, C24-, and C26-carnitine.

3b

The impact of long-term storage on the molar ratios of acylcarnitine species is depicted in **Figure 2**. The C14:1/C12:1 was excluded from statistical analysis because of infinite values in > 50% of the data entries. Only the C4/C8-, C8/C10-, and C14:1/C16-ratio did not demonstrate a significant trend upon long-term storage, as determined by Jonckheere's trend test. The C14:1/C10 ratio, though statistically significant, did not show a visual trend. The percent decays of the individual acylcarnitines in these ratios were similar (i.e. the absolute difference in the estimated percent decays was < 5%). Therefore, these ratios were assessed as robust with regard to the impact of metabolite instability upon long-term storage at room temperature.

The metabolite instability increases the likelihood of wrong interpretation of acylcarnitine biomarkers for IEMs in DBS stored at room temperature. **Table 1** and **2** present the potential risks for the detection of IEMs upon retrospective MS/MS analysis, and for validation studies in stored control DBS to determine cut-off targets for NBS programs.



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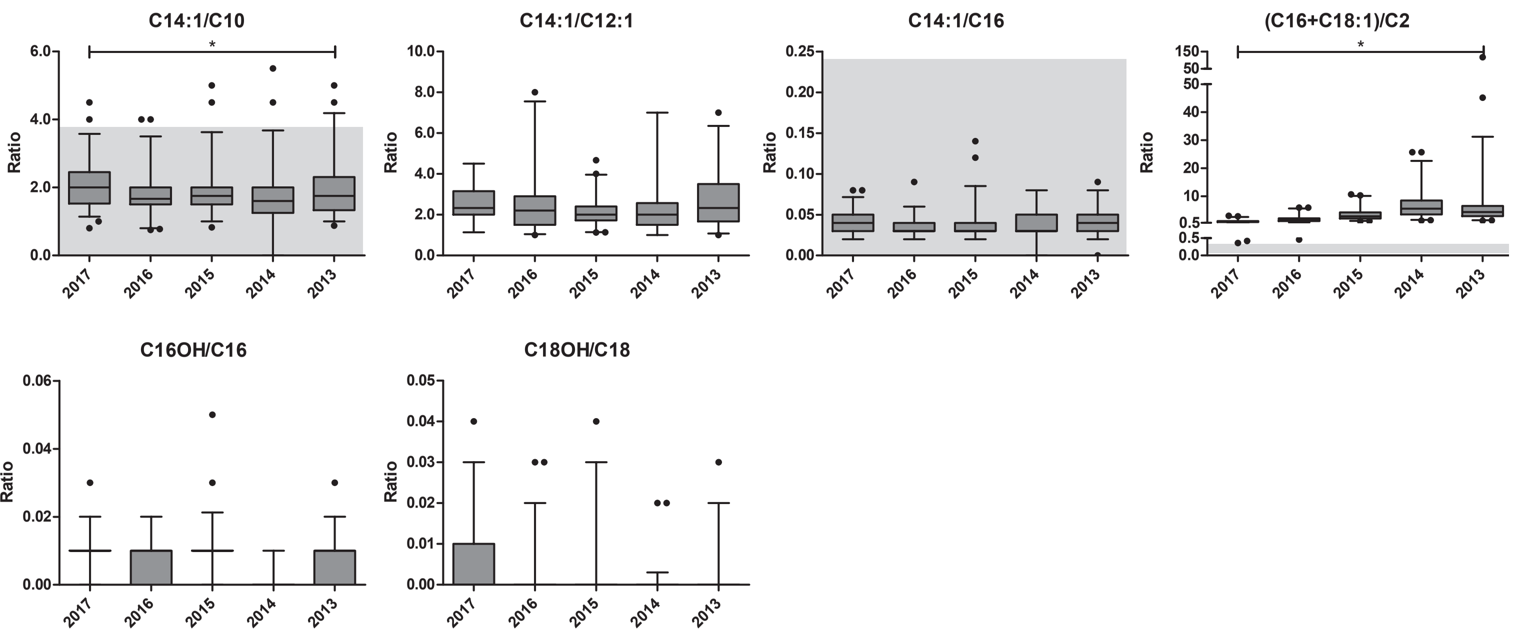

Figure 2. The changes in the molar ratios of carnitine and acylcarnitines. The impact of metabolite instability upon long-term storage at room temperature. The boxplots represent the first quartile, median and third quartile. The whiskers extend to the 2.5th and 97.5th percentiles. Shaded areas represent the reference ranges as determined by our centre for the neonatal population. For C14:1/C12:1, no reference range is available because of too many infinite values. For C16OH/C16 and C18OH/C18, the upper reference limit is 0.0. *Statistically significant trend in the molar ratio upon storage duration, as determined by Jonckheere's trend test. The following ratios were excluded from statistical analysis: C5/C0, C14:1/C12:1, C16OH/C16, and C18OH/C18.

Table 1. The potential impact of metabolite instability on the interpretation of carnitine and acylcarnitine concentrations.

Parameter	Disorder	Retrospective analysis of IEMs	Validation studies for NBS programs
		Risk category	Potential effect on cutoff target
C0 (low)	CUD	False-negative	Too high
C0 (high)	CPT-I	False-positive	Too high
C2 (low)	CUD, CPT-II	False-positive	Too low
C3 (low)	CUD	False-positive	Too low
C3 (high)	PROP, MUT, Cbl A-D	False-negative	Too low
C4	SCAD, EE, IBG, FIGLU[a], MADD	False-negative	Too low
C5	IVA, MADD, 2MBG, EE	False-negative	Too low
C6	MCAD, MADD	False-negative	Too low
C8	MCAD, MADD	False-negative	Too low
C10:1	MCAD	False-negative	Too low
C10	MADD, MCAD	False-negative	Too low
C12:1	MADD, VLCAD	False-negative	Too low
C12	MADD, CPT-II, CACT, VLCAD	False-negative	Too low
C14:1	VLCAD, MADD, LCHAD/TFP	False-negative	Too low
C14	MADD, CPT-II, VLCAD, CACT, LCHAD/TFP	False-negative	Too low
C16:1	VLCAD, LCHAD/TFP, CACT, CPT-II	False-negative	Too low
C16 (low)	CPT-I, CUD	False-positive	Too low
C16 (high)	CACT, CPT-II	False-negative	Too low
C18:2 (low)	CPT-I	False-positive	Too low
C18:2 (high)	CPT-II, CACT	False-negative	Too low
C18:1 (low)	CPT-I, CUD	False-positive	Too low
C18:1 (high)	CPT-II, CACT	False-negative	Too low
C18 (low)	CPT-I, CUD	False-positive	Too low
C18 (high)	CPT-II, CACT	False-negative	Too low
C5OH+C4DC	3MCC, HMG, MCD, 3MGA, BTD, BKT, 2M3HBA	False-negative	Too low
C18:1OH	LCHAD/TFP	False-negative	Too low
C16DC	PBD	False-negative	Too low

The potential consequences associated with 1) the interpretation of retrospectively analysed carnitine and acylcarnitine concentrations in DBS upon long-term storage at room temperature for the detection of IEMs, and 2) using control DBS stored at room temperature for validation studies on cut-off targets for NBS programs. [a]FIGLU is only associated with a high C4-carnitine when butylation is applied. Abbreviations (in alphabetical order): 2M3HBA, 2-methyl-3-hydroxybutyric aciduria (i.e. alpha-methylacetoacetic aciduria; online mendelian inheritance

in man (OMIM) #203750); 2MBG, 2-short/branched chain acyl-CoA dehydrogenase deficiency (# 610006); 3MCC, 3-methylcrotonyl-CoA carboxylase deficiency (#210200, #210210); 3MGA, 3-methylglutaconic aciduria (#250950, #302060); BKT, beta-ketothiolase deficiency (#203750); BTD, biotinidase deficiency (#253260); CACT, carnitine-acylcarnitine translocase deficiency (#212138); Cbl, cobalamin deficiency (complementation group); CPT-I, carnitine palmitoyltransferase I deficiency (#255120); CPT-II, carnitine palmitoyltransferase II deficiency (#255110); CUD, carnitine uptake defect (#212140); EE, ethylmalonic encephalopathy (#602473); FIGLU, formiminoglutamic aciduria (#229100); IBG, isobutyryl-CoA dehydrogenase deficiency (#611283); IVA, isovaleryl-CoA dehydrogenase deficiency (#243500); HMG, 3-hydroxy-3-methylglutaric aciduria (#246450); LCHAD, long-chain L-3-Hydroxy dehydrogenase deficiency (#609016); MADD, multiple acyl-CoA dehydrogenase deficiency (#231680); MCAD, medium-chain acyl-CoA dehydrogenase deficiency (#607008); MCD, holocarboxylase synthetase deficiency (#253270); MUT, methylmalonic aciduria (#251000, 251100, 251110); PBD, peroxisome biogenesis disorder (complementation group); PROP, propionic aciduria (#606054); SCAD, short-chain acyl-CoA dehydrogenase deficiency (#201470); TFP, trifunctional protein deficiency (#609015); VLCAD, very long-chain acyl-CoA dehydrogenase deficiency (#201475).

Table 2. The potential impact of metabolite instability on the interpretation of carnitine and acylcarnitine ratios.

Molar ratio	Disorder[a]	Retrospective analysis of IEMs	Validation studies
		Risk category	Potential effect on cutoff target
C0/(C16+C18) (low)	CPT-II, CACT	False-negative	Too high
C0/(C16+C18) (high)	CPT-I	False-positive	Too high
C3/C2	PROP, MUT, Cbl A-D, MCD	False-positive	Too high
C3/C16	PROP, MUT, Cbl A-D, CPT-I, MCD	False-negative	Too low
C4/C2	SCAD, MADD, IBG, EE, FIGLU[b]	False-positive	Too high
C4/C3 (low)	MCD, Cbl A-D, PROP	False-negative	Too high
C4/C3 (high)	EE, IBG, FIGLU[b], MADD, SCAD	False-positive	Too high
C4/C8	IBG, SCAD, EE, FIGLU[b]	None, similar percent decay of the involved acylcarnitine species	
C5/C2	IVA, MADD, 2MBG, EE	False-positive	Too high
C5/C3 (low)	MCD, MUT, Cbl A-B, PROP	False-negative	Too high
C5/C3 (high)	IVA, MADD, EE, 2MBG	False-positive	Too high
C8/C2	MCAD, MADD	False-positive	Too high
C8/C10	MCAD	None, similar percent decay of the involved acylcarnitine species	
C14:1/C2	VLCAD, MADD, LCHAD/TFP	False-positive	Too high
C14:1/C10	VLCAD	Appears negligible, negative statistical trend, but no visual trend and similar percent decay of the involved acylcarnitine species	
C14:1/C16	VLCAD, MADD, LCHAD/TFP	None, similar percent decay of the involved acylcarnitine species	
(C16+C18:1)/C2 (low)	CPT-I	False-negative	Too high
(C16+C18:1)/C2 (high)	CPT-II, CACT	False-positive	Too high

The potential consequences associated with 1) the interpretation of retrospectively analysed carnitine and acylcarnitine ratios in DBS upon long-term storage at room temperature for the detection of IEMs, and 2) using control DBS stored at room temperature for validation studies on cut-off targets for NBS programs. Shaded rows represent the ratios that appear robust for the metabolite instability. The percent decays were defined as similar if the absolute difference in the estimated percent decays was <5%. The C14:1/C12:1 ratio was excluded from analysis because of infinite values in > 50% of the data entries. [a]See caption of Table 1 for the defined abbreviations. [b]FIGLU is only associated with a high C4-carnitine when butylation is applied.

DISCUSSION

Since the development of MS/MS analysis, several studies have reported on the instability of acylcarnitines upon long-term storage at room temperature. However, limited data is available on the decay in original samples, and the impact on acylcarnitine ratios and the associated risks of using common cut-off values for the interpretation of acylcarnitine profiles, have never been systematically assessed.[12-15] In this study, upon long-term storage at room temperature, we found a decrease in the measured concentrations of short-, medium-, and long-chain acylcarnitines of saturated and unsaturated fatty acids, while free carnitine increased. The total carnitine concentration remained similar throughout the years of storage. Likely, the acylcarnitine species are hydrolysed to free carnitine upon long-term storage at room temperature. The different decay rates of acylcarnitine species have a substantial impact on many molar ratios. In fact, only the C4/C8-, C8/10-, and C14:1/C16-ratio, and C14:1/C10, appeared robust for the metabolite instability.

In case of retrospective, diagnostic analysis, metabolite instability may provoke false-positive and false-negative test results. Feasible identification of IEMs depends on the stability of the disease-related metabolite combined with its initial concentration and the significance of the elevation of the characteristic metabolite to the corresponding cut-off value. For many acylcarnitine biomarkers, at least the 1st to 10th percentile of the disorder range (or the 90th to 100th percentile in case of low cut-off targets) overlaps with reference intervals for the healthy population.[3] This interference, and thus the likelihood of overlooking an IEM, increases upon greater metabolite instability. Based on our results, the most adverse effects for reliable detection are expected for IEMs associated with accumulations of C3- and C5OH+C4DC-carnitine and free carnitine (e.g. propionic aciduria and methylmalonic aciduria; and 3-methylcrotonyl-CoA carboxylase deficiency, 3-hydroxy-3-methylglutaric aciduria, holocarboxylase synthetase deficiency, beta-ketothiolase deficiency; carnitine palmitoyltransferase I deficiency), and in case molar ratios are used as primary screening test such as C14:1/C2, C0/(C16+C18) and (C16/C18:1)/C2 (e.g. very long-chain acyl-CoA dehydrogenase deficiency; carnitine palmitoyltransferase I deficiency; and carnitine palmitoyltransferase II deficiency, carnitine-acylcarnitine translocase deficiency). Additionally, since > 75% of the C5DC-carnitine measurements were below the detection limit, there appears to be a considerable impact on the retrospective diagnosis of glutaric aciduria type I. The detection of a carnitine uptake defect seems complicated by the substantial rise in free carnitine, but the sum of acylcarnitines (and free carnitine) may still enable the diagnosis.[13] In line with previous studies, the impact of metabolite instability appears

relatively limited for the detectability of IEMs associated with saturated medium- and long-chain acylcarnitine accumulations, and when compared to the degradation of galactose-1-phosphate uridyltransferase and biotinidase activities.[13,20] In case of retrospective, diagnostic analysis, examination of the complete acylcarnitine profile may allow for an estimate of the metabolite instability. This would improve the interpretation of potential deviations and could reveal outliers that would otherwise not have been discovered. Especially in areas where the NBS panel is small and/or diagnostic resources are limited, this may be relevant. For diagnostic, retrospective cohort studies, we recommend to include control DBS.

Caution is warranted when control DBS stored at room temperature are used for validation studies of NBS programs, because of the risk on incorrect estimation of reference ranges and cut-off targets. This particularly applies when, given their rarity, the storage conditions for samples of IEM cases for comparison analysis are different (e.g. frozen, different storage time). For validation studies, we therefore advocate to use fresh control samples, and to compare them to carefully stored samples of IEM cases and controls. Additionally, we emphasize the importance of repeated re-evaluation of a metabolite's cut-off target after its introduction to the NBS panel.

Metabolite stability is affected by storage conditions. Increased temperature, humidity and sunlight exposure are known to influence sample integrity. This also accounts for other analytes used in NBS programs, such as amino acids, endocrine and enzyme markers.[13,16,20-22] Our results emphasize the importance of obtaining background information on a sample's storage conditions. Whereas storage at room temperature over a two-year period results in substantial changes in the concentrations of several acylcarnitine and amino acid species, these changes are attenuated or not present upon storage at -20 C° or -80 C°.[16] To minimize metabolite instabilities, DBS storage at low humidity (< 30%) and under frozen conditions (≤ -20 C°) is recommended.[13,16,20,22,23] Also during transport, the temperature and humidity should preferentially be minimized as much as possible.[20] Additionally, for optimal interpretation, the condition of the patient at the time of sample collection need to be taken into account. For example, the subject's age, metabolic stress and post mortem sample obtainment can have considerable impact on a metabolite profile.[9,24]

Some study limitations deserve discussion. First, the initial acylcarnitine profiles are unknown due to the required anonymization procedures. This limited calculation of the true percent decays, which were instead estimated from the median concentrations in 2017. Second, there is a variation in the stability data because 1) the exact storage time of the individual DBS is unknown due to the required anonymization, and 2) the longitudinal timing of our analyses. There is

evidence that suggests a more rapid degradation during the first months of storage at room temperature, which eventually slows down.[12,13] Therefore, the total decay might even be larger than the presented estimations, especially since the samples in our cohort were stored in a refrigerator without silica during the first year. Third, disparity in DBS quality and punch location are also important contributors to variation in analyte concentrations.[25] Fourth, it is unknown whether the efficiency of extraction of metabolites from filter paper cards changes upon long term storage of DBS. Possible effects on extractability do however not invalidate our findings, as the potential consequences coincide with the impact of metabolite instability. Finally, we had no access to NBS samples of diagnosed patients that were stored under the same conditions in order to validate the claimed risks presumably associated with the detection of IEMs in DBS upon long-term storage at room temperature.

In conclusion, acylcarnitine profiles in DBS stored at room temperature are subject to metabolite instability. In case of diagnostic, retrospective cohort studies, we recommend to include control DBS. For validation studies, we recommend to use fresh samples and to repeatedly re-evaluate cut-off targets.

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SUPPLEMENTARY APPENDIX

Supplementary Table 1.
Supplementary Table 2.

Supplementary Table 1. The inter-assay precision per acylcarnitine species.

Parameter	Low QC			High QC		
	Average (µmol/L)	SD	CV (%)	Average (µmol/L)	SD	CV (%)
Total	48.75	3.38	6.9	69.02	4.68	6.8
C0	25.50	2.09	8.2	29.84	2.38	8.0
C2	16.74	1.06	6.3	21.49	1.36	6.3
C3	1.94	0.13	6.6	6.17	0.36	5.9
C4	0.18	0.02	10.6	0.40	0.04	9.6
C5:1	ND			*0.01*	*0.01*	*161.3*
C5	0.08	0.01	15.4	0.24	0.03	11.8
C6	0.02	0.00	18.5	0.28	0.03	10.4
C8	*0.04*	*0.01*	*22.6*	1.94	0.17	8.6
C10:1	*0.02*	*0.01*	*38.4*	0.36	0.04	10.8
C10	0.05	0.01	18.9	0.48	0.04	8.1
C12:1	*0.01*	*0.01*	*191.8*	0.09	0.02	17.4
C12	*0.05*	*0.01*	*27.8*	0.29	0.03	10.8
C14:1	*0.05*	*0.01*	*23.1*	0.37	0.04	9.7
C14	0.15	0.02	14.2	0.30	0.02	7.7
C16:1	0.08	0.01	14.2	0.18	0.02	10.9
C16	0.96	0.08	7.9	1.14	0.08	7.1
C18:2	0.31	0.03	10.2	0.42	0.04	9.0
C18:1	1.26	0.11	8.9	1.73	0.14	8.1
C18	0.49	0.05	9.8	1.00	0.09	8.8
C4OH+C3DC	*0.10*	*0.06*	*61.6*	*0.20*	*0.14*	*70.4*
C5OH+C4DC	0.32	0.03	10.4	0.51	0.07	13.3
C5DC	*0.05*	*0.12*	*231.7*	0.92	0.16	17.8
C6DC	*0.01*	*0.01*	*141.2*	0.08	0.01	17.4
C12OH	ND			ND		
C14OH	ND			ND		
C16OH	*0.01*	*0.01*	*120.5*	*0.01*	*0.01*	*141.1*
C18:1OH	*0.01*	*0.01*	*136.6*	*0.02*	*0.01*	*43.9*
C18OH	ND			ND		
C16DC	*0.02*	*0.01*	*51.4*	*0.03*	*0.01*	*28.8*
C18DC	ND			ND		
C24	ND			*0.01*	*0.01*	*63.4*
C26	ND			*0.01*	*0.01*	*95.0*

Calculated from quality control samples (n = 30 in ten experiments). The coefficients of variation (CV) were defined as the ratio of the standard deviation to the mean. For C5:1-, C12OH-, C14OH-, C18OH-, C18DC-, C24- and C26-carnitine, concentrations were below the limit of detection (shown as not detectable: ND). Acylcarnitines in the low or high QC samples with concentrations below the limit of quantification are in *italic*.

Supplementary Table 2. The estimated decay rates of free carnitine and acylcarnitine species upon long-term storage.

Parameter	Estimated annual decay rate (µmol/L (95% CI))	Estimated annual percent decay from 2017 (% (95% CI))	Estimated percent decay 2017 – 2013 (%(95% CI))
C0	ND	ND	-20.0%
C2	ND	ND	84.4%
C3	ND	ND	65.3%
C4	0.010 (0.006 – 0.014)	6.7% (4.3 – 9.1 %)	26.7% (17.1 – 36.3%)
C5	0.005 (0.003 – 0.008)	5.5% (3.2 – 7.7%)	21.8% (12.7 – 30.9%)
C6	0.004 (0.004 – 0.005)	20.8% (17.8 – 23.9%)	83.4% (71.1 – 95.6%)
C8	0.002 (0.001 – 0.003)	6.0% (3.5 – 8.5%)	24.0% (13.9 – 34.1%)
C10:1	0.002 (0.002 – 0.003)	7.6% (5.1 – 10.1%)	30.4% (20.4 – 40.4%)
C10	0.004 (0.002 – 0.005)	7.1% (5.0 – 9.2%)	28.4% (19.9 – 36.8%)
C12:1	0.003 (0.002 – 0.004)	10.4% (5.9 – 14.9%)	41.5% (23.4 – 59.7%)
C12	0.006 (0.004 – 0.007)	6.3% (4.5 – 8.1%)	25.2% (17.8 – 32.5%)
C14:1	0.007 (0.005 – 0.009)	7.6% (5.7 – 9.5%)	30.4% (22.8 – 37.9%)
C14	0.016 (0.011 – 0.022)	5.8% (3.8 – 7.7%)	23.1% (15.2 – 30.9%)
C16:1	0.009 (0.006 – 0.012)	9.4% (6.5 – 12.4%)	37.7% (26.0 – 49.5%)
C16	0.158 (0.114 – 0.202)	6.9% (5.0 – 8.8%)	27.6% (20.0 – 35.2%)
C18:2	0.019 (0.014 – 0.024)	9.5% (11.8 – 7.1%)	38.0% (47.4 – 28.6%)
C18:1	0.116 (0.095 – 0.137)	8.6% (7.1 – 10.2%)	34.6% (28.3 – 40.9%)
C18	0.062 (0.050 – 0.073)	8.5% (6.9 – 10.2%)	34.2% (27.6 – 40.7%)
C5OH+C4DC	0.024 (0.021 – 0.028)	15.2% (13.3 – 17.2)	60.9% (53.1 – 68.8%)
C18:1OH	0.003 (0.003 – 0.004)	15.4% (18.1 – 12.6%)	61.4% (72.3 – 50.6%)
C16DC	0.003 (0.003 – 0.004)	11.3% (9.3 – 13.4%)	45.3% (37.0 – 53.5%)

Estimated decay rates and percent decays for carnitine and the acylcarnitine concentrations with a significant trend upon storage duration, as determined by Jonckheere's trend test. For free carnitine, the trend type was defined as polynomial trend, whereas for C2- and C3-carnitine the trend appeared exponential. For the remaining acylcarnitine species, the trend type was defined as linear. Abbreviation: ND, not determined.

3b

PART II

Toward population newborn screening for multiple acyl-CoA dehydrogenase deficiency

4

Prediction of disease severity in multiple acyl-CoA dehydrogenase deficiency: a retrospective and laboratory cohort study

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ABSTRACT

Introduction:

Multiple acyl-CoA dehydrogenase deficiency (MADD) is an ultra-rare inborn error of mitochondrial fatty acid oxidation (FAO) and amino acid metabolism. Individual phenotypes and treatment response can vary markedly. We aimed to identify markers that predict MADD phenotypes.

Methods:

We performed a retrospective nationwide cohort study; then developed an MADD -disease severity scoring system (MADD-DS3) based on signs and symptoms with weighed expert opinions; and finally correlated phenotypes and MADD-DS3 scores to FAO flux (oleate and myristate oxidation rates) and acylcarnitine profiles after palmitate loading in fibroblasts.

Results:

Eighteen patients, diagnosed between 1989 and 2014, were identified. The MADD-DS3 entails enumeration of eight domain scores, which are calculated by averaging the relevant symptom scores. Lifetime MADD-DS3 scores of patients in our cohort ranged from 0 to 29. FAO flux and [U-^{13}C]C2-, C5- and [U-^{13}C]C16-carnitines were identified as key variables that discriminated neonatal from later onset patients (all $p < 0.05$) and strongly correlated to MADD-DS3 scores (oleate: $r = -0.86$; myristate: $r = -0.91$; [U-^{13}C]C2-carnitine: $r = -0.96$; C5-carnitine: $r = 0.97$; [U-^{13}C]C16-carnitine: $r = 0.98$, all $p < 0.01$).

Conclusion:

Functional studies in fibroblasts were found to differentiate between neonatal and later onset MADD-patients and were correlated to MADD-DS3 scores. Our data may improve early prediction of disease severity in order to start (preventive) treatment and follow-up appropriately. This is especially relevant in view of the inclusion of MADD in population newborn screening programs.

INTRODUCTION

Multiple acyl-CoA dehydrogenase deficiency (MADD, or glutaric aciduria type II; OMIM #231680) is an ultra-rare (i.e. <1:50,000)[1] mitochondrial fatty acid oxidation (FAO) disorder caused by pathogenic variants in the genes encoding the electron transfer flavoproteins (ETF; ETFA or ETFB) or ETF dehydrogenase (ETFDH). The disrupted transfer of reduced flavin adenine dinucleotides towards the mitochondrial respiratory chain results in an impaired mitochondrial FAO and accumulation of toxic metabolites.[2] MADD-patients are historically classified into three groups: neonatal onset patients with/without congenital anomalies (type I/II) and patients with a later onset, relatively mild phenotype (type III).[2] Patients with a neonatal onset suffer from life-threatening symptoms such as metabolic derangements, cardiomyopathy, leukodystrophy, and hypotonia. The clinical course of later onset patients ranges from recurrent hypoglycemia to cyclic vomiting, lipid storage myopathy, exercise intolerance and chronic fatigue.[2] Symptoms in later onset patients can also be fatal, but only in rare cases and usually associated with metabolic stress.[3-5] Patients are identified through clinical presentation and in some countries also via population newborn bloodspot screening (NBS).[6,7] Treatment options include dietary fat- and protein- restrictions, fasting avoidance, and supplementation with carnitine, glycine, and riboflavin. Despite early identification and treatment, neonatal mortality remains high.[2,7,8]

Several laboratory studies can be used to characterize MADD-patients, including urine organic acid analysis, plasma acylcarnitine profiling, and ultimately molecular studies to pinpoint the genetic defect.[2,9,10] Unfortunately, prognostic biomarkers that may predict disease severity are not available. In fibroblasts, FAO flux activities provide an estimate of the rate of mitochondrial FAO, whereas acylcarnitine profiling improves insight on both the site and the severity of the enzymatic block.[11] In very long-chain acyl-CoA dehydrogenase deficiency, long-chain FAO flux analysis in fibroblasts[12,13] has been shown to correlate with the phenotype in patients using a clinical severity score.[14] Comparable studies in fibroblasts of neonatal onset MADD-patients demonstrated a markedly reduced FAO activity, in contrast to a less diminished or even normal flux in fibroblasts of later-onset patients.[8,15,16] To date, outcomes of functional studies in fibroblasts have not been correlated with standardized MADD disease severity.

To identify markers that predict disease phenotypes, we retrospectively studied a nationwide cohort of MADD-patients, developed an MADD-disease severity scoring system (DS3) as described previously for other IEMs[14,17-19], and correlated phenotypes and MADD-DS3 scores to the results of functional studies in fibroblasts.

METHODS

Retrospective cohort study

The medical care of Dutch pediatric patients with inborn errors of metabolism (IEM) is centralized in the metabolic divisions of six university hospitals. The pediatric metabolic divisions of all university hospitals and their affiliated metabolic laboratories were asked to participate. The Medical Ethical Committee of the University Medical Center Groningen stated that the Medical Research Involving Human Subjects Act was not applicable and that official study approval by the Medical Ethical Committee was not required (METc code 2014/249).

Patients with an MADD phenotype or biochemical profile (plasma acylcarnitines or urinary organic acids), supported by at least one identified variant in ETFA, ETFB or ETFDH, were included. Outcome parameters included data on clinical history, follow-up, and outcomes of laboratory studies performed according to certified, standardized protocols. All data were obtained by examining the medical files and documented in case record forms which were discussed by WR and TD. Data collection was completed in December 2014.

Multiple acyl-CoA dehydrogenase deficiency-disease severity scoring system

A systematic literature review and a meta-analysis were performed to establish MADD associated disease symptoms and –domains and to identify their occurrence rates. The “PRISMA-IPD”-guidelines were followed as accurately as possible.[20] Data extraction included reported clinical symptoms and general patient characteristics. Disease domains were defined based on organ systems involved in MADD. Occurrence rates were expressed as numbers and percentages.

The relative importance of disease domains and symptoms to be included in the MADD-DS3 was determined using the online survey software Qualtrics (Qualtrics, Provo, Utah, USA). Health care professionals attending “INFORM 2017” (annual conference of the International Network for Fatty Acid Oxidation Research and Management, Rio de Janeiro, Brazil), healthcare providers of MADD(-like)-patients treated with sodium-D,L-3-hydroxybutyrate and co-authors of this study, were invited to prioritize and select disease domains and symptoms based on their influence on the disease burden in patients.

Results of the previous steps provided an outline for the scoring system. The MADD-DS3 was composed according to the average scoring method, as described previously.[19] Contribution of disease domains and symptoms to the total MADD-DS3 score was weighed using their relation to MADD morbidity and mortality.

Functional studies in cultured skin fibroblasts

The functional fibroblast studies were performed within the context of the "Human Tissue and Medical Research: Code of Conduct for Responsible Use" (Federation of Dutch Medical Scientific Societies, 2011, *https://www.federa.org/codes-conduct*). Patient fibroblasts were cultured in HAM F-10 at 37 °C. FAO flux analysis was performed in fibroblasts from patients by measuring both [9,10-^{3}H]oleic acid and [9,10-^{3}H]myristic acid oxidation rates, essentially as described previously.[12,13] Oxidation rates were calculated as nanomoles of fatty acid oxidized per hour per milligram of cellular protein. Results are expressed as percentage of the mean activity measured in fibroblasts of two control subjects in the same experiment. Acylcarnitine profiling by tandem mass spectrometry was performed after incubating the fibroblasts for 96 hours in minimum essential medium supplemented with 120 μM [U-^{13}C]palmitate and 0.4 mM L-carnitine at 37°C, 5% CO_2, as described previously.[14,21] All incubations were performed in quadruplicate (FAO flux) or duplicate (acylcarnitine profiling) in least two independent experiments for each functional test. The presented results are the mean of independent experiments.

Statistical analysis

Data analysis was performed using GraphPad Prism v7.02 (GraphPad Software, La Jolla, California, USA) and SIMCA Software, v14.0 (Umetrics, Umea, Sweden). Categorical variables are presented as numbers and percentages. Remaining continuous variables are presented as median (range). Fisher's exact test or Mann-Whitney U test were used to test for significant differences between neonatal and later onset patients. P-values of <0.05 were considered statistically significant. A principal component analysis and discriminant analysis was used for visualization of the multi-parameter dataset in order to identify key variables. After passing D'Agostino-Pearson omnibus test for normality, Pearson's correlation analysis was used to test the correlation between MADD-DS3 scores and key variables from functional studies in fibroblasts. The Pearson correlation coefficient, r, defines the correlation's strength. Patients identified after population NBS or family screening were excluded from inferential and correlation analysis because early instituted treatment may have affected the natural history of the disease.[14]

RESULTS

Retrospective cohort study

In total, 18 patients diagnosed between 1989 and 2014 were identified. Eight

additional patients with (biochemical) phenotypes suggestive for MADD were excluded because the diagnosis was not supported by DNA analysis. Six out of 18 patients (33%) were classified as neonatal onset MADD, all with a clinical onset within the first week of life. Structural congenital anomalies were reported in one patient (6%). Six patients (33%) were only identified after population NBS or family screening. Affected organ systems included the heart, central nervous system, liver, and muscle. Respiratory insufficiency requiring mechanical ventilation was reported in four patients (22%). The summarized patient characteristics are presented in **Table 1A** and **1B**.

In total, 16 different genetic variants were detected of which nine have not been described previously. All reported plasma acylcarnitine profiles and 15 urinary organic acid profiles (83%) at diagnosis demonstrated abnormalities corresponding to MADD (i.e. ≥1 increased metabolite indicative of MADD). The glutaric aciduria type II-index, as defined by the New England Newborn Screening Program7, could be calculated in four neonatal onset patients who all demonstrated values >0.005, corresponding to “high risk” MADD. The index score was also >0.005 in three later onset patients, while in two later onset patients it was <0.005. The summarized diagnostic parameters are shown in **Table 2**.

Table 1A. Summarized patient characteristics.

PT	Sex	Age at onset	Age at death	Presentation	Structural congenital anomalies
1	F	0 d	-	clinical	-
2[a]	M	0 d	6 m	clinical	-
3[a]	F	1 d	-	clinical	-
4	F	1 d	1.5 y	clinical	-
5	M	<7 d	3.5 y	clinical	-
6	F	7 y	-	NBS	-
7[b]	M	3 y	-	clinical	-
8[b]	M	-	-	FS	-
9	F	childhood	-	clinical	-
10	M	1 d	3 d	clinical	+[d]
11[c]	M	3 y	3 y	SUD	-
12[c]	M	-	-	FS	-
13	M	childhood	-	clinical	-
14	M	<1 y	-	clinical	-
15	F	-	-	NBS	-
16	M	-	-	NBS	-
17	M	childhood	-	clinical	-
18	M	childhood	-	NBS	-

Table 1B. Summarized patient characteristics.

PT	Signs and symptoms									MADD-DS3 score
	Cardiac		CNS			Liver		Muscle	Respiratory insufficiency requiring mechanical ventilation	
	CMP	Arrhythmias	LD	Other brain defect	EPS	Dys*function* or failure[f]	Gluc < 2.6	Muscle weakness/ hypotonia or ≥2 PRO[g]		
1	+	+	+	+		+	+	+	+	29
2[a]				+		+	+	+		11
3[a]				+		+	+			10
4	+			+		+	+	+	+	23
5		+				+	+	+	+	19
6							+			2
7[b]						+	+			3
8[b]										0
9						+		+		7
10	+			+		+	+		+	23
11[c]						+[e]				4
12[c]										0
13								+		2
14								+		2
15										0
16										0
17						+		+		4
18						+		+		4

The patient characteristics (A) and signs and symptoms (B). [a,b,c]Sibling pairs; [d]hypospadia; [e]obduction demonstrated periportal hepatic steatosis; [f]including hyperammonemia, hyperbilirubinemia, hypoalbuminemia, coagulation disorders and encephalopathy; [g]including muscle weakness, myalgia, exercise intolerance and fatigue. Abbreviations (in alphabetical order): CMP, cardiomyopathy; EPS, extrapyramidal symptoms; FS, family screening; gluc, glucose; LD, leukodystrophy; NBS, newborn bloodspot screening; PRO, patient-reported outcome; PT, patient; SUD, sudden unexpected death.

Table 2A. Summarized diagnostic parameters and outcome of functional studies in fibroblasts.

PT	Gene	Mutation allele 1		Mutation allele 2	
		DNA	Protein	DNA	Protein
1	*ETFA*	c.1-40G>A		c.1-40G>A	
2[a]	*ETFA*[d]	c.797C>T	p.T266M	**c.73delA**	**p.Ile25X**
3[a]	*ETFA*	c.797C>T	p.T266M	**c.73delA**	**p.Ile25X**
4	*ETFA*	c.797C>T	p.T266M	**c664+1_664+2 delGT**	
5	*ETFA*	c.797C>T	p.T266M	c.797C>T	p.T266M
6	*ETFA*	**c.242A>C**	**p.H81P**	**c.242A>C**	**p.H81P**
7[b]	*ETFA*	c.797C>T	p.T266M		
8[b]	*ETFA*	c.797C>T	p.T266M		
9	*ETFB*	**c.187G>A**	**p.A63T**		
10	*ETFDH*	c.1414G>A	p.G472R	c.1414G>A	p.G472R
11[c]	*ETFDH*[d]	c.79C>T	p.P27S	**c.1842C>A**	**p.Y614X**
12[c]	*ETFDH*	c.79C>T	p.P27S	**c.1842C>A**	**p.Y614X**
13	*ETFDH*	c.1130T>C	p.L377P	c.1130T>C	p.L377P
14	*ETFDH*	**c.881C>T**	**p.T294I**	**c.881C>T**	**p.T294I**
15	*ETFDH*	c.79C>T	p.P27S	**c.1118C>T**	**p.S373F**
16	*ETFDH*	c.1351G>C	p.V451L	**c.1768A>T**	**p.K590X**
17	*ETFDH*	**c.606+1G>A**			
18	*ETFDH*	c.51dupT	p.A18Cfs		

Table 2B. Summarized diagnostic parameters and outcome of functional studies in fibroblasts.

PT	FAO flux (% of controls)		Acylcarnitine profiling (nmol/mg protein/96 hrs)										MADD profile at diagnosis	
	C18:1	C14	C2	C3	C4	C5	C6	C8	C10	C12	C14	C16	Plasma AC	UOA
1	13%	7%	1.6	0.1	0.2	5.0	0.1	0.1	0.1	0.1	0.3	30.1	+[e]	+
2[a]													+	+
3[a]													+[e]	+
4													NR	+
5	11%	1%	1.9	0.4	0.0	4.1	0.1	0.3	0.7	2.3	6.2	16.5	+[e]	+
6	56%	21%	1.2	0.6	0.2	7.5	0.6	2.4	3.0	2.4	3.4	8.4	+[e]	+
7[b]	48%	57%	17.2	0.6	0.8	0.5	0.4	1.1	1.6	1.6	0.7	3.9	NR	+
8[b]	77%	50%	16.8	0.5	0.2	0.4	0.8	2.0	1.4	0.3	0.2	2.8	NR	+
9	94%	64%	11.8	1.7	1.7	1.3	1.5	4.0	3.3	0.5	0.2	2.6	+	+
10	13%	0%	0.2	0.1	0.0	5.8	0.0	0.0	0.0	0.0	0.6	18.6	+[e]	+
11[c]													+	+
12[c]	29%	10%	4.9	0.4	0.0	4.2	0.8	2.4	4.4	4.0	4.7	12.2	+	-
13	94%	108%	16.1	0.5	0.4	0.3	0.4	0.5	0.4	0.2	0.1	1.6	+	-
14	86%	92%	16.3	0.4	0.5	0.4	0.5	0.8	0.7	0.2	0.2	1.3	+[f]	+
15	36%	20%	5.8	0.5	0.3	2.1	1.0	3.1	3.8	2.4	2.3	6.3	+[e]	+
16													+	+
17	103%	70%	13.0	0.9	0.5	0.7	0.8	1.3	1.0	0.2	0.1	1.1	+[f]	-
18	65%	60%	13.7	0.2	0.3	0.2	0.3	0.6	0.4	0.2	0.1	3.6	+[e]	+

Genetic defects (A) and outcomes of functional studies in fibroblasts and biochemical profiles in plasma and urine (B). Novel mutations are in bold. Aberrant outcomes of the functional studies in fibroblasts are shaded in gray. FAO flux activities below 60% of controls were defined as abnormal. The outcomes of acylcarnitine profiling concern [U-^{13}C]-labeled acylcarnitines except for C3- and C5-carnitine. Control values of acylcarnitine profiling in fibroblasts are presented in Supplementary Table 3. Biochemical profiles indicative of MADD are indicated with a '+' sign. [a,b,c]Sibling pairs; [d]molecular studies only performed in sibling; the glutaric aciduria type II-index, as defined by the New England Newborn Screening Program, was [e]above 0.005 or [f]below 0.005. Abbreviations (in alphabetical order): AC, acylcarnitines; FAO, fatty acid oxidation; NR, not reported; PT, patient; UOA, urinary organic acid.

Multiple acyl-CoA dehydrogenase deficiency-disease severity scoring system
The extensive literature search strategy, screening protocol, and a flowchart of the screening process are presented in **Supplementary Data 1**. In short, the search strategy identified 776 publications of which 78 were included. Data of 413 patients were extracted for further analysis. Age at onset was reported in 396 patients of whom 50 with a neonatal onset (13%). Neonatal onset patients more often had genetic variants in ETFA (neonatal onset patients: 33% versus later onset patients: 3%, $p < 0.0001$) and ETFB (18% versus 1%, $p < 0.0001$). In contrast, ETFDH variants were more frequently identified in later onset patients (48% versus 96%, $p < 0.0001$). The occurrence of two genetic variants expected to have a large effect on protein function (e.g. nonsense and stop-loss variants, deletions, insertions, duplications, and splicing defects) was increased in neonatal compared to later onset patients (45% versus 1%, $p < 0.0001$). This was also significantly related to the incidence of congenital anomalies (85% versus 20%, $p = 0.0004$). In contrast, compound heterozygous missense variants were more frequently identified in later onset patients (30% versus 82%, $p < 0.0001$).

Based on the reported MADD associated symptoms, six disease domains were defined including a cardiac-, central nervous system-, peripheral nervous system-, respiratory system-, liver- and muscle domain. The following clinical symptoms were more frequently reported in neonatal onset patients compared to later onset patients: cardiac (42% versus 3%, $p < 0.0001$; i.e. cardiomyopathy, arrhythmias), central nervous system (12% versus 2%, $p = 0.0041$; i.e. leukodystrophy), hepatic (92% versus 21%, $p < 0.0001$; i.e. hypoglycemia, liver dys*function*/failure), and respiratory problems (38% versus 14%, $p = 0.0001$). Muscle related symptoms including muscle weakness, exercise intolerance and myalgia were more frequently reported in later onset patients compared to neonatal onset patients (60% versus 93%, $p < 0.0001$), except for hypotonia which was reported more often in neonatal onset patients, as described in **Supplementary Table 1**.

Nine health care professionals participated in our survey. **Supplementary Table 2** presents the data on the prioritization and selection of disease domains and –symptoms to be included in the MADD-DS3. This resulted in 1) addition of the domains “congenital anomalies”, “patient reported”, and “age at onset”, and the symptom “cognitive impairment”, and 2) respiratory symptoms being included within the muscle domain. Next, the MADD-DS3 was composed of eight domains with one to five symptoms each. The final MADD-DS3 score is the sum of the individual domain scores, which are calculated by averaging the available symptom scores per domain. **Figure 1** presents the working model of the MADD-DS3 with a total score of 51. An automated tool of the MADD-DS3 is presented in **Supplementary Appendix**

2 (accessible online).

The lifetime MADD-DS3 score of the MADD-patients included in the retrospective cohort ranged from 0 to 29, as presented in Table 1B. Scores of 11 patients were included in the inferential analysis. MADD-DS3 scores differed significantly between neonatal and later onset patients (median 23 (range 11 - 29) versus 4 (2 - 7), p = 0.0043).

Instructions for use
The total MADD-DS3 score is the sum of all domain scores with a maximum of 51.
Calculate the domain scores by averaging the available symptom scores per domain.
In case of unavailable data, data from previous evaluations may be used if the patient's overall clinical status has remained stable. If not, skip that specific item.
Regarding symptom specific assessments:
[a]Left or right ventricular wall thickness of at least one segment > 2 SD, corrected for age, measured via imaging techniques. [b]Clinical value of ≥ NYHA III and/or functional values as ejection fraction ≤ 44% and/or shortening fracture ≤ 21%. [c]Thickness of at least one segment > 2 SD, corrected for age, measured via imaging techniques. [d]Presence of at least one of the following criteria: hyperammonemia; hyperbilirubinemia and/or symptoms as cholestasis; hypoalbuminemia and/or symptoms as ascites; abnormal coagulation and/or symptoms as bleeding disorders; metabolic acidosis. [e]Physician-reported muscle weakness (medical research council grade ≤ 4) or hypotonia, or ≥ 2 patient-reported outcomes including muscle weakness, myalgia, exercise intolerance and fatigue. [f]CK (>1000 U/L).

Figure 1. Multiple acyl-CoA dehydrogenase deficiency-disease severity scoring system. *Continues on the next page*

DOMAIN	ITEM	DISEASE SEVERITY SCORE				SYMPTOM SCORE	DOMAIN SCORE
		0	3	6	9		
AGE AT ONSET	First onset < 1 month of age	No	Yes				
CONGENITAL ANOMALIES	Polycystic kidneys, hypospadias, neuronal migration defects	No		Yes			
CARDIAC	Cardiomegaly[a]	No			> 2 SD		
	Cardiomyopathy[b]	No			Yes		
	Arrhythmias	No			Yes		
CNS	Leukodystrophy	No			Yes		
	Other structural brain defects	No			Yes		
	Extrapyramidal symptoms/dystonia	No			Yes		
	Cognitive impairment	No			Yes		
PNS	Sensory neuropathy	No	Yes				
	Neuropathic EMG	No	Yes				
LIVER	Hepatomegaly[c]	No		> 2 SD			
	Hypoglycemia	No		Glucose < 2.6 mmol/L			
	Dysfunction/failure[d]	No		Yes			
	Encephalopathy	No		Yes			

MUSCLE	Muscle symptoms[e]	No				Yes					
	Rhabdomyolysis[f]	No				Yes					
	Lipid storage myopathy	No				Yes					
	Myopathic EMG	No				Yes					
	Respiratory insufficiency requiring mechanical ventilation	No				Yes					
PATIENT REPORTED OUTCOME	"Considering how MADD affects you/ your child, rate influence on overall well-being during the last 3 months or since the most recent management change"	No influence		Minor influence		Moderate influence		Major influence			
										Total MADD-DS3 score	

Figure 1. Multiple acyl-CoA dehydrogenase deficiency-disease severity scoring system. The total MADD-DS3 score is the sum of all domain scores with a maximum of 51. An automated tool is presented in Supplementary Appendix 2 (accessible online). Abbreviations: CK, creatinine kinase; CNS, central nervous system; EMG, electromyogram; NYHA, New York heart association classification; PNS, peripheral nervous system; SD, standard deviation.

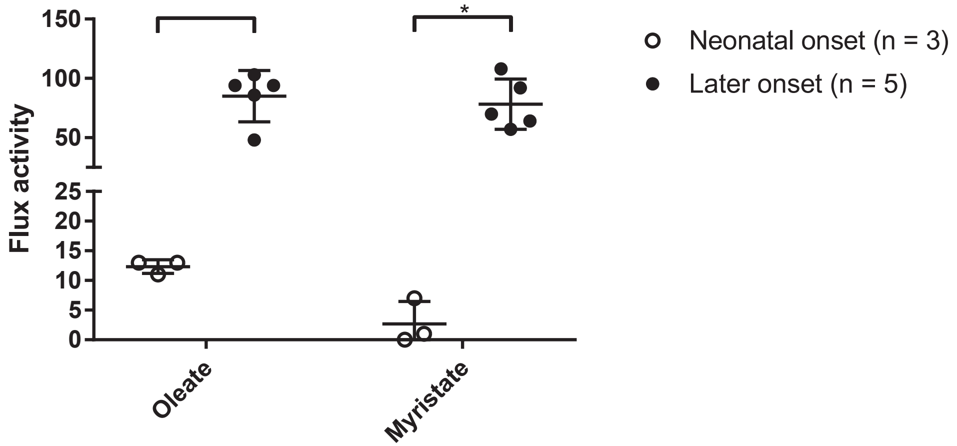
Functional studies in cultured skin fibroblasts

Cultured skin fibroblasts of 13 patients were available for functional studies. Three neonatal and five later onset index patients were included in the inferential analyses. Oleate and myristate flux rates were significantly lower in fibroblasts from neonatal onset patients compared to patients with a later onset (median 13% (range 11 - 13%) vs. 94% (48 - 103%), p = 0.0357; 1% (0 - 7%) vs. 70% (57 - 108%), p = 0.0357, respectively). Acylcarnitine profiling in fibroblasts loaded with [U-^{13}C]palmitate demonstrated significantly increased C5- and [U-^{13}C]C16-carnitine concentrations in neonatal onset patients compared to later onset patients (5 (4.1-5.8) vs. 0.5 (0.3 - 1.3) nmol/mg protein/96 hrs, p = 0.0357; 18.6 (16.5 - 30.1) vs. 1.6 (1.1 - 3.9) nmol/mg protein/96 hrs, p = 0.0357, respectively). [U-^{13}C]C2-, [U-^{13}C]C4-, [U-^{13}C]C6- and [U-^{13}C]C8-carnitine were significantly decreased in neonatal onset patients compared to later onset patients (1.6 (0.2 - 1.9) vs. 16.1 (11.8 - 17.2) nmol/mg protein/96 hrs, p = 0.0357; 0.0 (0.0 - 0.2) vs. 0.5 (0.4 - 1.7) nmol/mg protein/96 hrs, p = 0.0179; 0.1 (0.0 - 0.1) vs. 0.5 (0.4 - 1.5) nmol/mg protein/96 hrs, p = 0.0357; and 0.1 (0.0 - 0.3) vs. 1.1 (0.5 - 4.0) nmol/mg protein/96 hrs, p = 0.0357, respectively). The principal component analysis model identified FAO flux activities, [U-^{13}C]C2-, C5-, and [U-^{13}C]C16-carnitine as key variables for differentiation between neonatal and later onset patients. Discrimination between neonatal and later onset patients by the identified key variables and the individual outcomes combined with the MADD-DS3 scores are presented in **Figure 2**.

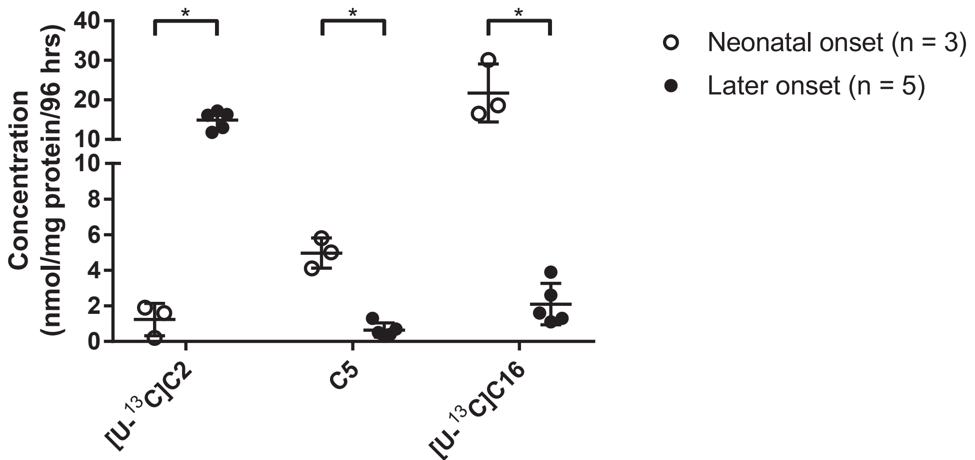
Correlation between disease severity and functional fibroblast studies

Three neonatal and five later onset patients were included in the correlation analyses between MADD-DS3 scores and the identified key variables. A strong association was found between oleate flux activity and myristate flux activity. This enabled differentiation between neonatal and later onset patients, as presented in Figure 3A. Strong negative correlations were observed between MADD-DS3 scores and oleate flux activity, and MADD-DS3 scores and myristate flux activity, as respectively demonstrated in Figure 3B and 3C. MADD-DS3 scores were also strongly associated with [U-^{13}C]C2-, C5-, and [U-^{13}C]C16-carnitine (Pearson r = -0.96; p = 0.0002; Pearson r = 0.97; $p < 0.0001$; and Pearson r = 0.98; $p < 0.0001$, respectively). Oleate and myristate flux activity strongly correlated to [U-^{13}C]C2- (Pearson r = 0.82; p = 0.0121; and Pearson r = 0.93; p = 0.0009, respectively), C5- (Pearson r = -0.88; p = 0.0044; and Pearson r = -0.93; p = 0.0009, respectively), and [U-^{13}C]C16-carnitine (Pearson r = -0.88; p = 0.0042; and Pearson r = 0.86; p = 0.0058, respectively).

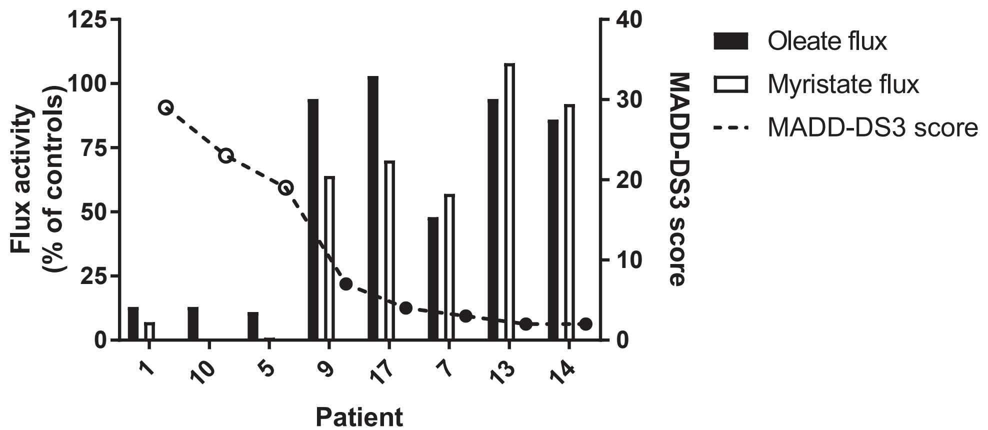
A)



B)



C)



4

D)

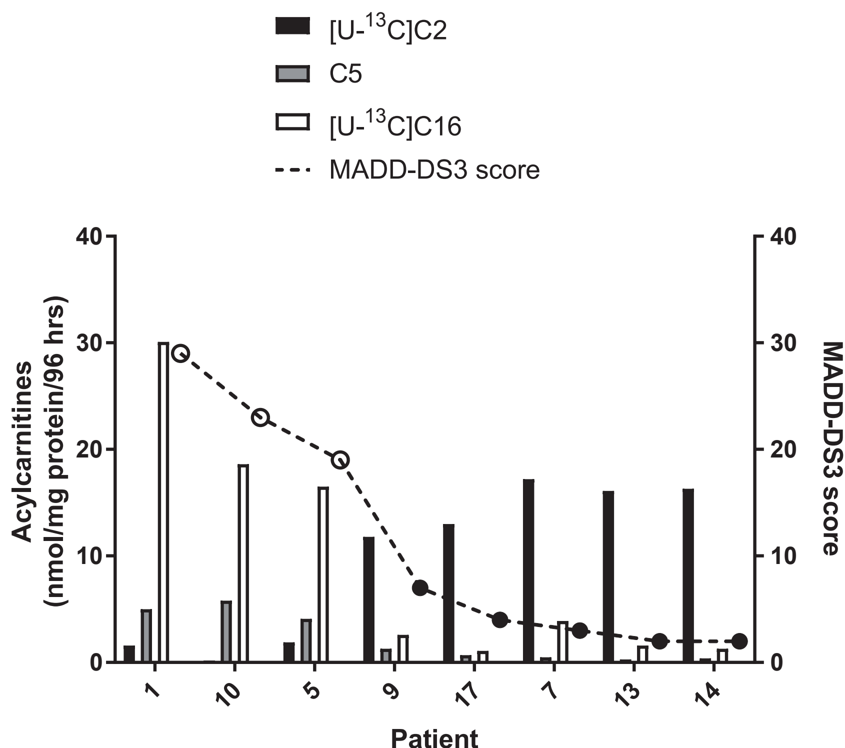
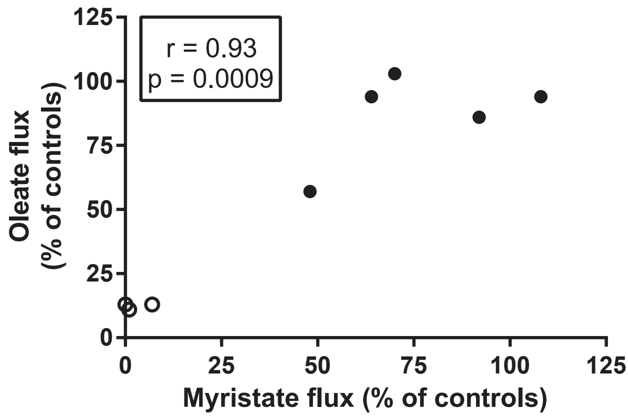
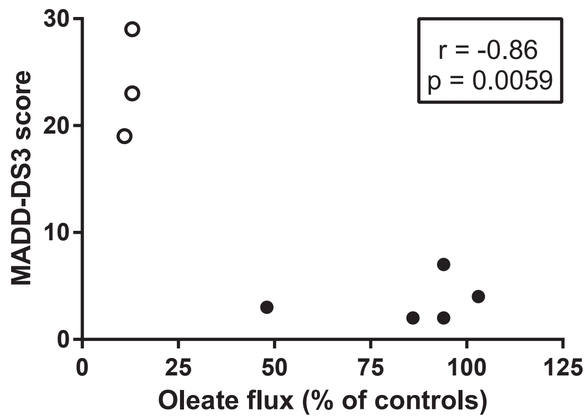


Figure 2. Differences in fatty acid oxidation flux activities and acylcarnitine profiling between neonatal and later onset multiple acyl-CoA dehydrogenase deficiency. Outcomes of functional studies in fibroblasts of three neonatal onset (○) and five later onset MADD-patients (●). Scatter dot plots (mean with SD) of FAO flux activities measured with ([9,10-^{3}H]oleate and [9,10-^{3}H]myristate (A), and concentrations of [U-^{13}C]C2-, C5 and [U-^{13}C] C16-carnitines in the medium after [U-^{13}C]palmitate loading for 96 hours at 37 °C (B). Individual outcomes of FAO flux activities (C), and acylcarnitine profiling (D) plotted against MADD-DS3 scores (right y-axis). Patient numbers refer to identification numbers in Table 1A and 1B and Table 2, with the order of display based on MADD-DS3 scores.

A)



B)



C)

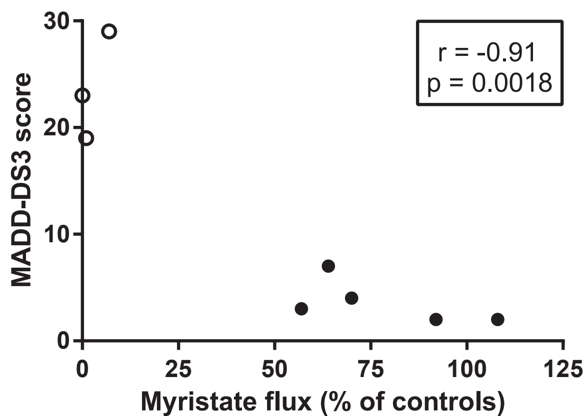


Figure 3. Correlation between disease severity and fatty acid oxidation flux activity. Correlation between [9,10-^{3}H]oleate and [9,10-^{3}H]myristate FAO flux activities (A), and correlation between disease severity as defined by the MADD-DS3 scores and FAO flux activities measured with [9,10-^{3}H]oleate (B) or [9,10-^{3}H]myristate (C) in fibroblasts of three neonatal onset (○) and five later onset MADD-patients (●). r, Pearson correlation coefficient.

4

DISCUSSION

Functional studies in fibroblasts can be used to predict the potential risk of clinical symptom development in MADD patients. Our study demonstrates that neonatal onset and later onset MADD patients could be distinguished based on their FAO flux activities and acylcarnitine profiling in the medium after palmitate loading in fibroblasts. There was a strong correlation between individual FAO flux activities and MADD-DS3 scores. Both functional tests provide useful information for (early) phenotype prediction in individual MADD patients.

Neonatal onset patients demonstrated low flux activities combined with particularly high [U-^{13}C]C16-carnitine levels and low medium- and short-chain acylcarnitine concentrations, indicating an almost complete block of FAO. In contrast, flux activities in later onset patients varied from normal to (mildly) decreased combined with normal to (mildly) increased acylcarnitine concentrations of variable chain lengths. The increase in (unlabeled) C5-carnitine concentration in neonatal onset patients suggests a profound deficiency of isovaleryl-CoA dehydrogenase. Computational studies already suggested that differences in acylcarnitine profiles and FAO flux capacities might be relevant to clinical phenotypes, and can be explained by substrate competition.[22] In this study, it was not possible to extrapolate the differences identified in fibroblast acylcarnitine profiles to plasma and dried blood spot samples due to limited sample availability and possible influence of interlaboratory, analytical differences. Since blood sampling is less invasive than a skin biopsy and could enable immediate risk prediction after identification, further studies are warranted.

Our results suggest that a low FAO flux is associated with the development of severe symptoms including leukodystrophy and cardiomyopathy. Hence these symptoms should be monitored in patients with a predicted severe phenotype. It should be noted that the functional studies in fibroblasts were only performed at 37 °C. In some very long-chain acyl-CoA dehydrogenase deficient-patients with mild phenotypes and a relatively high oleate flux activity at 37 °C, performing the assays at 40 °C resulted in a 40% decrease in flux activity.[14] It is very well possible that FAO flux in fibroblasts is also temperature sensitive at least in a subset of MADD patients. Although generalization of these in vitro studies towards in vivo observations remains debatable, it can be hypothesized that an increased body temperature, for example during intercurrent illness, may cause a drop in FAO flux activity which poses a risk for symptom development. A previous in vitro study demonstrated an activity decay in ETFA variants induced by physiological thermal stress.[23] Thus, even in patients with a relatively high flux activity and low MADD-DS3 scores, the risk to develop

potential, life-threatening symptoms should still be considered.

To enable standardized clinical description of disease severity in patients from our cohort, we developed an MADD-DS3 based on existing literature and weighed expert opinions. DS3's provide a method for systematic assessment of disease burden and have been developed for only a few other IEMs.[14,17-19] The used average scoring method eliminates biased estimates in case of missing items when completing the score.[19] The system is designed to be easy to use with no required assessments beyond standard patient care. However, in order to facilitate clinical use during follow-up, prospective, longitudinal validation is warranted, for instance during monitoring of MADD patients on (prophylactic) treatment with sodium-D,L-3-hydroxybutyrate.[24,25]

The present study has several methodological limitations. First, an inclusion bias was introduced because we only included patients via pediatric metabolic centers. Second, the retrospectively cohort data covers a period of >20 years, causing a risk of information bias. Third, the interferential and correlation analysis comprises a relatively small sample. Therefore, the authors propose confirmation and validation in a larger (international) patient population, possibly with the help of international networks such as "INFORM" and "MetabERN" (European Reference Network for Hereditary Metabolic Disorders). Finally, genetic defects in at least five other metabolic pathways dependent of flavin adenine dinucleotides are recognized to cause clinical and biochemical MADD-like profiles.[26-33] Although promotor region- or intronic variants might have been overlooked, it can also not be excluded that patients in whom DNA analysis only demonstrated one genetic variant, actually suffer from an MADD-like disease.

4

CONCLUSION

This study shows the value of functional studies in fibroblasts and an MADD-DS3 for characterization and risk stratification of MADD-patients. Our data can be used to improve (early) identification of patients at risk for severe symptoms and metabolic derangements in order to start preventive treatment and follow-up appropriately. This is especially relevant in view of the inclusion of MADD in population NBS programs.

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SUPPLEMENTARY APPENDIX 1

Supplementary Data 1
Supplementary Table 1
Supplementary Table 2
Supplementary Table 3

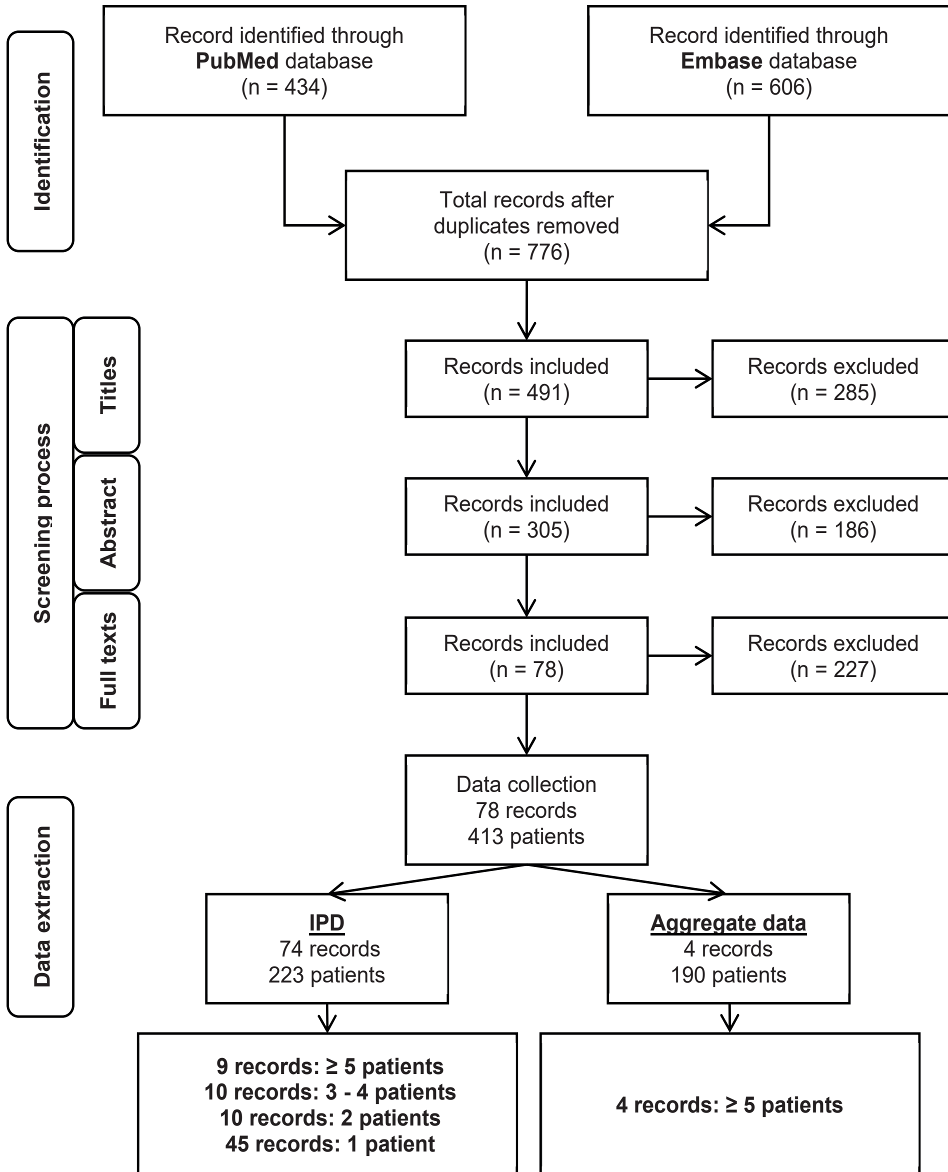
Supplementary Data 1. Detailed description of the systematic literature review methods.

Presentation of the search strategy. Implementation of the search strategy in PubMed database resulted in 434 hits on February 27th, 2017.

(“Multiple Acyl Coenzyme A Dehydrogenase Deficiency”[Mesh] OR Multiple Acyl Coenzyme A Dehydrogenase Deficien*[tiab] OR Multiple Acyl-CoA Dehydrogenase Deficien*[tiab] OR Multiple Acyl-Coenzyme A Dehydrogenase Deficien*[tiab] OR Multiple Acylcoenzyme A Dehydrogenase Deficien*[tiab] OR Multiple Acyl-Co A Dehydrogenase Deficien*[tiab] OR Multiple Acyl Coenzyme A Dehydrogenation Deficien*[tiab] OR Multiple Acyl-CoA Dehydrogenation Deficien*[tiab] OR Multiple Acyl-Coenzyme A Dehydrogenation Deficien*[tiab] OR Multiple Acylcoenzyme A Dehydrogenation Deficien*[tiab] OR Multiple Acyl-Co A Dehydrogenation Deficien*[tiab] OR Multiple FAD Dehydrogenase Deficien*[tiab] OR Electron Transfer Flavoprotein Deficien*[tiab] OR Electron Transfer Flavoprotein Dehydrogenase Deficien*[tiab] OR Electron Transfer Flavoprotein Alpha Subunit Deficien*[tiab] OR Electron Transfer Flavoprotein Beta Subunit Deficien*[tiab] OR ETFDH Deficien*[tiab] OR ETFA Deficien*[tiab] OR ETFB Deficien*[tiab] OR “Glutaric Aciduria type II”[tiab] OR “Glutaric Aciduria type 2”[tiab] OR “Glutaric Aciduria, type 2”[tiab] OR “Glutaric Aciduria II”[tiab] OR “Glutaric Aciduria 2”[tiab] OR “Glutaric Acidemia type II”[tiab] OR “Glutaric Acidemia type 2”[tiab] OR “Glutaric Acidemia, type 2”[tiab] OR “Glutaric Acidemia II”[tiab] OR “Glutaric Acidemia 2”[tiab] OR “Glutaric Acidemia type IIA”[tiab] OR “Glutaric Acidemia type IIB”[tiab] OR “Glutaric Acidemia type IIC”[tiab] OR Ethylmalonic-Adipic Aciduria*[tiab] OR Ethylmalonic Adipic Aciduria*[tiab] OR Mitochondrial Fatty Acid Oxidation Disorder*[tiab] OR Mitochondrial Fatty-Acid Oxidation Disorder*[tiab] OR Mitochondrial Fatty Acid Beta Oxidation Disorder*[tiab] OR Mitochondrial Fatty Acid Beta-Oxidation Disorder*[tiab] OR Mitochondrial Fatty-Acid Beta Oxidation Disorder*[tiab] OR Mitochondrial Fatty-Acid Beta-Oxidation Disorder*[tiab] OR Mitochondrial Fatty Acid β-Oxidation Disorder*[tiab] OR Mitochondrial Fatty-Acid β-Oxidation Disorder*[tiab] OR mFAO Disorder*[tiab] OR FAO Disorder*[tiab] OR FAOD*[tiab]) NOT ((“Animals”[Mesh] NOT “Humans”[Mesh]) OR Animal[ti] OR Mouse[ti] OR Mice[ti] OR Rodent*[ti] OR Rat[ti] OR Rats[ti])

Protocol of the screening process and data extraction.
Two reviewers (WvR & PG) independently performed title-, abstract- and full text screening to identify publications with detailed patient data on MADD phenotypes. Records were excluded in case of 1) no confirmed diagnosis through DNA-analysis with two mutations identified in ETFA, ETFB or ETFDH or enzymatic assay; 2) only detailed patient data of previously published patients; 3) not available in English or Dutch language; 4) published before 1984, corresponding with the year in which diagnosis confirmation via enzymatic assay was introduced and/or 5) no available publications. Consensus on eligibility was reached through regular meetings. 79 records were included for data extraction after completion of the screening process. Subsequently, general study characteristics and individual patient data (IPD) on clinical symptoms (primary outcome parameters) and general patient characteristics (secondary outcome parameters) were extracted. If applicable, only one patient per pedigree was considered for data extraction. In case of a lack of IPD, aggregate data was collected and analyzed if possible. If necessary, a third, independent reviewer (TD) was consulted to make the final call on eligibility during the screening process and data extraction.

Identified disease symptoms were categorized according disease domains based upon organ systems. Subsequent meta-analysis resulted in determination of the general occurrences of disease symptoms and –domains associated with MADD in numbers and percentages. Additionally, the relation between reported genotypes and phenotypes was investigated.



Flowchart of the literature screening process and data extraction. Implementation of the search strategy in PubMed and Embase databases on February 27th, 2017 resulted in 434 and 606 hits, respectively. After deduplication, a total of 776 records remained.

Supplementary Table 1. Reported symptoms in neonatal- and later onset multiple acyl-CoA dehydrogenase deficiency.

Disease domains and –symptoms	Total (n = 413)	Neonatal onset (n = 50)	Later onset (n = 346)	p-value
Cardiac	**34 (8%)**	**21 (42%)**	**9 (3%)**	<0.0001
Cardiomyopathy	20	12	5	<0.0001
Arrhythmia(s)	13	6	4	0.0004
Fatty infiltration	9	8	1	<0.0001
Central nervous system	**14 (3%)**	**6 (12%)**	**8 (2%)**	0.0041
Leukodystrophy	7	3	4	0.0460
Other structural brain abnormalities	6	4	2	0.0028
Extrapyramidal symptoms/dystonia	4	1	3	ns
Peripheral nervous system	**27 (7%)**	**0 (0%)**	**27 (8%)**	0.0352
Neuropathic electromyographic abnormalities	23	0	23	ns
Sensory neuropathy	4	0	4	ns
Respiratory system[a]	**66 (16%)**	**19 (38%)**	**47 (14%)**	0.0001
Required mechanical ventilation	18	8	10	0.0006
Liver	**133 (32%)**	**46 (92%)**	**72 (21%)**	<0.0001
Hypoglycemia	74	39	22	<0.0001
Metabolic acidosis	59	28	17	<0.0001
Liver dysfunction/failure	58	21	25	<0.0001
Fatty infiltration	43	9	29	0.04
Hepatomegaly	31	9	8	<0.0001
Reye syndrome/(transient) encephalopathy	21	1	12	ns
Muscle	**358 (87%)**	**30 (60%)**	**323 (93%)**	<0.0001
Muscle weakness	303	2	301	<0.0001

Fatty infiltration	242	4	238	<0.0001
Myopathic electromyographic abnormalities	140	0	140	<0.0001
Exercise intolerance	179	0	179	<0.0001
Myalgia	76	0	76	<0.0001
Hypotonia	44	26	18	<0.0001
Fatigue	21	1	20	ns
(Rhabdo)myolysis	16	0	11	ns

Aggregate and individual patient data were extracted to describe occurrence rates. The results are presented as numbers and percentages (%). Data from 14 patients without (complete) diagnostic confirmation and from five siblings could not be excluded because only aggregate data was presented. Data from 17 patients was excluded from the comparison analysis between neonatal- and later onset patients because age at onset was not reported. [a]Reported symptoms include dyspnea, pulmonary dysfunction, respiratory arrest, respiratory distress, respiratory failure, respiratory insufficiency, and tachypnea.

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Supplementary Table 2. Relevance of disease domains and disease symptoms to the multiple acyl-CoA dehydrogenase deficiency-disease severity scoring system as determined by nine health care professionals.

Disease domains and –symptoms	Experts selecting item
Cardiac	**100% (9/9)**
Cardiomyopathy	100% (9/9)
Arrhythmia(s)	100% (9/9)
Cardiomegaly	100% (9/9)
Central nervous system	**100% (9/9)**
Leukodystrophy	100% (9/9)
Other structural brain abnormalities	100% (9/9)
Extrapyramidal symptoms/dystonia	89% (8/9)
Cognitive impairment[a]	22% (2/9)
Peripheral nervous system	**89% (8/9)**
Neuropathic electromyographic abnormalities	63% (5/8)
Sensory neuropathy	75% (6/8)
Liver	**100% (9/9)**
Hypoglycemia	89% (8/9)
Metabolic acidosis	89% (8/9)
Liver dysfunction/failure	100% (9/9)
Hepatomegaly	89% (8/9)
(Transient) encephalopathy	56% (5/9)
Hyperammonemia[a]	33% (3/9)
Muscle	**89% (8/9)**
Muscle weakness	100% (8/8)
Myopathic electromyographic abnormalities	63% (5/8)
Fatty infiltration	63% (5/8)
Exercise intolerance	75% (6/8)
Myalgia	88% (7/8)
Hypotonia	100% (8/8)
Fatigue	75% (6/8)
(Rhabdo)myolysis	100% (8/8)
Chronic respiratory insufficiency requiring mechanical ventilation[b]	78% (7/9)
Patient reported	**78% (7/9)**
"Influence MADD on overall well-being rated from 0 (no influence) to 5 (worst)"	86% (6/7)
Congenital anomalies[a]	**33% (3/9)**

Disease domains- and symptoms selected by > 1 expert. [a]Included based on expert opinions; [b]preferred inclusion within the muscle domain (n = 3) or within a combined neuromuscular (n = 1), central nervous system (n = 1) or independent domain (n = 2).

Supplementary Table 3. Control values of acylcarnitine profiling in fibroblasts.deficiency.

Acylcarnitine profiling (nmol/mg protein/96 hrs)									
C2	C3	C4	C5	C6	C8	C10	C12	C14	C16
22.8 (2.3 - 3.3)	1.0 (0.0 - 2.3)	0.7 (0.0 - 2.0)	1.1 (0.0 - 2.5)	0.7 (0.0 - 1.4)	1.3 (0.1 - 2.6)	1.6 (0.2 - 3.1)	0.4 (0.0 - 0.8)	0.1 (0.0 - 0.4)	2.1 (0.0 - 4.3)

Data is presented as mean (two standard deviations below and above the mean) after loading with labeled palmitate in fibroblasts of healthy controls. The outcomes concern [U-^{13}C]-labeled acylcarnitines except for C3- and C5-carnitine.

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Efficacy and safety of D,L-3-hydroxybutyrate treatment in multiple acyl-CoA dehydrogenase deficiency

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ABSTRACT

Purpose:
Multiple acyl-CoA dehydrogenase deficiency (MADD) is a life-threatening, ultra-rare inborn error of metabolism. Case reports described successful D,L-3-hydroxybutyrate (D,L-3-HB) treatment in severely affected MADD-patients, but systematic data on efficacy and safety is lacking.

Methods:
A systematic literature review and an international, retrospective cohort study on clinical presentation, D,L-3-HB treatment method, and outcome in MADD(-like)-patients.

Results:
Our study summarizes 23 MADD(-like)-patients, including 14 new cases. Median age at clinical onset was two months (interquartile range (IQR): 8 months). Median age at starting D,L-3-HB was seven months (IQR: 4.5 years). D,L-3-HB doses ranged between 100 and 2600 mg/kg/day. Clinical improvement was reported in 16 patients (70%) for cardiomyopathy, leukodystrophy, liver symptoms, muscle symptoms, and/or respiratory failure. D,L-3-HB appeared not effective for neuropathy. Survival appeared longer upon D,L-3-HB compared to historical controls. Median time until first clinical improvement was one month, and ranged up to six months. Reported side effects included abdominal pain, constipation, dehydration, diarrhoea, and vomiting/nausea. Median D,L-3-HB treatment duration was two years (IQR: 6 years). D,L-3-HB treatment was discontinued in 12 patients (52%).

Conclusion:
The strength of the current study is the international pooling of data demonstrating that D,L-3-HB treatment can be effective and safe in MADD(-like)-patients.

INTRODUCTION

Multiple acyl-CoA dehydrogenase deficiency (MADD; also known as glutaric aciduria type II, OMIM #231680) is an ultra-rare (i.e. < 1 : 50,000) inborn error of metabolism (IEM). MADD can be primary, caused by a genetic defect in the electron transfer flavoproteins (ETF) or in ETF dehydrogenase (ETFDH) or secondary, resulting from genetic defects of riboflavin transport (RFVT) or flavin adenine dinucleotide (FAD) synthesis (i.e. MADD-like disease). The impairment of mitochondrial fatty acid oxidation (FAO) and amino acid metabolism causes energy deficiency and the accumulation of toxic metabolites, such as medium-chain and long-chain length plasma acylcarnitines, urinary organic acids (e.g., isovaleric, isobutyric, 2-methylbutyric, glutaric, ethylmalonic, 3-hydroxyisovaleric, 2-hydroxyglutaric, 5-hydroxyhexanoic, and several dicarboxylic acids) and urinary acylglycines (e.g., isovalerylglycine, isobutyrylglycine, and 2-methylbutyrylglycine).[1]

Historically, MADD-patients are classified into three categories: patients with a severe, neonatal onset with or without congenital anomalies (type I or II, respectively), and patients with a relatively mild, later onset (type III).[1] Type I and II patients often demonstrate life-threatening symptoms including metabolic derangements, cardiomyopathy, leukodystrophy, and severe hypotonia. The clinical course in type III patients can vary from recurrent hypoglycemia to lipid storage myopathy and exercise intolerance.[1] Treatment options include dietary fat- and protein restriction, fasting avoidance, and supplementation with carnitine, glycine, and/or riboflavin, when riboflavin responsive. Despite early diagnosis and treatment, morbidity and mortality remain high in neonatal onset patients.[1]

Upon prolonged fasting, hepatic mitochondrial FAO fuels synthesis of ketone bodies (KB) acetoacetate and 3-hydroxybutyrate, as important alternative energy sources for the brain, skeletal muscle, and heart.[2-4] Patients with mitochondrial FAO disorders, such as MADD, demonstrate multi-organ dysfunction especially during catabolism.[2] Administration of exogenous KB might bypass the disturbed ketogenesis. Several case reports described successful treatment of severely affected MADD-patients with racemic D,L-3-hydroxybutyrate (D,L-3-HB).[5-9] The lack of systematic data on efficacy and safety of D,L-3-HB hampers the treatment of seriously ill patients and prevents D,L-3-HB reimbursement. Therefore, we performed a twofold study including a systematic literature review and an international, retrospective cohort study to describe the clinical presentations of MADD(-like)-patients treated with D,L-3-HB, the details of D,L-3-HB treatment methods, and outcomes.

PATIENTS AND METHODS

The Medical Ethical Committee of the University Medical Center Groningen confirmed that the Medical Research Involving Human Subjects Act does not apply and that official approval of this study by the Medical Ethical Committee was not required (METc code 2016/470). The study protocol was performed in compliance with the Declaration of Helsinki and approved for waiver of consent by all participating institutes or performed conform the laws and regulations of the respective countries and institutes.

Systematic literature review

To identify all reported IEM-patients treated with D,L-3-HB and their health care providers, a comprehensive search strategy for relevant publications before 21 December 2016 was performed in PubMed and EMBASE public databases. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed as accurately as possible. The detailed search strategy, a protocol of the screening process and data extraction, a flowchart, and the "PRISMA-P" 2015 checklist are presented in **Supplementary Data 1** ("PRISMA-P" 2015 checklist accessible online). Articles were included based on the presence of detailed patient data concerning D,L-3-HB or KB treatment, as well as a confirmed diagnosis by biochemical (acylcarnitine or urinary organic acid profile), DNA, or enzymatic analysis. Exclusion criteria were 1) no detailed patient data described, 2) lack of accessibility of the abstracts or articles, and 3) no availability in English or Dutch language.

Retrospective cohort study

In February 2017 health care providers with experience in D,L-3-HB treatment of MADD(-like)-patients were invited to collaborate in this study by contacting 1) the first and/or corresponding authors of former publications, identified in our systematic literature study, 2) clinicians who have previously contacted the authors (JVH or TD) on this topic, 3) several professional organizations and networks, including a list server for the metabolic community (Metab-l), Society for the Study of Inborn Errors of Metabolism (SSIEM), Society for Inherited Metabolic Disorders (SIMD), and the European Reference Network for Hereditary Metabolic Diseases (MetabERN).

D,L-3-hydroxybutyrate treatment has been reported in at least two MADD-like patients who were later found to have RFVT defects.[10,11] Patients with genetic defects of RFVT (i.e. SLC52A1, SLC52A2, SLC52A3 (alias C20orf54)) and FAD metabolism (i.e. SLC25A32, FLAD1) were, therefore, included in this study in

addition to those with ETF or ETF dehydrogenase defects. MADD(-like)-patients were eligible for enrolment in case of a diagnosis confirmed by biochemical, DNA or enzymatic analysis, performed conform local protocols. Outcome parameters included data on clinical presentation, laboratory and molecular parameters, D,L-3-HB treatment method, and (long-term) outcome. Data was collected via an anonymous questionnaire in Microsoft Word to be completed by health care providers involved. Data inclusion was concluded in December 2018, after which data from all completed questionnaires was summarized. The STROBE checklist for reporting observational studies is presented in **Supplementary Data 2** (accessible online).

Statistical analysis

Data analysis was performed using Microsoft Excel and GraphPad Prism, version 5.0 (GraphPad Software, La Jolla, California, USA). Descriptive statistics were used to summarize the data. Categorical and continuous variables are presented as numbers (percentages) or median (interquartile range (IQR)), respectively. Kaplan-Meier plots were used to estimate the survival and visualize the data on time till first reported clinical improvement and D,L-3-HB treatment duration. The survival of MADD(-like)-patients treated with D,L-3-HB was compared to survival data from historical controls who were not reported to have been treated with D,L-3-HB, as collected in a previous meta-analysis.[12] Mann-Whitney U test was used to analyze the significance of differences between groups. If data were missing, the analysis was performed on data from the remaining patients. A p-value of <0.05 was considered statistically significant.

RESULTS

Systematic literature review

Supplementary Table 1 summarizes data from 14 references on D,L-3-HB treatment in 16 patients with MADD(-like)-disease.[2,5-8,10,11,13-19] Additionally, D,L-3-HB treatment was reported in 18 patients with other IEMs in which ketogenesis is disturbed, demonstrating potential indications of the compound. These IEMs included carnitine-acylcarnitine translocase deficiency (n = 1),[17,18] glycogen storage disease type III (n = 3),[20,21] 3-hydroxy-3-methylglutaryl-coenzyme A lyase deficiency (n = 4),[17,18,22,23] mitochondrial complex IV deficiency (n = 2),[20] persistent hyperinsulinemic hypoglycemia of infancy (n = 6),[24,25] propionic acidemia (n = 1),[20] and very long-chain acyl-CoA dehydrogenase deficiency (n = 1).[20]

Retrospective cohort study

Patient characteristics

In total, 23 MADD(-like)-patients treated with D,L-3-HB treatment were identified, including 14 novel cases. The individual patient characteristics are presented in **Table 1**. Median age at clinical ascertainment was two months (IQR: 8 months). Nine patients (39%) had a neonatal disease onset and all presented clinically during the first week of life. Structural congenital anomalies were not reported. Hence, they were classified as type II patients. The 14 remaining patients (61%) could be categorized as type III patients, including two with a clinical onset during adulthood. Abnormal population newborn screening results were observed in 14 patients (61%) of whom eight (57%) developed clinical symptoms and signs during the first week of life.

Diagnosis was molecularly confirmed in 20 cases (87%) (pathogenic variants in ETFA (n = 4); ETFDH (n = 6); compound heterozygosity in ETFA (n = 2); ETFDH (n = 6); SLC52A3 (n = 2)). In one patient in whom DNA analysis was inconclusive, the results of an enzyme assay were indicative of MADD. All reported acylcarnitine profiles (n = 18) and urinary organic acid profiles (n = 22) at diagnosis demonstrated at least mild abnormalities consistent with MADD.

D,L-3-hydroxybutyrate treatment method

In our cohort of patients, D,L-3-HB was prescribed as a food supplement (KetoForce) in one patient, and as hospital pharmacy constituted formulation or prepared by a caregiver in others, after being obtained from various suppliers including Huddersfield Pharmacy Specials, Inresa, M2i, Sigma-Aldrich, and Special Products Ltd (Veriton Pharma). The most reported formulation involved a racemic sodium salt. Currently, in The Netherlands, the D,L-3-HB is magistrally prepared as 593.3 mg/mL (4.7M) solution in distilled water. The D,L-3-HB is acquired by Sigma-Aldrich and this treatment costs € 0.0040 per mg (price for the active ingredient only and a simple product formulation) averaging approximately €3.60 per kg/day at an assumed starting dose of 900 mg/kg/day.

The median age at start of D,L-3-HB treatment was seven months (IQR: 4.5 years). Prescribed doses ranged between 100 and 2600 mg/kg/day, divided in one to six daily doses. Five patients (22%) received D,L-3-HB continuously during the night and one patient (4%) continuously for 24h per day. The D,L-3-HB was administered orally or via nasogastric/gastrostomy tube, usually combined with nutrition or before/after the meal.

Table 1. Diagnostic characteristics of patients included in the retrospective cohort study.

Patient	Sex	Age at clinical onset	Affected gene	Variant allele 1		Variant allele 2		Enzyme activity (nmol/min/mg protein)
				cDNA	Protein	cDNA	Protein	
1[a(7)b]	F	0 d	*ETFA*	c.1-40G>A		c.1-40G>A		
2	M	0 d	*ETFA*	c.797C>T	p.T266M	c.797C>T	p.T266M	ETF: < 0.01 (< 1% of C)
3[c]	F	1 d	*ETFA*	c.797C>T	p.T266M	c.797C>T	p.T266M	
4[a(17,18)bc]	M	1 d	*ETFA*	c.370G>A	p.A124T	c.370G>A	p.A124T	
5	F	1 d	*ETFA*	c.200T>C	p.L67P	c.854A>T	p.Q285L	
6[a(17,18)]	M	7 d	*ETFA*[e]	c.365G>A	p.R122K	c.809-811del	p.V270del	
7	F	1 d	*ETFDH*	c.896T>C	p.L299S	c.1842C>A	p.Y614X	
8[bc]	F	3 d	*ETFDH*	c.1141G>C	p.G381R	c.1141G>C	p.G381R	ETF-QO: 0.05 (C: 0.22 ± 0.09)
9	F	1 m	*ETFDH*	c.34G>C	p.A12P	c.1234G>T	p.E412X	
10	M	2 m	*ETFDH*	c.1001T>C	p.L334P	c.1074G>C	p.R358S	ETF-QO: 0.71 (C: 0.8 - 2.4)
11[c]	M	4 m	*ETFDH*	c.820G>T	p.G274X	c.1601C>T	p.P534L	ETF: 1.23 (C: 0.79 - 2.1) ETF-QO: 0.96 (C: 0.8 - 2.4)
12[a(17,18)]	M	5 m	*ETFDH*	c.858G>A	p.W286X	c.1099A>G	p.N367D	
13[a(15)]	F	6 m	*ETFDH*	c.51dupT	p.A18Cfs	c.940G>A	p.E314K	ETF-QO: 0.44 (C 0.8 - 2.4)
14	M	10 m	*ETFDH*	c.463A>G	p.R155G	c.463A>G	p.R155G	
15	F	1 y 3 m	*ETFDH*	c.665A>C	p.Q222P	c.665A>C	p.Q222P	ETF-QO: 0.07 (C: 0.31 ± 0.19)[f]
16[bd]	F	1 y 8 m	*ETFDH*	c.1693G>C	p.V565L	c.1693G>C	p.V565L	
17[a(8)b]	M	2 y 7 m	*ETFDH*	c.1106G>C	p.G369A	c.1106G>C	p.G369A	
18	M	25 y 11 m	*ETFDH*	c.1367C>T	p.P456L	c.1367C>T	p.P456L	

Continues on the next page

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Patient	Sex	Age at clinical onset	Affected gene	Variant allele 1		Variant allele 2		Enzyme activity (nmol/min/mg protein)
				cDNA	Protein	cDNA	Protein	
19	F	19 y	*ETFDH*	c.1774T>C	p.C592R			ETF: 1.82 (C: 0.79 - 2.1) ETF-QO: 0.21 (C: 0.8 - 2.4)
20[a(17,18)c]	F	7 d	NF					
21[c]	F	1 m						
22[a(10)]	M	5 m	*SLC52A3*	c.639C>G	p.Y213X	c.678-680del	p.L227del	ETF: 1.27 (C: 1.25 ± 0.32) ETF-QO: 0.06 (C: 0.22 ± 0.09)
23[a(11)]	F	6 m	*SLC52A3*	c.49T>C	p.W17R	c.639C>G	p.Y213X	ETF: 1.11 (C: 1.25 ± 0.32) ETF-QO: 0.17 (C: 0.22 ± 0.09)

[a]Patient has been published before in relation to D,L-3-HB treatment, see corresponding reference; [b]consanguinity; cdeceased; [d]diagnosed prenatally due to family history; [e]DNA analysis also demonstrated compound heterozygous variants in *ETFB* (c.217-4G>T and c.438+20C>T), classified as variant of uncertain significance and likely benign, respectively; [f]analysis only performed in sister. Abbreviations (in alphabetical order): C, control; cDNA, complementary DNA; NF, no variant found.

D,L-3-hydroxybutyrate treatment outcome

Patient and treatment characteristics according to outcome are summarized in **Table 2**. Individual D,L-3-HB treatment characteristics and outcome are presented in **Supplementary Table 2**. In total, clinical improvement upon D,L-3-HB was reported in 16 patients (70%) for cardiomyopathy, leukodystrophy, liver symptoms (i.e. hyperammonemia, hypoglycemia, liver dysfunction or failure, and metabolic acidosis), muscle symptoms (i.e. exercise intolerance, hypotonia, myopathy, and rhabdomyolysis), and/or respiratory failure. D,L-3-HB treatment was effective in six out of nine type II patients (67%). The efficacy was questionable in two type II patients (22%) in whom D,L-3-HB was used as a preventive measure which complicated the interpretation of treatment outcomes. Clinical improvement was also reported in ten out of 14 type III patients (71%), including a patient with RFVT3 deficiency. The efficacy was questionable in one type III patient (7%) because the duration of treatment was only three months at the time of data analysis. Additional follow-up demonstrated that the patient remained clinically stable without further deterioration of the leukodystrophy. D,L-3-HB was ineffective in one type II (11%) and three type III patients (21%), of whom one patient was diagnosed with RFVT3 deficiency. The treatment indications in those patients included muscle and liver symptoms, respiratory failure and neuropathy with the maximum prescribed doses ranging between 750 to 1800 mg/kg/day. **Figure 1A** presents the summarized organ based D,L-3-HB treatment indications and efficacy. Symptom-specific indications and efficacy are demonstrated in **Supplementary Figure 1**. Compared to data from historical controls (i.e. 26 type II patients), the survival appeared longer in type II MADD(-like)-patients treated with D,L-3-HB, as depicted in **Figure 1B**. The median interval from start of D,L-3-HB to first reported clinical improvement was one month (IQR: 3 months) and ranged up to six months, as demonstrated in **Figure 1C**. Three out of four patients (75%) in the group of non-responders and one out of three patients (33%) in the group with questionable efficacy, had a treatment duration of more than six months.

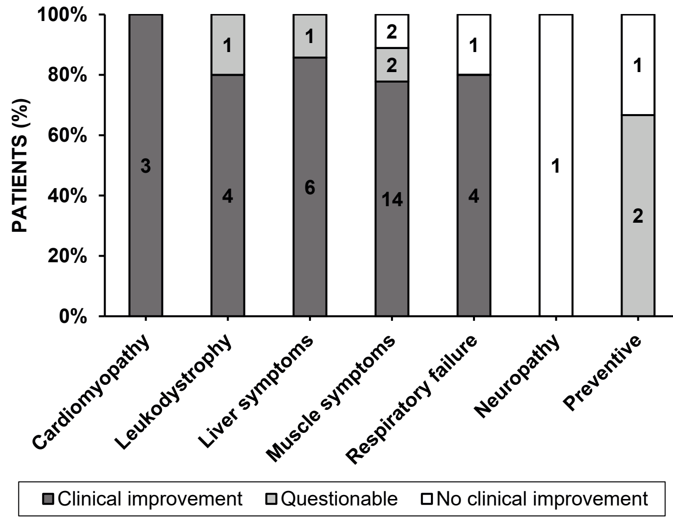
Table 2. Summarized patient and D,L-3-hydroxybutyrate treatment characteristics according to outcome.

	Clinical improvement upon D,L-3-HB treatment		
	Yes (n = 16; 70%)	**Questionable (n = 3; 13%)**	**No (n = 4; 17%)**
Gender	M:F = 9:7	M:F = 1:2	M:F = 0:4
Alive	12 (75%)	2 (67%)	3 (75%)
Current age	13 y (6.5 y)	3 y (1.5 y)	13.5 y (10.5 y)
Age at death	1.5 y (8 y)	8 m	10 d
Age at onset	3 m (8 m)	3 d (5 m)	3 m (5 y)
Congenital anomalies	-	-	-
Positive newborn bloodspot screening	8 (50%)	3 (100%)	2 (50%)
Genetic analysis	14 (88%)	3 (100%)	4 (100%)
ETFA	5[a]	-	1
ETFB	-	-	-
ETFDH	8	3	2[b]
SLC52A3	1	-	1
Enzyme assay	6 (38%)	1 (33%)	2 (50%)
ETF deficiency	1	-	-
ETF-QO deficiency	4[c]	1	1
D,L-3-HB treatment			
Age at start	1.5 y (6 y)	6 m (2 y)	5 m (6.5 y)
Minimum D,L-3-HB dose (mg/kg/day)	330 (215)	200 (105)	490 (215)
Maximum D,L-3-HB dose (mg/kg/day)	650 (400)	395 (925)	905 (330)
Maximum number of gifts/day	4 (0.3)[d]	4 (1.5)	4 (0.5)[e]
D,L-3-HB discontinuation	7 (44%)	2 (33%)	3 (75%)
Age at discontinuation	6 y (17 y)	1 y (5 m)	3.5 y (13 y)

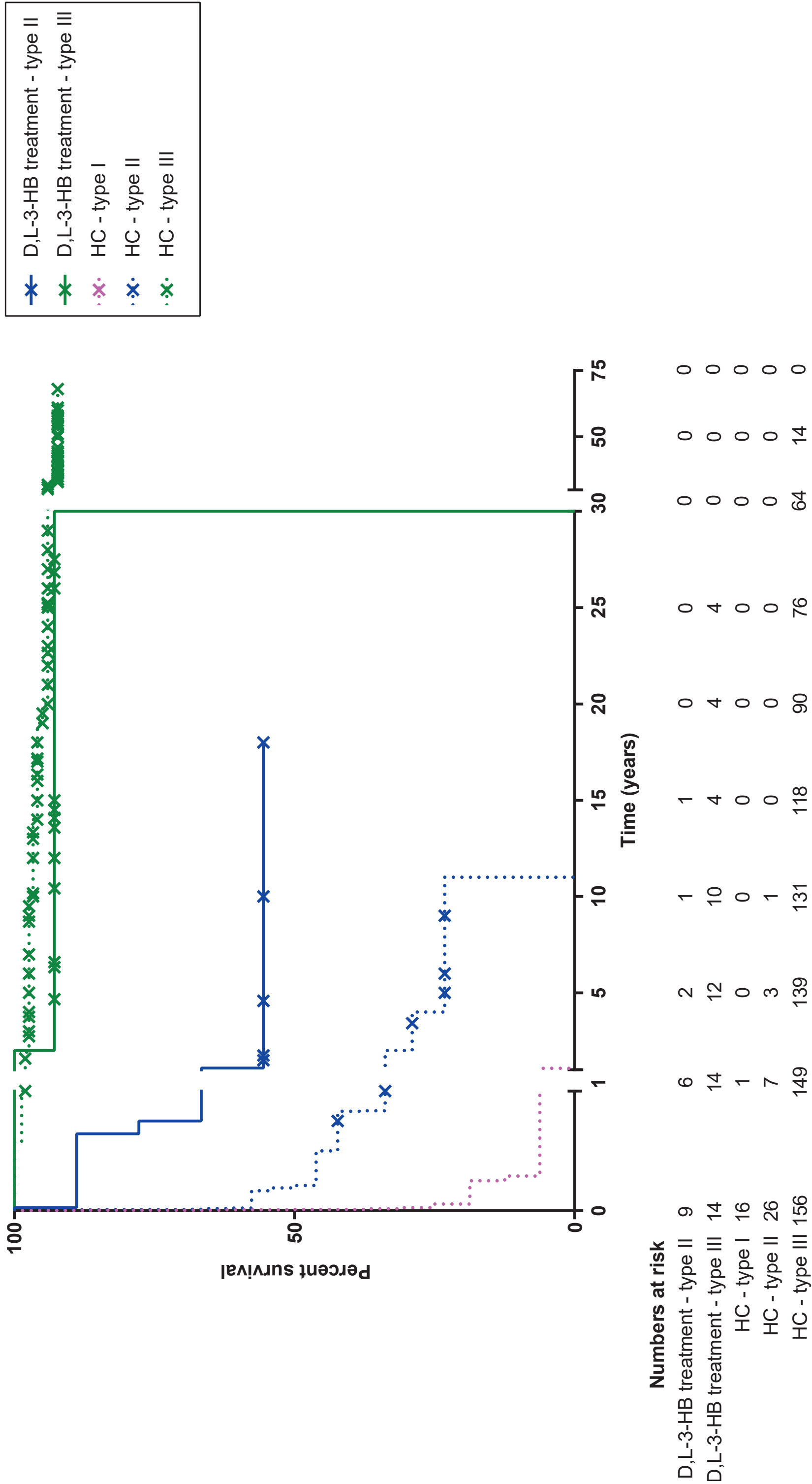
D,L-3-HB treatment duration	3 y (7.5 y)	6 m (5 m)	2 y (3.5 y)

Values are presented as number of patients or median (IQR). [a]In one patient, DNA analysis also demonstrated compound heterozygous variants in *ETFB* (c.217-4G>T and c.438+20C>T) which were classified as variant of uncertain significance and likely benign, respectively; [b]in one patient only one pathogenic variant identified; [c]in one patient only performed in sister; [d]continuous nocturnal administration (n = 4); [e]continuous nocturnal administration (n = 1) and continuous 24h administration (n = 1).

A)



B)



5

C)

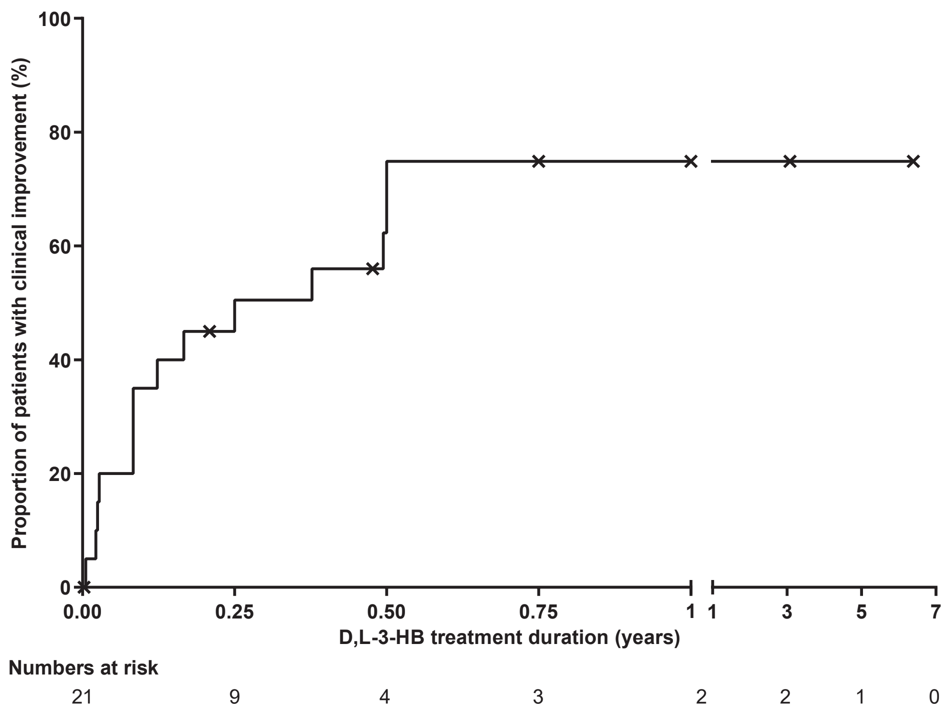


Figure 1. Efficacy of D,L-3-hydroxybutyrate treatment. (A) Proportion of MADD(-like)-patients with organ based indication and efficacy of D,L-3-hydroxybutyrate treatment, with the numbers presented in the columns. Clinical improvement regarding liver symptoms included hyperammonemia, hypoglycemia, and/or metabolic acidosis; clinical improvement regarding muscle symptoms included exercise intolerance, hypotonia, myopathy, and/or rhabdomyolysis. (B) Kaplan-Meier curve of the survival in type II (n = 9) and type III (n = 14) MADD(-like)-patients treated with D,L-3-HB compared to the survival in historical controls from literature (type I (n = 16), type II (n = 26) and type III (n = 156) MADD) who were not reported to have been treated with D,L-3-HB. (C) Kaplan-Meier plot that demonstrates the cumulative proportion of MADD(-like)-patients with reported clinical improvement upon initiation of D,L-3-HB treatment over time. Calculated from a total of 21 patients with sufficient data. Abbreviations (in alphabetical order): HC, historical control. x = censored patient.

The following side effects of D,L-3-HB were reported in eight patients (35%): abdominal pain, constipation, dehydration, diarrhea, and vomiting or nausea. Detailed data on D,L-3-HB safety is presented in **Figure 2**. D,L-3-HB treatment related (treatment duration > 1 d) side effects appeared dose dependent with a median maximum dose of 600 mg/kg/day (IQR: 410 mg/kg/day) in patients without side effects compared to 950 mg/kg/day (IQR: 555 mg/kg/day) in patients with side effects ($p = 0.0544$). In four patients (17%), the D,L-3-HB dose was titrated based upon biochemical parameters, including (peak) concentrations of D-3-HB in plasma, blood and urine, and ammonia concentrations.

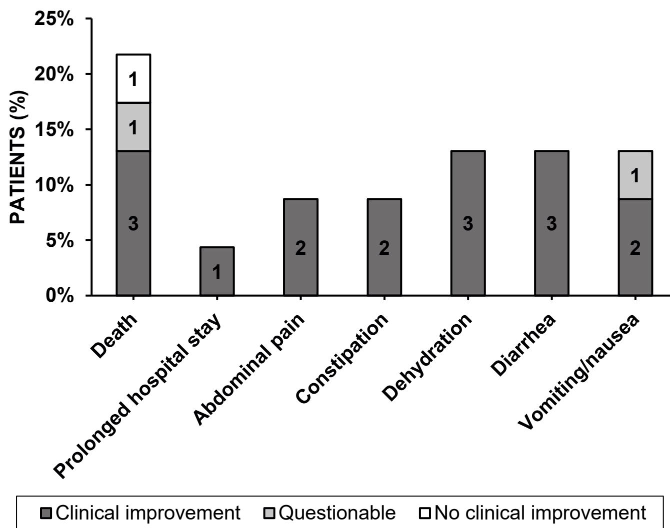


Figure 2. Safety of D,L-3-hydroxybutyrate treatment. The (serious) adverse effects that occurred in a total of 13 MADD(-like)-patients during the course of D,L-3-HB treatment.

The median duration of D,L-3-HB treatment in the whole cohort was two years (IQR: 6 years) and is demonstrated in **Figure 3**, categorized according to D,L-3-HB treatment efficacy. Treatment was discontinued in 12 patients (52%) due to (combinations of) clinical improvement after which further D,L-3-HB treatment was deemed unnecessary (n = 2), lack of clinical improvement (n = 2), death irrespective of the cause (n = 5), side effects (n = 1), non-compliance (n = 1), and cost (n = 2). The median age at D,L-3-HB discontinuation was three years (IQR: 13.5 years). The median treatment duration before D,L-3-HB discontinuation was 10 months (IQR: 1.5 years), while in the patients who continued D,L-3-HB treatment, the median treatment duration was 6.5 years (IQR: 7 years). In patients who died during the course of D,L-3-HB treatment, the median D,L-3-HB treatment duration was 8 months (IQR: 4 months).

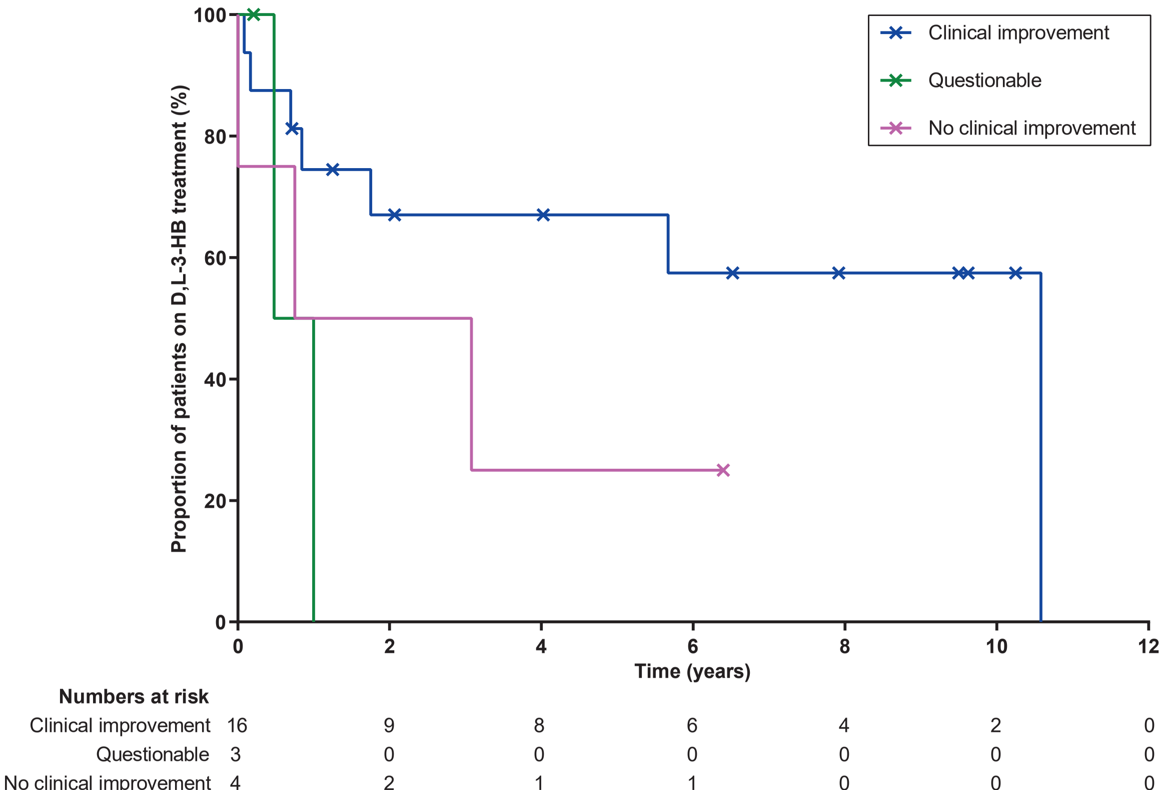


Figure 3. D,L-3-Hydroxybutyrate treatment duration. Kaplan-Meier plot that demonstrates the cumulative proportion of MADD(-like)-patients receiving D,L-3-HB treatment over time, categorized according to D,L-3-HB treatment efficacy. Calculated from a total of 23 patients with sufficient data; x = censored patient.

DISCUSSION

D,L-3-HB treatment is unlicensed but has been reported in at least eight IEMs in which exogenous KB treatment may be indicated. In 70% of the presented cohort of 23 MADD(-like)-patients, we observed clinical improvement of cardiomyopathy, leukodystrophy, liver symptoms, muscle symptoms and respiratory failure upon start of D,L-3-HB treatment. D,L-3-HB treatment appeared to be ineffective for neuropathy. Side effects occurred in 35% of the patients but were never a reason to discontinue supplementation in patients who experienced clinical improvement.

To date, there are no clinical or laboratory parameters predicting clinical efficacy of D,L-3-HB treatment, such as phenotype, genotype or age at start of D,L-3-HB treatment. In our study, symptom improvement is observed up to six months after commencing D,L-3-HB. It was not possible to relate the (timespan of) D,L-3-HB treatment efficacy to age at clinical onset, D,L-3-HB dosing or to a specific organ because in a number of patients there were several concurrent treatment indications. The authors emphasize the importance of a relatively long evaluation period for assessment of efficacy, because clinical improvement occurs in weeks or months rather than days after starting D,L-3-HB treatment. Furthermore, studies are warranted to identify MADD (bio)markers that correlate with clinical severity and can be used as outcome parameters during prospective trials.[12]

Biochemical monitoring of D,L-3-HB treatment was performed in only four patients (17%), all of whom experienced clinical improvement. D,L-3-HB dose titration towards at least detectable concentrations in blood, plasma, or urine can indicate that a sufficient amount of exogenous KB is supplied. Stable isotope infusion studies demonstrated an increased endogenous KB production in fasting healthy newborns compared to healthy adults.[26,27] Thus, when *endogenous* KB production rates are insufficient, it may be hypothesized that *exogenous* requirements are higher in infants. Prospective *in vitro* and (stable isotope) *in vivo* metabolic flux studies may help guide the (individualized) dose response curves and relations to symptoms and signs.

In this study, the risk of side effects due to D,L-3-HB appeared to increase with dose. However, frequently reported side effects such as abdominal pain, constipation, dehydration, diarrhea, and vomiting or nausea are difficult to discriminate from the natural course of the underlying disorder and from intercurrent illness. In addition, it is important to realize that the salt-free dose between different compounds can differ. It is unclear whether the adverse effects would be caused by the high amount of D,L-3-HB or by the associated cation load. A relatively low dose of 600 mg/kg/day of sodium-D,L-3-HB provides 4.8 mmol/kg/day of sodium, and a high dose of

2600 mg/kg/day provides 20.6 mmol/kg/day of sodium, compared to the normal sodium intake of 1 and 3 - 4 mmol/kg/day for adults and infants and young children, respectively. Nevertheless, it should be emphasized that in our study the benefits of D,L-3-HB appeared to outweigh the side effects. Recently, D,L-3-HB treatment in the form of a sodium or calcium salt was described in an MADD-patient. Severe alkalosis and hypernatremia were reported after D,L-3-HB doses above 1400 mg/kg/day.[9] Hypothetically, the alkalosis might be caused by the high cation load or the conjugate base excess of dissolved D,L-3-HB.[9,28] The high sodium load is also associated with increased calcium loss and alkalization of urine, which can lead to nephrocalcinosis and renal stones.[9,28] Sufficient hydration is recommended for these associated electrolyte challenges. Future studies are warranted to investigate the influence of D,L-3-HB on fluid, electrolyte, and acid-base homeostasis.

The mode of action of D,L-3-HB treatment is incompletely understood and several mechanisms likely act simultaneously. In mitochondrial FAO disorders, next to intracellular energy deficiency and accumulation of toxic metabolites, shortage of KB impairs cholesterol synthesis, which is required for myelination.[29] Endogenous 3-hydroxybutyrate also has several direct and indirect signaling functions including gene expression and activation of hydroxycarboxylic acid receptor 2 which is associated with reduced lipolysis as well as anti-inflammatory and neuroprotective effects.[30,31] Presumably, this all targets the complex pathophysiology and clinical manifestations in MADD-patients, such as cardiomyopathy, leukodystrophy, and myopathy. In MADD-like disorders, a different working mechanism can be proposed. D,L-3-HB treatment was also effective in one patient suffering from RFVT3 deficiency in whom the treatment indication included respiratory failure due to diaphragm paralysis and muscle symptoms. It can be hypothesized that D,L-3-HB treatment acts on the glutamate excitotoxicity and generation of reactive oxygen species which are potentially induced by riboflavin deficiency and mitochondrial dysfunction.[30,32-35] Additionally, exogenous D,L-3-HB can provide a therapeutic option in selected cases of other IEMs in which ketogenesis is impaired, such as mitochondrial FAO disorders, defects of FAD metabolism, glycogen storage disease, mitochondrial respiratory chain disorders, organic acidurias, hyperinsulinism and as an additive for patients using a ketogenic diet.

3-Hydroxybutyrate is a chiral molecule with two enantiomers: D-3-hydroxybutyrate and L-3-hydroxybutyrate. Compared to D-3-hydroxybutyrate, utilization of L-3-hydroxybutyrate appears slower and through different routes.[28,36] Metabolism of D-3-hydroxybutyrate yields two molecules of acetyl-CoA which enter the Krebs cycle.[31] After mitochondrial import likely via monocarboxylate transporter 1[37], L-3-hydroxybutyrate is activated to L-3-hydroxybutyryl-CoA by a specific coenzyme

A ligase and becomes a substrate for short-chain acyl-CoA dehydrogenase.[38-41] Although effects that depend on 3-hydroxybutyrate catabolism might primarily be induced by D-3-hydroxybutyrate, L-3-hydroxybutyrate may have its own specific utility. The primary use of L-3-hydroxybutyrates seems to be in the central nervous system, where the key enzymes are most expressed.[36] In rats, L-3-hydroxybutyrate seems the preferred substrate for synthesis of fatty acids and sterols in spinal cord, brain and kidney, while D-3-hydroxybutyrate is favored for oxidation.[36,39] Future studies should evaluate if L-3-hydroxybutyrate may have a specific therapeutic role towards neurological symptoms. In addition to optimal dosing, the most advantageous ratio between D-3-hydroxybutyrate and L-3-hydroxybutyrate may need to be determined based on (organ specific) treatment indications.

Several methodological limitations concerning this study should be considered. As this study concerned a retrospective study collecting data over a period of > 20 years, it was not possible to consistently capture all detailed data, for instance regarding specific time points. Second, the lack of standardized clinical and biochemical outcome parameters made a detailed study of efficacy difficult. Third, the assignment of outcome to D,L-3-HB treatment was complicated by concurrent other treatment options, and by a fluctuating natural disease course. Finally, despite their best efforts, the authors have been unable to include all centers with experience in this field.

Since the first publication in 2003[6], the story of D,L-3-HB for MADD has become an excellent example of the long and complex journey towards possible orphan drug designation and registration of an unlicensed compound for the treatment of an ultra-rare, life-threatening disease. The strength of the current study is the international pooling of data on the efficacy and safety profile of D,L-3-HB in MADD(-like)-patients. FAIR (i.e. Findable, Accessible, Interoperable, Reusable)[42], evidence-based and transparant approaches are essential in order to establish sustainable orphan drugs.[43,44] Therefore, the authors included information on preparation and pricing of D,L-3-HB. Our findings may be useful in the pursuit of orphan drug designation and registration. To this aim, organisations such as Fair Medicine[45], which introduces a coalition model that involves all stakeholders in the pharmaceutical development process, can perhaps bring a fresh impetus.

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SUPPLEMENTARY APPENDIX

Supplementary Data 1
Supplementary Table 1
Supplementary Table 2
Supplementary Figure 1

Supplementary Data 1. Detailed description of the systematic literature review methods.
Presentation of the original search strategy. Original search strategy implemented in PubMed database retrieved 310 hits on September 27th, 2016.

(“Hydroxybutyrates”[Mesh] OR “3-Hydroxybutyric Acid”[Mesh] OR “Ketone Bodies”[Mesh] OR 3-Hydroxybutyr*[tiab] OR beta-Hydroxybutyr*[tiab] OR 3hydroxybutyr*[tiab] OR b-hydroxybutyr*[tiab]) AND (“Metabolism, Inborn Errors”[Mesh] OR “Multiple Acyl Coenzyme A Dehydrogenase Deficiency”[Mesh] OR MADD[tiab] OR Multiple Acyl-CoA Dehydrogenase Deficien*[tiab] OR “Glutaric Aciduria”[tiab] OR ETFDH Deficien*[tiab] OR ETFA Deficien*[tiab] OR ETFB Deficien*[tiab]) NOT ((“Animals”[Mesh] NOT “Humans”[Mesh]) OR animal[ti] OR mouse[ti] OR mice[ti] OR rodent*[ti] OR rat[ti] OR rats[ti])

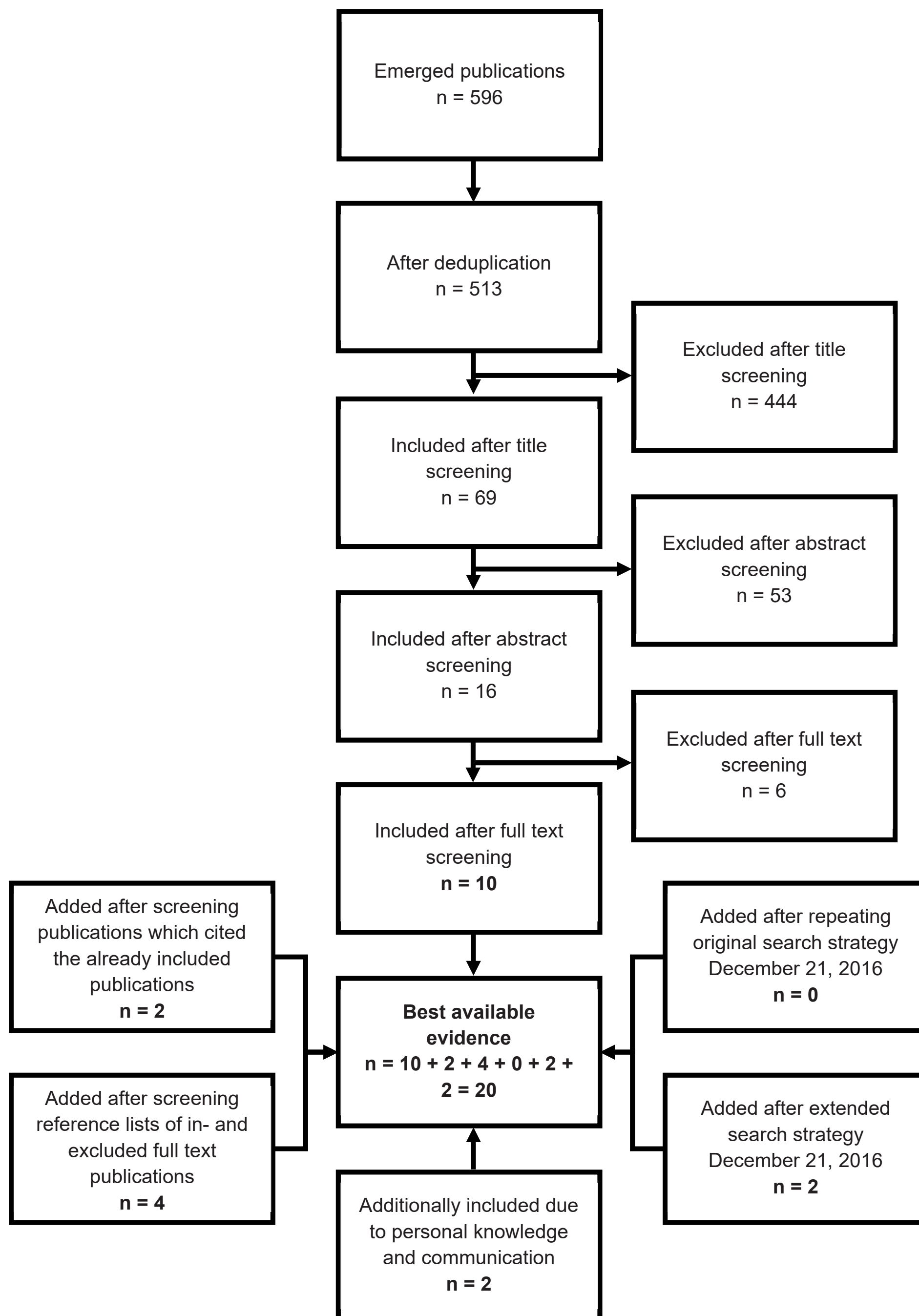
Protocol of the screening process and data extraction. The screening process was performed independently by two reviewers (WvR & EJ). Articles were included based upon the presence of detailed patient data concerning D,L-3-HB or ketone body treatment as well as a confirmed diagnosis by biochemical (acylcarnitine or organic acid profile), DNA, or enzymatic analysis. The following exclusion criteria were maintained: a) no detailed patient data described, b) lack of accessibility of the abstracts or articles, c) no availability in English or Dutch language.

Nine articles were included as search outcome after completion of the screening process and one was added due to personal knowledge of the literature.[1] To prevent missing any publications, we extended our search with two approaches: 2) screening publications, identified via Web of Science, which cited the already included publications, and 3) screening the reference lists of already previously retrieved (included and excluded) full text publications. This resulted in the addition of two and four publications to our search outcome, respectively (n = 16 in total). To minimize the risk of missing data, we expanded our original search strategy with terms used in abstracts or articles which were only found through the 2nd and 3rd search approach. This lead to the inclusion of two more publications (n = 18 in total). Finally, two more publications were added due to personal knowledge and communication (n = 20 in total).[2,3]

General study characteristics and data on clinical presentation and D,L-3-HB treatment per inborn error of metabolism were collected independently by two reviewers (WvR & EJ). Consensus on eligibility during the screening process and data extraction was reached through regular meetings. If necessary, a third, independent reviewer (TD) was consulted to make the final call on eligibility.

Presentation of the extended search strategy. Extended search strategy implemented in PubMed database retrieved 333 hits on December 21st, 2016.

(“Hydroxybutyrates”[Mesh] OR “3-Hydroxybutyric Acid”[Mesh] OR “Ketone Bodies”[Mesh] OR 3-Hydroxybutyr*[tiab] OR beta-Hydroxybutyr*[tiab] OR 3hydroxybutyr*[tiab] OR b-hydroxybutyr*[tiab] OR D,L-3-hydroxybutyr*[tiab] OR OH-B[tiab] OR 3-HB[tiab] OR B-OHB[tiab] OR BOHB[tiab]) AND (“Metabolism, Inborn Errors”[Mesh] OR “Multiple Acyl Coenzyme A Dehydrogenase Deficiency”[Mesh] OR MADD[tiab] OR Multiple Acyl-CoA Dehydrogenase Deficien*[tiab] OR Multiple Acyl CoA Dehydrogenase Deficien*[tiab] OR “Glutaric Aciduria”[tiab] OR ETFDH Deficien*[tiab] OR ETFA Deficien*[tiab] OR ETFB Deficien*[tiab] OR “3-Hydroxy-3-Methylglutaryl-CoA Lyase Deficiency”[Mesh] OR “HMG-CoA lyase”[tiab] OR “HMG Co-A lyase”[tiab] OR 3-hydroxy-3-methylglutaryl-coenzyme A lyase deficien*[tiab] OR “Congenital Hyperinsulinism”[Mesh] OR “persistent hyperinsulinemic hypoglycemia”[tiab] OR PHHI[tiab] OR “persistent hypoglycemia and hyperinsulinism”[tiab] OR “Glycogen Storage Disease Type III” [MESH] OR “Glycogenosis type III”[tiab] OR “Glycogen storage disease type 3” [tiab] OR “Cytochrome-c Oxidase Deficiency”[MESH] OR “Deficiency in complex IV of respiratory chain”[tiab] OR “Mitochondrial complex IV deficiency” [tiab] OR “VLCAD deficiency”[MESH] OR “Deficiency on very long chain acyl-CoA dehydrogenase”[tiab] OR “Very long-chain acyl-coa dehydrogenase deficiency”[tiab] OR “Propionic academia”[MESH] OR “Ketotic hyperglycinemia”[tiab]) NOT ((“Animals”[Mesh] NOT “Humans”[Mesh]) OR animal[ti] OR mouse[ti] OR mice[ti] OR rodent*[ti] OR rat[ti] OR rats[ti])



Flowchart of the screening process. Implementation of the original search strategy in PubMed and EMBASE databases on September 27th, 2016 resulted in 310 and 286 hits, respectively.

References to Supplementary Data 1

1. Bosch AM, Abeling NG, Ijlst L, et al. Brown-vialetto-van laere and fazio londe syndrome is associated with a riboflavin transporter defect mimicking mild MADD: A new inborn error of metabolism with potential treatment. J Inherit Metab Dis. 2011;34(1):159-164.
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Supplementary Table 1. Overview of D,L-3-hydroxybutyrate treatment in 34 patients with inborn errors of metabolism; results from the systematic literature review.

Reference	IEM (OMIM #)	n (M/F)	Age onset	Age start D,L-3-HB	D,L-3-HB dose range (mg/kg/d)	D,L-3-HB indication: X Clinical improvement: (+/-/NR)					Side effect(s)
						CMP	LD	Liver[a]	Muscle[b]	RF	
Francois, 1981	HMG-CoA lyase deficiency (246450)	1 (1/0)	1 d	8 m	760			X (+)			NR
Bougneres, 1983	PHHI (256450)	4 (4/0)	1 - 10 d	3 - 32 m	115 - 150			X (+)			No
Bonham, 1999; Olpin, 2004	MADD (231680)	1 (0/1)	NS	14 m	1 - 10 g/d			X (+)	X (+)		No
Van Hove, 2001, 2003; Grunewald, 2008	MADD (231680)	3 (2/1)	6 d - 5 m	2 - 28 m	80 - 900	X (+)	X (+)	X (+)	X (+)		No
Plecko, 2002	PHHI (256450)	2 (1/1)	0 - 1 d	6 m	880 - 4000			X (NR)			No
Van Spronsen, 2005	RFVT3 deficiency[c] (211530)	1 (1/0)	5 m	5 m[c]	700				X (+)[c]	X (+)	Constipation, diarrhea[c]
Al-Hertani, 2008	MADD (231680)	1 (0/1)	6 m	NR	NR		X (+)[c]				No[c]
Marquardt, 2009	MADD (231680)	2 (NR)	NR	NR	NR			X (NR)			Metabolic alkalosis, nephron-calcinosis
Bhattacharya, 2010	HMG-CoA lyase deficiency (246450)	1 (1/0)	1 d	1.5 m	300		X (+)				No

Dalkeith, 2010, 2013	MADD (231680)	4 (NR)	<1 m - 5 m	7 d - 5 m	300 - 900	X (+)[c]	X (+)	X (+)	X (+)[c]	NR
	HMG-CoA lyase deficiency (24645)	2 (NR)	NR	3 m - 16 y	300 - 900	Youngest patient was treated empirically (NR);the oldest for liver symptoms (NR).				NR
	CACT deficiency (212138)	1 (NR)	NR	<1 m	300 - 900	X (+)	X (+)			
Van, 2010	GSD type III (232400)	2 (NR)	NR	1 - 9 y	600 - 900	X (+)[d]		X (+)[d]		No
	Mitochondrial complex IV deficiency (220110)	2 (NR)	NR	1 - 9 y	600 - 900					
	VLCAD deficiency (201475)	1 (NR)	NR	1 - 9 y	600 - 900					
	PA (606054)	1 (NR)	NR	1 - 9 y	600 - 900					
Bosch, 2011	RFVT3 deficiency (211530)	1 (0/1)	6 m[c]	7 m[c]	480 - 860[c]			X (-)[c]	X (-)[c]	NR
Hale, 2011	Presumptive MADD (231680)	1 (0/1)	1 d	7 m	300 - 1200	X (-)			X (-)	NR
Valayannopoulos, 2011	GSD type III (232400)	1 (1/0)	NR	2 m	400 - 800	X (+)	X (+)			No

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Reference	IEM (OMIM #)	n (M/F)	Age onset	Age start D,L-3-HB	D,L-3-HB dose range (mg/kg/d)	D,L-3-HB indication: X Clinical improvement: (+/-/NR)					Side ef fect(s)
						CMP	LD	Liver[a]	Muscle[b]	RF	
Van Rijt, 2014	MADD (231680)	1 (0/1)	0 d	7 m	450 - 2600	X (+)	X (+)[c]		X (+)[c]	X (+)[c]	*Mild* dehydration, diarrhea, vomiting[c]
Gautschi, 2015	MADD (231680)	1 (1/0)	2.5 y	4 y	300 - 900		X (+)		X (+)		Indigestion, dehydration

Phenotype OMIM #: *http://www.omim.org/*. The presence (+) or absence (-) of clinical improvement regarding [a]liver symptoms including hepatic/hyperammonemic encephalopathy (+), hypoglycemia (+) and poor metabolic control (+); [b]muscle symptoms including myopathy (+) and hypotonia (+). [c]Personal knowledge or communication; [d]unclear which patient(s). Abbreviations (in alphabetical order): CACT, carnitine-acylcarnitine translocase; CMP, cardiomyopathy; GSD, glycogen storage disease; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IEM, inborn error of metabolism; LD, leukodystrophy; multiple acyl-CoA dehydrogenase deficiency; NR, not reported; OMIM, online mendelian inheritance in man; PA, propionic acidemia; PHHI, persistent hyperinsulinemic hypoglycemia of infancy; RF, respiratory failure; RFVT, riboflavin transport; VLCAD, very long-chain acyl-CoA dehydrogenase.

Supplementary Table 2. Individual D,L-3-hydroxybutyrate treatment characteristics and outcome; results from the retrospective cohort study.

PT	Age start D,L-3-HB	D,L-3-HB indication: X Clinical improvement reported: (+/-)						Range of D,L-3-HB dose (mg/kg/d)	Administration method(s)	Safety	D,L-3-HB dis continued
		CMP	LD	Liver[a]	Muscle[b]	RF	Neuro-pathy				
1[c(7)d]	7 m	X (+)	X (+)		X (+)	(+)		450 - 2600	Gastrostomy/ nasogastric tube, oral	Prolonged hospitalization, mild dehydration, diarrhea, vomiting	No
2	10 + 1/12 y				X (+)			250 - 500	Oral	Infrequent abdominal pain	No
3[e]	9 d	Preventive treatment aspect (-), deceased after 1 d.						900 - 1800	Nasogastric tube	Death	Yes, death
4 [c(17, 18)de]	19 d			X (+)	X (+)	X (+)		300 - 600	Oral	Death	Yes, death
5	9 m			X (+)	X (+)			130 - 1000	Nasogastric tube	Mild vomiting	No
6 [c(17, 18)]	6 m	X (+)		X (+)	X (+)			200 - 650	Gastrostomy tube, oral	-	No
7	6 m	Questionable due to preventive treatment aspect.						200 - 2000	Gastrostomy/ nasogastric tube	Chronic nausea and vomiting	Yes, side effects
8[de]	2 m	Liver, muscle and preventive treatment; questionable due to very early start and preventive treatment aspect, patient seemed more stable.						355 - 395	Nasogastric tube	Death	Yes, death
9	2 m	Low C2 carnitine (-).						100 - 950	Oral	-	No

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PT	Age start D,L-3-HB	D,L-3-HB indication: X Clinical improvement reported: (+/-)						Range of D,L-3-HB dose (mg/kg/d)	Administration method(s)	Safety	D,L-3-HB dis continued
		CMP	LD	Liver[a]	Muscle[b]	RF	Neuro-pathy				
10	2 + 5/12 y				X (+)	X (+)		650 - 1200	Oral	-	No
11[e]	28 + 6/12 y			X (+)	X (+)			450	Oral	-	Yes, costs
12 c(17, 18)	5 m				X (+)			200 - 300	Oral	-	Yes, no treatment compliance
13 c(15)	4 + 3/12 y		X (+)					500	Oral	-	No
14	4 + 6/12 y	Leukodystrophy and muscle treatment; questionable due to short treatment duration of only three months at time of data collection.						150	Oral	-	No
15	24 + 9/12 y		X (+)		(+)			560 - 600	Oral	Mild dehydration, abdominal pain, constipation	No
16[df]	5 + 1/12 y				X (+)			300 - 1000	Oral	Mild diarrhea	No
17[c] (8)d	3 + 11/12 y		X (+)		X (+)			480 - 900	Gastrostomy tube	Mild dehydration	No
18	25 + 11/12 y			X (+)	X (+)			900	Nasogastric tube	-	Yes, clinical improvement

19	25 y			X (-)		X (-)	500 - 750[g]	Oral	-	Yes, ineffectiveness and costs
20[c] (17,18)[e]	3 m		X (+)	X (+)			300 - 600	Oral	Death	Yes, death
21[e]	3 m	X (+)					NR	Oral	Death	Yes, death
22[c] (10)	5 m			X (+)	X (+)		330 - 730	Nasogastric tube, oral	Constipation, diarrhea	Yes, clinical improvement
23[c] (11)	7 m			X (-)	X (-)		480 - 860	Nasogastric tube	-	Yes, ineffectiveness

The presence (+) or absence (-) of clinical improvement regarding [a]liver symptoms including hyperammonemia (+), hypoglycemia (+), liver dysfunction/failure (+) and metabolic acidosis (+); [b]muscle symptoms including exercise intolerance (+), hypotonia (+), myopathy (+) and rhabdomyolysis (+). [c]Patient has been published before in relation to D,L-3-HB treatment, see corresponding reference in manuscript; [d]consanguinity; [e]deceased; [f]diagnosed prenatally due to family history; [g]prescribed as food supplement (Ketoforce). Abbreviation: CMP, cardiomyopathy; LD, leukodystrophy; NR, not reported; PT, patient; RF, respiratory failure.

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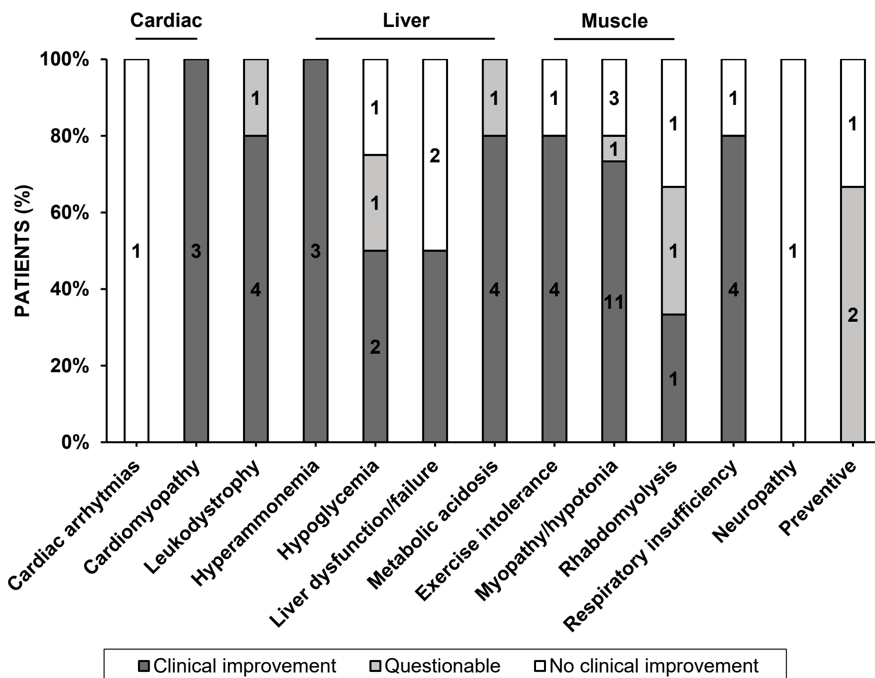

Supplementary Figure 1. Symptom based indication and efficacy of D,L-3-hydroxybutyrate treatment. Proportion of MADD(-like)-patients with symptom based indication and efficacy of D,L-3-HB treatment, with the numbers presented in the columns.

6

Enantiomer-specific pharmacokinetics of D,L-3-hydroxybutyrate: implications for the treatment of multiple acyl-CoA dehydrogenase deficiency

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Under review

ABSTRACT

Background

Treatment with racemic D,L-3-hydroxybutyrate (D,L-3-HB, a ketone body) has been described in several inborn errors of metabolism, including severe multiple acyl-CoA dehydrogenase deficiency (MADD; glutaric aciduria type II). We aimed to improve the understanding of enantiomer-specific pharmacokinetics (PK) of D,L-3-HB.

Methods

Using UPLC-MS/MS, we analyzed D-3-HB and L-3-HB concentrations in blood samples from three MADD patients, and blood and tissue samples from healthy rats, upon D,L-3-HB salt administration (patients: 736-1123 mg/kg/day; rats: 1579-6317 mg/kg/day of salt-free D,L-3-HB). PK parameters were calculated manually and modeled using MW/Pharm PK software.

Results

D,L-3-HB administration caused substantially higher L-3-HB concentrations than D-3-HB. In MADD patients, both enantiomers peaked at 30-60 minutes, and approached baseline after three hours. In rats, D,L-3-HB administration significantly increased the C_{max} and AUC of D-3-HB in a dose-dependent manner (controls vs. ascending dose groups for C_{max}: 0.10 vs. 0.30-0.35-0.50 mmol/L, and AUC: 14 vs. 58-71-106 min*mmol/L), whereas for L-3-HB the increases were significant compared to controls, but not dose proportional (C_{max}: 0.01 vs. 1.88-1.92-1.98 mmol/L, and AUC: 1 vs. 380-454-479 min*mmol/L). The PK model performance was adequate for the low dose group. L-3-HB concentrations increased extensively in brain, heart, liver and muscle, whereas the most profound rise in D-3-HB was observed in heart and liver.

Conclusions

Our study provides important knowledge on the absorption and distribution of D-3-HB and L-3-HB upon a single, oral dose of D,L-3-HB salt. The enantiomer-specific PK implies differential metabolic fates of D-3-HB and L-3-HB.

INTRODUCTION

Upon prolonged fasting, increased mitochondrial fatty acid oxidation (FAO) fuels energy production in many tissues and hepatic ketogenesis in healthy individuals. When glucose availability is limited, ketone bodies (KB) acetoacetate (AcAc) and 3-hydroxybutyrate (3-HB) serve as an important alternative energy source for extrahepatic tissues such as the brain, muscle, and heart.[1,2]

Multiple acyl-CoA dehydrogenase deficiency (MADD; or glutaric aciduria type II; OMIM #231680) is an ultra-rare (i.e. < 1:50,000) disorder of mitochondrial FAO and amino acid metabolism.[3] Treatment options include dietary fat and protein restrictions, fasting avoidance, and supplementation with riboflavin, glycine, coenzyme Q10 and L-carnitine. Although this treatment is generally sufficient in milder MADD, severely affected patients often develop life-threathening symptoms as cardiomyopathy, leukodystrophy and myopathy.[3] Since patients with mitochondrial FAO disorders may exhibit impaired ketogenesis, KB supplementation potentially provides a therapeutic option to ensure an adequate supply.[1,4] Racemic D,L-3-HB salt supplementation (i.e. ratio D-3-HB:L-3-HB is 1:1) has been shown to result in clinical improvement of cardiomyopathy, leukodystrophy and hypotonia in severe MADD.[5,6] The enterally administered quantities ranged between 100 to 2600 mg/kg D,L-3-HB salt in one to six daily doses. D,L-3-HB can be prescribed as food supplement or as magistral formula prepared by pharmacists, and is currently available as sodium salt, sodium/calcium salt, or mixed salt formulation.[5,6] Recently, continuous administration of D,L-3-hydroxybutyric acid has been advocated as well.[7]

3-hydroxybutyrate is a chiral molecule and its D- and L-enantiomer can have different pharmacokinetic (PK) and pharmacodynamic (PD) properties.[8] To date, the lack of a clear clinical and biochemical dose-efficacy relationship[6], and the absence of PK and PD insights of the individual enantiomers, hamper optimal treatment. To bridge this gap, we analyzed circulating D-3-HB and L-3-HB concentrations in three MADD patients, and characterized the enantiomer-specific PK in terms of absorption and distribution in healthy rats, following single dose administration of racemic D,L-3-HB.

MATERIALS AND METHODS

Exploratory measurements in three patients with multiple acyl-CoA dehydrogenase deficiency

We identified three MADD patients who received enteral D,L-3-HB for

cardiomyopathy, leukodystrophy and myopathy, in addition to their standard (dietary) treatments. The patient characteristics and D,L-3-HB treatment details are described in **Supplementary Data 1**. Blood samples were obtained at several time points post-administration and analyzed for D-3-HB and L-3-HB concentrations. The results of longitudinal urinary metabolite monitoring in patient 1 were analyzed retrospectively.

The exploratory patient studies were performed according to the principles of the Helsinki Declaration, as revised latest in 2013. For patient 1, the Medical Ethical Committee of the University Medical Center Groningen confirmed that the Medical Research Involving Human Subjects Act did not apply for the retrospective data analysis and that official approval of this study by the Medical Ethical Committee was not required (METc 2019/119). The measurements were conducted with parental informed consent, within the context of standard patient care and following the codes of conduct of the FEDERA (Federation of Medical Scientific Institutions). For patient 2 the measurements and clinical review were performed after obtaining informed consent on an IRB-approved research protocol (COMIRB# 16-0146). In patient 3, the studies were done within the context of standard patient care.

Animal study

The animal procedures were performed in compliance with EU legislation (Directive 2010/63/EU) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The study protocol was approved by the Dutch Central Committee Animal Testing (#AVD1050020172265). All institutional and national guidelines for the care and use of laboratory animals were followed.

Sodium-D,L-3-HB (≥ 99.0%) was purchased from Sigma-Aldrich and prepared as solutions in demineralized water (range: 260 - 1030 mg sodium-D,L-3-HB/mL ([$C_4H_7O_3Na$ (aq)] = 2.1 - 8.2 M)). The concentrations ensured dosing with weight-based volumes (range: 2.6 - 3.4 mL) between 70 - 80% of the recommended maximum volumes for administration via oral gavage.

Twenty-five healthy, male Wistar rats (Envigo, the Netherlands) were group housed on a reversed 12h light - 12h dark cycle (lights on at 20.00 PM). Drinking water and standard chow (RM1 diet, Special Diets Services) were provided ad libitum. Experimental studies were performed after two weeks of acclimatization. The experimental timing was accommodated to the rat's active period, with the procedures operated under infrared light, starting one hour after onset of the dark period.

Experimental design of the animal study

A flowchart and timeline diagram of the experimental design is presented in

Supplementary Figure 1. The animals (mean weight: 414 gram, SD: 30 gram) were randomly assigned to three experimental groups and one control group. The rats were housed individually. There were no food or drinking water restrictions in order to mimic the patient setting. The animals in the three experimental groups received a single, oral dose of sodium-D,L-3-HB via oral gavage. To allow correct data interpretation, the doses are described in salt-free amount (i.e. approximately 82% of the sodium-D,L-3-HB dose). The low (n = 6), medium (n = 7), and high dose (n = 6) groups received 1579 mg/kg, 3159 mg/kg, and 6317 mg/kg, respectively (i.e. 1926, 3852, and 7704 mg/kg of sodium-D,L-3-HB). These doses correspond to the most commonly prescribed doses in humans of 369 mg/kg, 738 mg/kg, and 1476 mg/kg (i.e. 450 mg/kg, 900 mg/kg, and 1800 mg/kg of sodium-D,L-3-HB), based on endogenous D-3-HB production rates, as described in **Supplementary Data 2**.[9,10] The control group (n = 6) received demineralized water in equivalent volumes, except for two animals in which oral gavage was not possible due to stress.

Before and post-administration (i.e. 20, 40, 60, 90, 120, 150, 180, 240, and 360 minutes), venous blood samples (about 200 µL) were collected into EDTA cups via tail vein nick bleeds. The rats were euthanized by decapitation after deep anesthesia with inhalation isoflurane. The mean time from experiment initiation to termination was six hours and 47 minutes (SD: 23 minutes). Brain, heart, liver, and muscle tissue were collected in 10 to 15 minutes.

Enantiomer-specific analysis of 3-hydroxybutyrate and 3-hydroxybutyrate-carnitine

A detailed description of the sample preparation procedures is presented in **Supplementary Data 3**. The concentrations of D-3-HB and L-3HB were determined in processed blood and tissue samples using reversed phase ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), as previously described for the analysis of D-lactate and L-lactate.[11] For patients 2 and 3, the analysis was performed in deproteinized plasma samples. In short, D-3-HB and L-3-HB were quantified using an isotopically-labelled internal standard (final concentration 25 µmol/L 3,4,4,4-$^{2}H_4$-3-HB). After derivatization with diacetyl-L-tartaric-anhydride, the samples were separated on a BEH-C_{18}-reversed phase column and detected on a Xevo TQ-S micro (Waters). The 3-HB enantiomer concentrations in blood and tissues were expressed in millimole per liter and micromole per gram of protein, respectively. For patient 2, plasma concentrations of D-3-HB and AcAc were also analyzed enzymatically using BDH1 as previously described.[12-15] The quantitative oxidation of D-3-HB to AcAc in the presence of excess BDH1 and NAD^+ at pH 8.5, was determined by the increase in absorbance of NADH at 340 nm. Conversely,

quantitative reduction of AcAc to D-3-HB in the presence of excess BDH1 and NADH at pH 7.0, was determined by the decrease in absorbance of NADH at 340 nm.

To study the involvement of the ketones in mitochondrial metabolism upon D,L-3-HB administration, we used high-performance liquid chromatography MS/MS to analyze the tissue concentrations of D-3-HB- and L-3-HB-carnitine, as derivatives of enantiomer specific 3-OH-butyryl-CoA.[16,17] The concentrations are expressed in micromole per gram of protein.

Statistical analysis

Patient data are presented in absolute concentrations. Animal data are presented as mean (SD) or in absolute concentrations. The absorption and elimination phases of D-3-HB and L-3-HB were depicted in (semi-log) concentration-time plots. First, the maximum concentration (C_{max}) and time to reach C_{max} (t_{max}) were derived directly from the experimental data. The area under the concentration-time curve (AUC_{0-6}) was calculated based on the linear trapezoidal method. Next, the PK parameters were modeled using MW/Pharm pharmacokinetic software version 3.86 (MwPharm, Zuidhorn, the Netherlands)[18], as described in **Supplementary Data 4**. The enantiomer-specific population PK models were applied to estimate Cmax, tmax and AUC0-6, using Bayesian fitting to the concentration-time and weight data. The oral absorption rate constant (k_A), volume of distribution to the central compartment (V_1), elimination half-life ($t_{1/2}$), and metabolic clearance (Cl_m) were derived from the individual parameters. Model performance was evaluated on basis of the Akaike Information Criterion (AIC), the root of the Weighed Sum of Squares (ΣWSS) and visual inspection of the overall goodness-of-fit. An adequate model performance allowed estimation of the distribution and elimination parameters.

Inferential statistical analysis of the absorption data from the animal experiment was performed using GraphPad Prism version 7.02 (GraphPad Software, La Jolla California, USA) and Analyse-it in Microsoft Excel (Analyse-it Software, Ltd, Leeds, UK). One-way analysis of variance test followed by post hoc Bonferroni's multiple comparison test as appropriate, was used to test for differences of the 3-HB enantiomer parameters between the experimental groups and the control animals. A paired t-test was used to analyze differences between parameters of both 3-HB enantiomers per dose group. A p-value of < 0.05 was considered statistically significant ($p < 0.01$ after Bonferroni correction for the analyses between both 3-HB enantiomers per dose group).

RESULTS

Exploratory measurements in three patients with multiple acyl-CoA dehydrogenase deficiency

In all patients, administration of oral D,L-3-HB resulted in ketonemia with higher L-3-HB- compared to D-3-HB concentrations, as shown in **Supplementary Table 1**. In patient 1, the peak concentrations of both enantiomers could not be determined because of measurements at two time points only. In patients 2 and 3, peak concentrations of both enantiomers were achieved between 30 to 60 minutes after administration and returned toward baseline after three hours, as shown in **Figure 1**. Peak total 3-HB, D-3-HB and L-3-HB concentrations ranged between 1.11-3.92 mmol/L, 0.21-1.54 mmol/L and 0.89-2.38 mmol/L, respectively. In patient 2, following administration of D,L-3-HB we also found an increase in AcAc with peak concentrations of 0.40-0.50 mmol/L, as shown in Supplementary Table 1.

Longitudinal urinary metabolite monitoring in patient 1, revealed highly elevated excretions of tricarboxylic acid (TCA) cycle intermediates including citrate, aconitate, alpha-ketoglutarate, malate, fumarate and succinate, as presented in **Supplementary Figure 2**.

Animal study

All animals were included in the analyses. Data from 16 out of 250 samples (control group: n = 12; experimental groups: n = 4) were missing due to inadequate blood collection or sample processing. The D-3-HB and L-3-HB concentrations in controls were low and stable. Single dose administration of D,L-3-HB induced ketonemia in all experimental groups. Maximum total 3-HB, D-3-HB and L-3-HB concentrations ranged between 2.1-2.4 mmol/L, 0.30-0.50 mmol/L and 1.88-1.98 mmol/L, respectively, as shown in **Figure 2**.

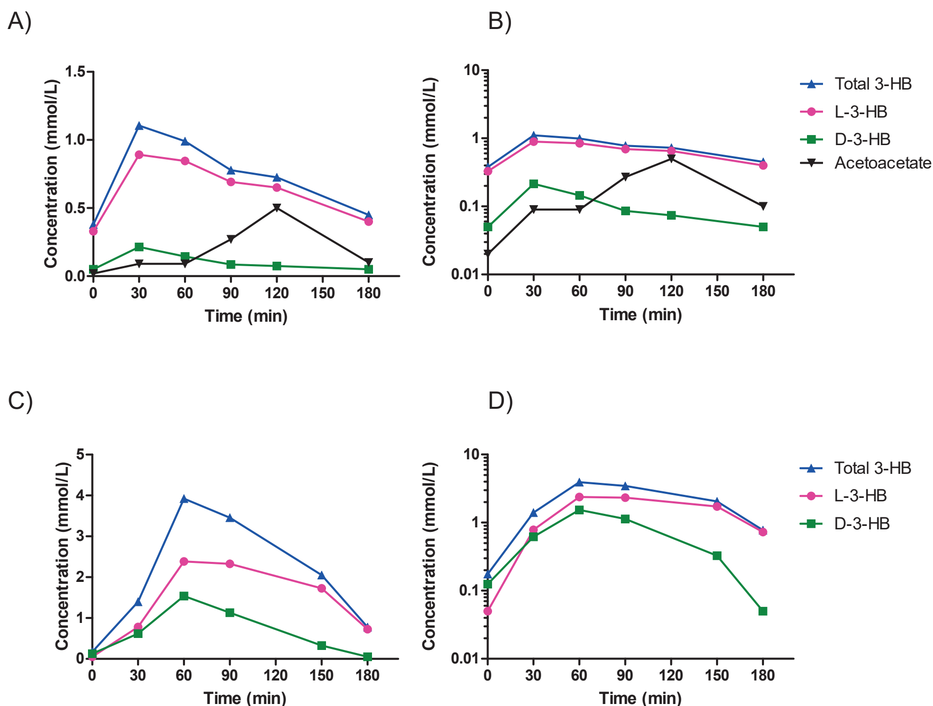

Figure 1. Exploratory measurements in two patients with multiple acyl-CoA dehydrogenase deficiency after an oral dose of D,L-3-hydroxybutyrate. The concentration-time and semi-log concentration-time plots of 3-HB, D-3-HB and L-3-HB, and AcAc (if available), determined in plasma after an oral dose of D,L-3-HB. For measurements of <0.050, a cutoff value of 0.050 was used. Data of patient 1 is not depicted because the peak concentrations of both enantiomers could not be determined due to measurements at two time points only; she received a salt-free D,L-3-HB dose of 1123 mg/kg/day divided in 5 daily doses; body weight 17.3 kg; 5 years old. Patient 2 (A/B) received a multiple salt solution of D,L-3-HB at a salt-free dose of 736 mg/kg/day divided in eight daily doses; body weight 4.5 kg; 3 months old. Patient 3 (C/D) received sodium-D,L-3-HB at a salt-free dose of 820 mg/kg/day of in four daily doses; body weight 40.0 kg; 16 years old. Data are presented in absolute concentrations.

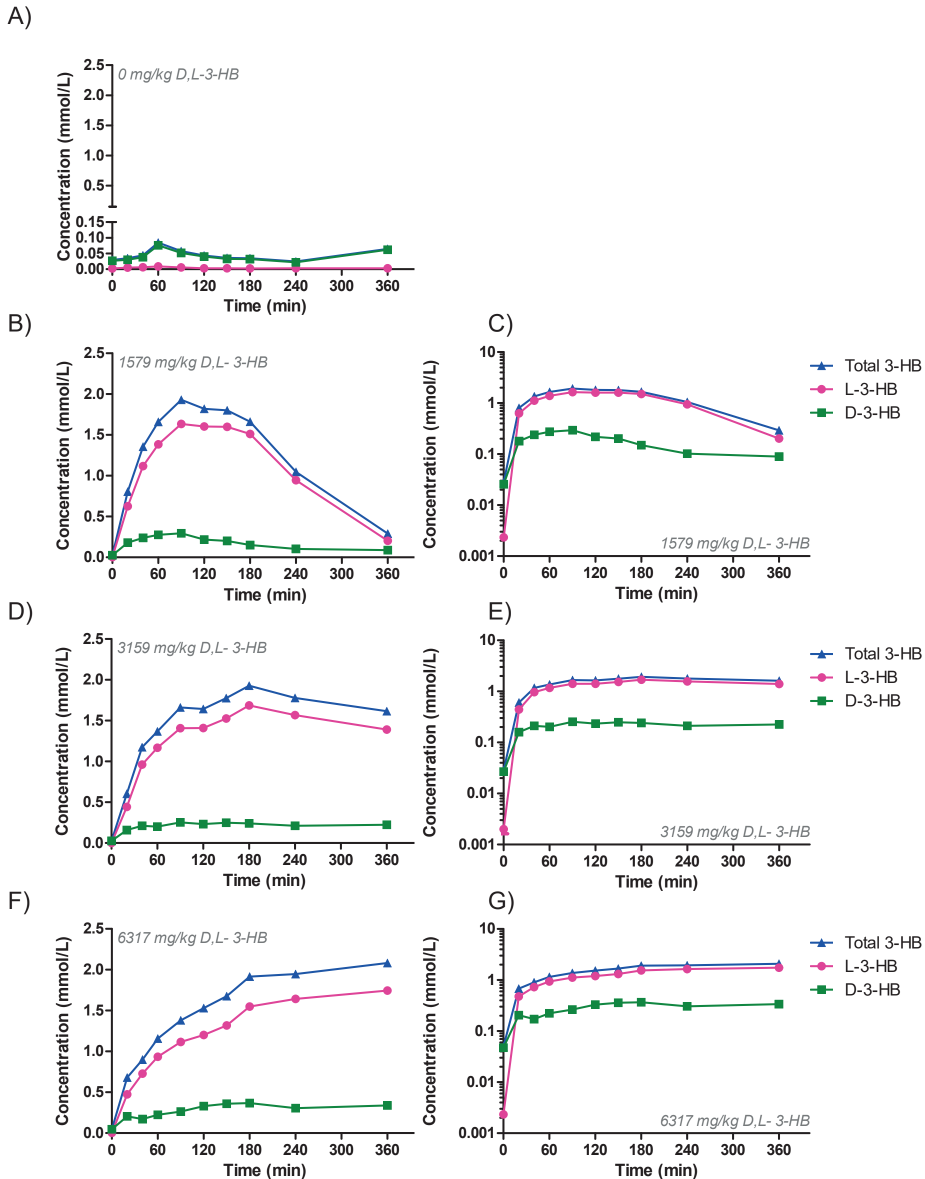

Figure 2. The enantiomer-specific absorption after a single, oral dose of D,L-3-hydroxybutyrate in rats. The blood concentration-time and semi-log concentration-time plots of 3-HB, D-3-HB and L-3-HB in the control group (n = 6) (A), and after oral sodium-D,L-3-HB at a salt-free dose of 1579 mg/kg (n = 6) (B/C), 3159 mg/kg (n = 7) (D/E), and 6317 mg/kg (n = 6) (F/G). Data are presented as mean of the respective groups.

The absorption of D,L-3-hydroxybutyrate

The 3-HB, D-3-HB and L-3-HB concentration-time curves are presented in Figure 2. The PK parameters per administered dose are listed in **Table 1**. Only for the low- and medium dose groups, the absorption and (part of) the elimination phases could be observed, with C_{max} reflecting peak concentrations. In all experimental groups, the obtained L-3-HB concentrations were substantially higher than D-3-HB, with significant differences for C_{max} and AUC. The C_{max} and AUC of both D-3-HB and L-3-HB increased significantly in all experimental groups as compared to the controls. For D-3-HB, the trend appeared dose-dependent (data of controls vs. the ascending dose groups for C_{max}: 0.10 vs. 0.30-0.35-0.50 mmol/L, and AUC: 14 vs. 58-71-106 min*mmol/L), with significant differences between the C_{max} and AUC of the low dose and high dose group, and the medium dose and high dose group. The increase in C_{max} and AUC of L-3-HB was not dose proportional (data of controls vs. ascending dose groups for C_{max}: 0.01 vs. 1.88-1.92-1.98 mmol/L, and AUC: 1 vs. 380-454-479 min*mmol/L). There was a significant increment in t_{max} of D-3-HB and L-3-HB upon increased D,L-3-HB dosing. The t_{max} of D-3-HB was lower than that of L-3-HB for all administered doses, however, the difference did not reach statistical significance. The elimination of D-3-HB and L-3-HB demonstrated signs of zero order kinetics (i.e. saturable elimination) upon ketonemia, as indicated by the linear parts in the concentration-time plots and the convex parts in the semi-log concentration-time plots in Figure 1 and 2.

The final enantiomer-specific population PK models and corresponding variables are presented in **Figure 3** and **Supplementary Table 2**, respectively. The model performance was adequate for the low dose group only, as also demonstrated by the similar curves of the individual- and population model data. The modeled PK parameters are presented in Table 1. The C_{max} and AUC of L-3-HB were significantly higher than those of D-3-HB in all experimental groups. For both 3-HB enantiomers, a dose-dependent increment in C_{max} was not observed (D-3-HB: 0.28-0.26-0.36; and L-3-HB: 1.64-1.61-1.71). The trend in AUC appeared dose proportional for D-3-HB (58-77-107 min*mmol/L), with a significant difference between the low and high dose group, whereas for L-3-HB this was not observed (338-416-475 min*mmol/L). Upon increased D,L-3-HB dosing, the t_{max} of D-3-HB and L-3-HB increased significantly. The t_{max} of D-3-HB was lower compared to L-3-HB for all dose groups, with the difference reaching statistical significance in the low dose group.

Table 1. Pharmacokinetic parameters of D-3-hydroxybutyrate and L-3-hydroxybutyrate after a single, oral dose of D,L-3-hydroxybutyrate in rats.

		D-3-HB		L-3-HB		Total 3-HB	
	Parameter	**Raw data**	**PK model**	**Raw data**	**PK model**	**Raw data**	**PK model**
Controls, n = 6 0 mg/kg	C_{max}	0.10 (0.07)	-	0.01 (0.00)	-	0.10 (0.07)	-
	AUC_{0-6}	14 (3)	-	1 (0)	-	16 (3)	-
Low dose, n = 6 1579 mg/kg	C_{max}	0.30 (0.03)[a]	0.28 (0.03)	1.88 (0.23)[a]	1.64 (0.24)	2.12 (0.23)	1.96 (0.27)
	t_{max}	85 (12)	64 (10)	135 (31)	101 (10)	135 (31)	97 (9)
	$t_{1/2}$	-	129 (93)	-	71 (6)	-	75 (25)
	AUC_{0-6}	58 (10)[a]	58 (10)	380 (55)[a]	338 (159)	438 (62)	406 (45)
	Cl_m	-	7.04 (2.25)	-	1.06 (0.11)	-	0.95 (0.14)
	V_1	-	0.42 (0.15)	-	0.04 (0.01)	-	0.04 (0.01)
	k_A	-	2.04 (1.11)	-	0.59 (0.05)	-	0.79 (0.34)
Medium dose, n = 7 3159 mg/kg	C_{max}	0.35 (0.11)[a]	0.26 (0.07)	1.92 (0.47)[a]	1.61 (0.50)	2.24 (0.50)	1.87 (0.57)
	t_{max}	167 (104)	106 (70)	227 (101)	233 (95)[b]	223 (104)	175 (87)
	AUC_{0-6}	71 (17)[a]	77 (18)	454 (140)[a]	416 (192)	526 (155)	467 (239)
High dose, n = 6 6317 mg/kg	C_{max}	0.50 (0.13)[abc]	0.36 (0.10)	1.98 (0.26)[a]	1.71 (0.41)	2.41 (0.37)	2.05 (0.45)
	t_{max}	245 (94)[b]	211 (120)[b]	340 (49)[bc]	332 (68)[b]	340 (49)	309 (80)
	AUC_{0-6}	106 (29)[abc]	107 (26)[b]	479 (87)[a]	475 (91)	585 (97)	581 (96)

Data are presented as mean (SD). For the low and medium dose group, the absorption and (part of) the elimination phases were observed, with C_{max} reflecting peak concentrations. Upon high dose D,L-3-HB, we only observed the absorption phase, with C_{max} possibly not reflecting peak concentrations. Based on the model performance, the distribution and elimination parameters could be estimated for the low dose group only. One-way analysis of variance test followed by post hoc Bonferroni's multiple comparison test was used to test the 3-HB enantiomer parameters for significant differences [a]from control data; [b]from the low dose group; [c]from the medium dose group. The results were considered significantly different if $p < 0.05$. Abbreviations (in alphabetical order): AUC, area under the concentration-time curve (min*mmol/L); Cl_m, metabolic clearance (L/h), C_{max}, maximum concentration (mmol/L); PK, pharmacokinetic; k_A, oral absorption rate constant (h^{-1}); t_{max}, time point at which C_{max} is reached (min); $t_{1/2}$, elimination half-life (min); V_1, volume of distribution to the central compartment (L/kg).

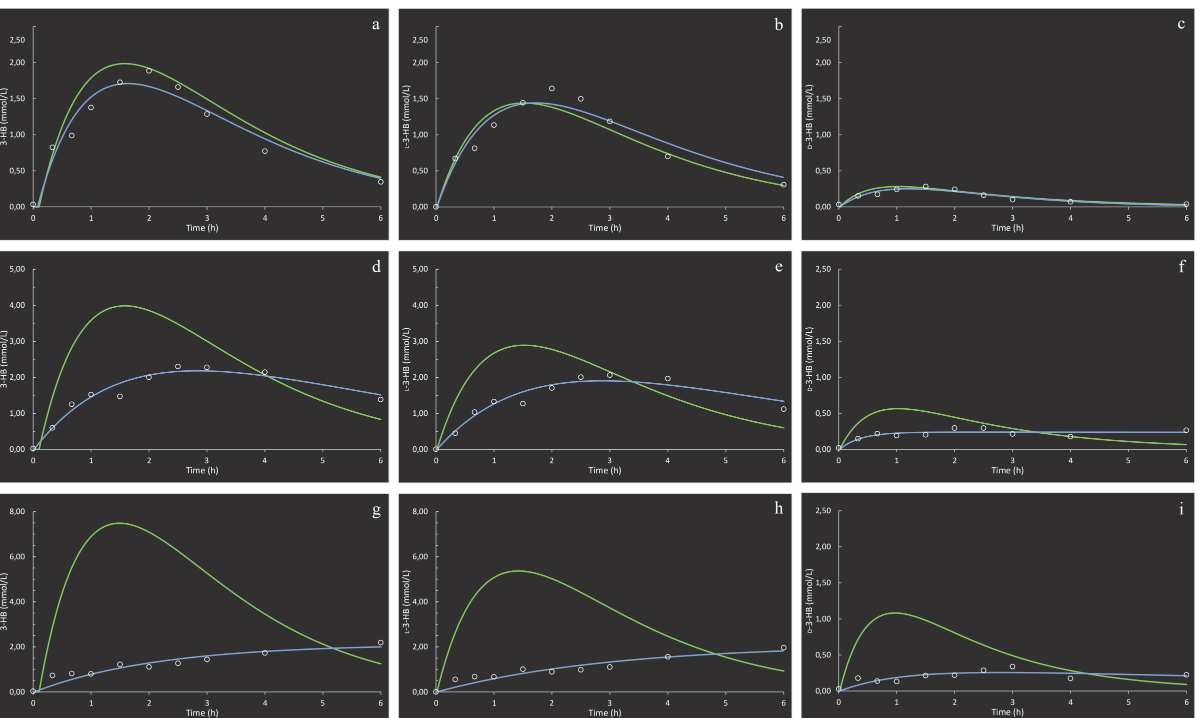

Figure 3. The final, enantiomer-specific population pharmacokinetic models after a single, oral dose of D,L-3-hydroxybutyrate in rats. The modeled blood-concentration time plots of 3-HB, L-3-HB, and D-3-HB after oral sodium D,L-3-HB at a salt-free dose 1579 mg/kg (n = 6) (a/b/c), 3159 mg/kg (n = 7) (d/e/f), and 6317 mg/kg (n = 6) (g/h/i). The green line represents the concentration-time curve from the population model. The blue line depicts the individual a posteriori Bayesian-estimated curve for one representative rat of the group, with the dots representing the measured concentrations.

The distribution of D,L-3-hydroxybutyrate

In control animals (n = 2), all tissue D-3-HB concentrations exceeded the L-3-HB concentrations (D-3-HB vs. L-3-HB brain: 1.32 (1.26, 1.39) vs. 0.15 (0.14, 0.16) µmol/g protein; in heart: 2.33 (2.02, 2.63) vs. 0.75 (0.51, 0.99) µmol/g protein; in liver: 1.85 (1.78, 1.92) vs. 0.13 (0.13, 0.13) µmol/g protein; and muscle tissue 1.81 (1.53, 2.08) vs. 0.10 (0.09, 0.10) µmol/g protein). This reversed upon a single, high dose of D,L-3-HB (n = 3) with a considerable increase of L-3-HB in all tissues, including notably in brain, whereas the increase in D-3-HB was most pronounced in heart and liver tissue, and barely noticeable in brain and muscle, as depicted in **Figure 4**.

Upon a single, high dose of D,L-3-HB, we observed an increase in D-3-HB- and L-3-HB-carnitine in heart tissue, as demonstrated in **Supplementary Figure 3**. The concentrations in brain, liver and muscle tissue were very low, and differences were therefore difficult to interpret. After D,L-3-HB administration, there appeared to be an increase of both 3-HB-carnitine esters in muscle tissue, and of L-3-HB-carnitine in brain tissue. In liver, we found no changes in D-3-HB- and L-3-HB-carnitine concentrations upon a single, high dose of D,L-3-HB. The percentage increase in D-3-HB and L-3-HB concentrations were not reflected in the relative changes in D-3-HB- and L-3-HB-carnitine in the respective tissues.

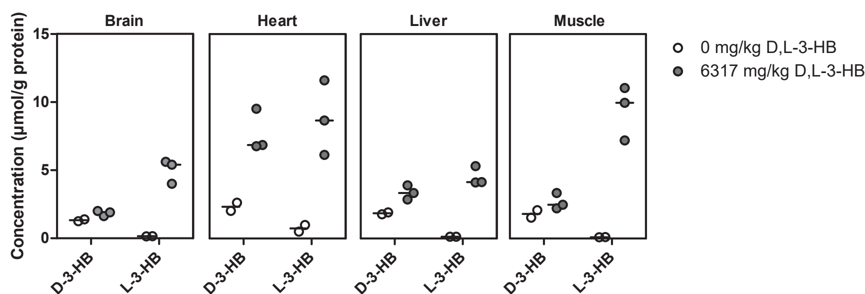

Figure 4. The enantiomer-specific tissue distribution after a single, oral dose of D,L-3-hydroxybutyrate in rats. The concentrations of D-3-HB and L-3-HB in brain, heart, liver, and muscle of control animals (n = 2) and after oral sodium-D,L-3-HB at a salt-free dose of 6317 mg/kg (n = 3). Data are presented as scatter dot plots including median values.

6

DISCUSSION

The field of KB management is rapidly expanding. Knowledge of D,L-3-HB PK and PD is essential for its further development, to enable general application, and to improve the chance of beneficial outcomes in patients. Here, we characterized the absorption and distribution of D-3-HB and L-3-HB following oral administration of racemic D,L-3-HB salt. To the best of our knowledge, this is the first UPLC-MS/MS-based enantiomer-specific PK study of D,L-3-HB.

Previous preclinical studies generated conflicting results on the capacity of D,L-3-HB salts to provoke ketonemia.[19,20] Here, we demonstrate in MADD patients and in healthy rats, that enteral D,L-3-HB salt produces ketonemia with substantially higher concentrations of L-3-HB than D-3-HB. In rats, the increment in C_{max} of L-3-HB was non-dose proportional, whereas a possible dose responsive trend could be noticed for D-3-HB. Combined with the prolonged t_{max}, this could indicate saturated absorption for L-3-HB. The heterogeneous patient PK data, with a considerably lower C_{max} of D-3-HB in the two younger patients, may imply a higher utilization and thus exogenous requirement at a younger age, although individual differences in the uptake capacity, underlying genetic defect and treatment characteristics may also play a role.[10,21] Timing of the peak 3-HB enantiomer concentrations in MADD patients appeared optimal between 30 and 60 minutes after administration. The elimination of both 3-HB enantiomers may be explained by a Michaelis-Menten process, with saturation upon ketonemia. This is substantiated by the dose-dependent kinetics in our PK models. Although confirmatory experiments are required, our findings are in line with earlier studies demonstrating a reduction in KB clearance in hyperketotic states, and non-linear elimination following administration of D-3-HB ester.[22,23] In MADD patients during chronic oral intake, we found that concentrations of D-3-HB and L-3-HB returned toward baseline concentrations after about three hours. Based on our limited patient measurements, we recommend a dosing schedule of six to eight daily administrations upon initiation of D,L-3-HB in MADD, which can subsequently be tailored to the individual patient. The place of continuous administration[6,7] and slow release preparations, for example in the form of a 1,3-butanediol- or glycerol-derived ester or a polymer[24-26], remains to be determined.

We observed low L-3-HB concentrations in blood and tissues of control animals compared to D-3-HB, with the highest content in the heart (i.e. L-3-HB made up about 10%, 10%, 24%, 6%, and 5% of the total 3-HB concentration in blood, brain, heart, liver and muscle, respectively). Whether this represents normal metabolism or is caused by hydrolysis of L-3-OH-butyryl-CoA originating from FAO cannot be determined based on our data. However, our results are in line with

previous studies.[27,28] Upon high dose D,L-3-HB, the most profound rise in D-3-HB was observed in heart and liver. The lower concentrations in brain and muscle may indicate an increased utilization of D-3-HB or a limited uptake. L-3-HB concentrations increased extensively in all tissues, with the highest concentrations in heart and muscle. Although the organ-specific effects of D-3-HB and L-3-HB remain to be elucidated, the distinct PK profiles suggest that D-3-HB and L-3-HB are differentially metabolized. **Figure 5** depicts a schematic representation of the proposed utilization routes. It can be hypothesized that upon oral D,L-3-HB administration, D-3-HB and L-3-HB are absorbed from the gut to the portal system. Transport into cells and mitochondria can occur via monocarboxylate transporters (MCT).[29] The higher increment in L-3-HB may be explained by a slower tissue import or metabolization, as has been demonstrated by labeled $^{14}CO_2$ expiration in rats.[30] As for the tissue import, enantiomer selectivity of MCT may also play a role.[31] Finally, the potential impact of hepatic first-pass metabolism on both 3-HB enantiomers remains unknown.

In mitochondrial FAO disorders, insufficient availability of *endogenous* KB renders patients vulnerable to energy deficiency and the accumulation of toxic metabolites, and furthermore impairs cholesterol synthesis, which is essential for myelin formation.[32] Administration of *exogenous* D,L-3-HB potentially bypasses the disturbed ketogenesis under these conditions. It can be hypothesized that in peripheral tissues, D-3-HB oxidation is mainly responsible for the beneficial actions of D,L-3-HB supplementation. L-3-HB may predominantly act as a substrate for sterol and fatty acid synthesis in the central nervous system, where the key enzymes responsible for its metabolism are highly expressed.[30,33] The presence of an increased concentration of L-3-HB in brain after exogenous administration indicates that this metabolite can cross the blood brain barrier and reach brain tissue, which would be necessary for a white matter therapeutic. L-3-HB-carnitine appeared to increase in heart, brain, and muscle tissue upon D,L-3-HB administration, indicating the use of L-3-HB in metabolism of these organs. Besides its roles in intermediate metabolism, endogenous 3-HB exhibits several intracellular signaling functions of which enantiomer-specificity is not fully known.[34,35] In addition to posttranslational histone modification that can modulate gene expression levels, endogenous 3-HB activates the hydroxycarboxylic acid receptor 2, which reduces lipolysis and has anti-inflammatory and neuroprotective effects.[34,35] It also inhibits the neuronal vesicular glutamate transporter 2, thereby potentially reducing excitatory glutamate neurotransmission and the generation of reactive oxygen species.[34-36] The possible downstream effects of D,L-3-HB on intercellular signaling remain to be systematically investigated. Currently, MADD patients are generally prescribed racemic D,L-3-HB salts. The most effective D-3-HB:L-3-HB ratio may depend on (organ-specific)

treatment indications. It can be hypothesized that an excess of D-3-HB might apply for fuel-derived symptoms, whereas increasing L-3-HB may be indicated for leukodystrophy. At present, the correlation between blood 3-HB enantiomer concentrations and organ-specific clinically meaningful outcomes remains to be established.

In patient 2, we demonstrated increased AcAc concentrations with a D-3-HB:AcAc ratio of below one, while it usually approximates 2:1 to 3:1.[21,37] We also found a profound rise in urinary TCA cycle intermediates in patient 1 upon higher dosing and long-term treatment with D,L-3-HB. These exploratory findings may indicate the oxidation of *exogenous* D,L-3-HB and its contribution to the TCA cycle, causing an increased flux. Other explanations for the increased excretion of TCA cycle intermediates include an inhibited renal reabsorption of carboxylic acids, or a disturbed entry of TCA intermediates into the mitochondria. Paradoxically, the risk of a dysfunctional TCA cycle upon excessive KB oxidation should also be considered. This could potentially be caused by 1) sequestration of CoA, 2) NAD^+ depletion, or 3) because KB oxidation is essentially a cataplerotic process without replenishment of TCA cycle intermediates. However, first, sequestration of CoA would lead to inhibition at the level of alpha-ketoglutarate dehydrogenase, and is therefore not expected to result in an overall increased excretion of TCA intermediates, including those after alpha-ketoglutarate. Second, D-3-HB metabolism toward acetyl-CoA has a 75% lower NAD+ consumption compared to glucose, rendering depletion less likely.[38] In fact, an increased NAD^+:NADH ratio has been proposed as a key mechanism for the beneficial effects of KB therapies.[39] Third, cataplerotic KB oxidation can be compensated by an adequate supply of anaplerotic carbohydrates and amino acids to maintain the TCA cycle flux.[40-44] Finally, D,L-3-HB supplementation has been clinically beneficial in MADD patients.[6] The potential acute harm of a dysfunctional TCA cycle therefore does not seem applicable. Nonetheless, the significance of these exploratory findings should be investigated in future studies, possibly combined with computational modeling.

Several study limitations deserve discussion. The patient measurements were exploratory. They reflect the 3-HB levels during chronic oral administration as would be typically found during its clinical use. They do not provide for the derivation of pharmacokinetic parameters after single oral dosing. Possible confounding factors, for example by the standard (dietary) treatments, have not been accounted for. In the preclinical study, first, oral gavage was not performed in two controls due to stress, and there was a low power for the tissue analyses. Second, to reflect the target population, the experiments were performed in the non-fasted state. Although the endogenous KB production is expected to be low, it may have confounded our

results.[2,35] Third, potential interconversion between D-3-HB, L-3-HB and AcAc has not been accounted for. Use of separate ^{13}C-labeled D-3-HB and L-3-HB would allow to study the enantiomer-specific 3-HB metabolism in depth. Fourth, for further validation of the PK models, it would be helpful to obtain data for > 360 minutes after D,L-3-HB administration and upon intravenous dosing to calculate the absorption rate. Finally, differences regarding the diseased versus healthy state, and chronic administration versus single dosing, prevented the comparison of the identified PK profiles in patients and rats. Execution of these studies in a preclinical model for MADD is key to investigate the consequences of endogenous KB shortage on the PK of D,L-3-HB, and to further establish the effects on clinical outcome.

In conclusion, our study provides a proof of principle on the absorption and distribution of D-3-HB and L-3-HB upon a single, oral dose of racemic D,L-3-HB. These findings will contribute to further improvement of D,L-3-HB treatment in patients with inborn errors of metabolism in which ketogenesis is disturbed, such as MADD.

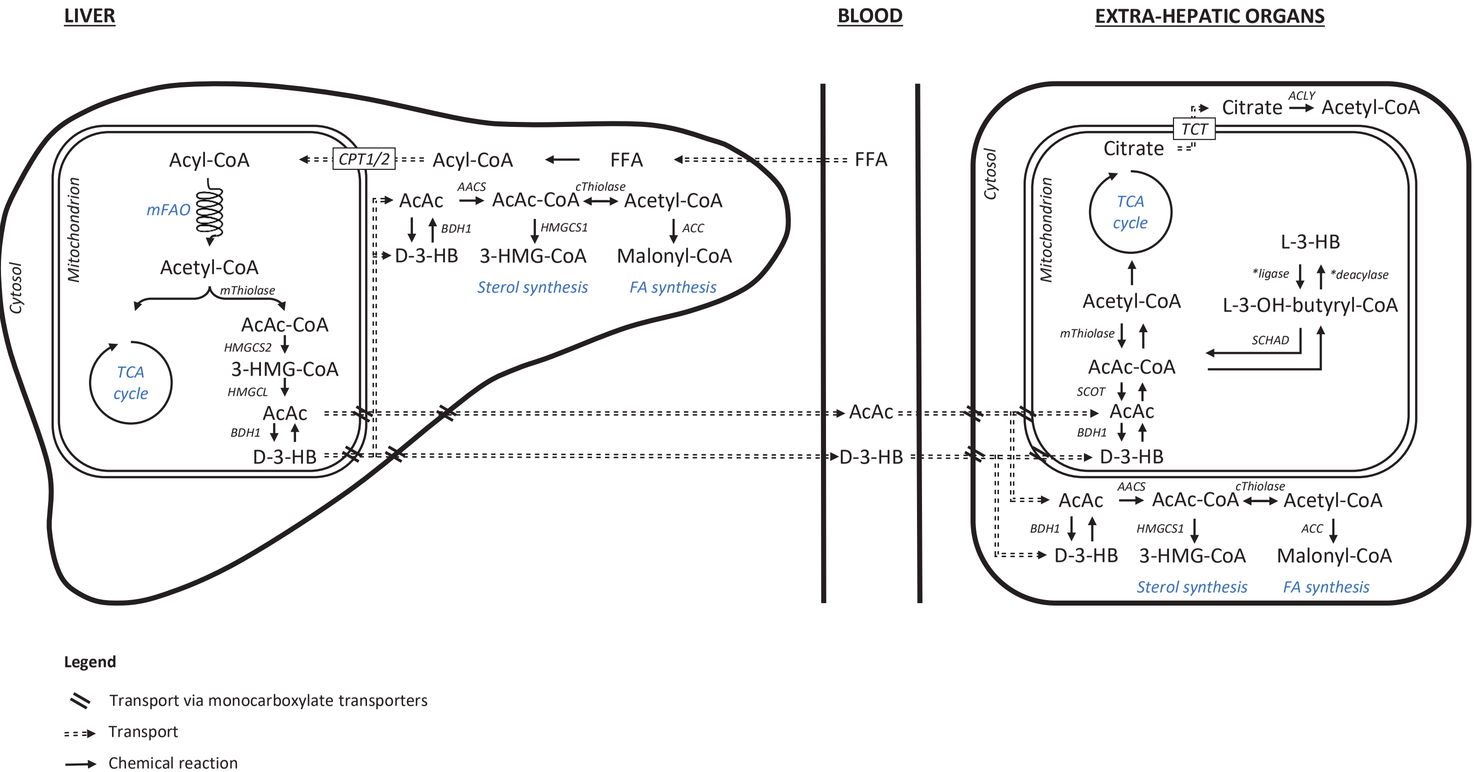

Figure 5. Schematic representation of the proposed production and utilization of ketone bodies. D-3-HB is primarily formed in the liver via the 3-hydroxy-3-methylglutaryl-CoA pathway, whereas the route of endogenous L-3-HB synthesis remains to be fully elucidated. In mitochondria, acetoacetyl-CoA can be converted to L-3-HB-CoA, and subsequently formed to L-3-HB via a specific CoA deacylase,[45] however this conversion does not appear to take place in the liver.[46] D-3-HB is oxidized to AcAc in extrahepatic tissues and subsequently reconverted to two molecules of acetyl-CoA which enter the Krebs cycle.[35] In contrast, a specific CoA ligase activates L-3-HB to L-3-hydroxybutyryl-CoA after mitochondrial import.[29] L-3-hydroxybutyryl-CoA is metabolized to acetyl CoA by short-chain acyl-CoA dehydrogenase.[33,45,47] Next to energy generation, non-oxidative fates of KB may include fatty acid and cholesterol synthesis. Abbreviations are defined on the next page.

Abbreviations (in alphabetical order): 3-HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; AcAc, acetoacetate; AcAc-Coa, acetoacetyl-CoA; AACS, acetoacetyl-CoA synthetase; ACC, acetyl-CoA carboxylase; ACLY, adenosine triphosphate citrate lyase; BDH1, D-3-hydroxybutyrate dehydrogenase; CoA, coenzyme A; CPT1, carnitine palmitoyltransferase 1; CPT2, carnitine palmitoyltransferase 2; cThiolase, cytosolic thiolase; D-3-HB, D-3-hydroxybutyrate; FA, fatty acids; FFA, free fatty acids; HMGCL, 3-hydroxy-3-methylglutaryl-CoA lyase; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; L-3-HB, L-3-hydroxybutyrate; L-3-OH-butyryl-CoA, L-3-hydroxybutyryl-CoA; mFAO, mitochondrial fatty acid oxidation; mThiolase, mitochondrial thiolase; SCHAD, short-chain 3-hydroxyacyl-CoA dehydrogenase; SCOT, succinyl-CoA-3-oxoacid-CoA transferase; TCA, tricarboxylic acid; TCT, tricarboxylate transport.

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SUPPLEMENTARY APPENDIX

Supplementary Data 1
Supplementary Data 2
Supplementary Data 3
Supplementary Data 4
Supplementary Table 1
Supplementary Table 2
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3

Supplementary Data 1. Summarized patient details and treatment characteristics.
We identified three MADD-patients that received enteral D,L-3-HB (i.e. oral or gastric) based on the best published clinical evidence.[1-3]

Indications for D,L-3-HB treatment in patient 1, with neonatal onset MADD, included cardiomyopathy, leukodystrophy and myopathy. She suffered from homozygous mutations in ETFA (c.1-40G>A). From the age of seven months, racemic sodium-D,L-3-HB (Sigma-Aldrich) was introduced at a salt-free D,L-3-HB dose of 369 mg/kg/day (i.e. approximately 82% of the sodium-D,L-3-HB dose of 450 mg/kg/day) which was increased to 738 mg/kg/day (sodium-D,L-3-HB dose of 900 mg/kg/day) after one week. The maximum salt-free dose during the course of treatment was 2132 mg/kg/day (sodium-D,L-3-HB dose of 2600 mg/kg/day). This dose was gradually weaned to 1123 mg/kg/day (sodium-D,L-3-HB dose of 1370 mg/kg/day), divided in five daily doses at the time of blood sampling.

In patient 2, D,L-3-HB treatment was started on the eight day of life for cardiomyopathy and leukodystrophy. Molecular analysis identified compound heterozygous mutations in ETFDH (c.121C>T and c.1117T>C). The infant received a mixed salt solution (sodium, potassium, calcium and magnesium) of racemic D,L-3-HB (Trumacro™ Nutrition), initially at a salt-free D,L-3-HB dose of 736 mg/kg/day (i.e. approximately 66% of the D,L-3-HB salt solution dose of 1115 mg/kg/day) provided in doses every three hours, and later 896 mg/kg/day (D,L-3-HB salt solution dose of 1357 mg/kg/day).

Patient 3 suffers from later onset MADD with compound heterozygous mutations in ETFDH (c.51dupT and c.940G>A).[3] From the age of four years and three months, racemic sodium-D,L-3-HB (Veriton Pharma, Fischer Scientific) was introduced at a salt-free D,L-3-HB dose 410 mg/kg/day (sodium-D,L-3-HB dose of 500 mg/kg/day) because of leukodystrophy with subsequent regression of the lesions. At the age of 18 years, the treatment was dosed at 820 mg/kg/day (sodium-D,L-3-HB dose of 1000 mg/kg/day) divided in four daily doses.

Supplementary Data 2. The conversion between human and animal doses of D,L-3-hydroxybutyrate based on endogenous ketone body production rates.
To allow correct data interpretation, the doses are described in salt-free amount (i.e. approximately 82% of the sodium-D,L-3-HB dose). Animal doses were based on the most commonly prescribed D,L-3-HB doses in MADD-patients of 369 mg/kg, 738 mg/kg, and 1476 mg/kg (i.e. 450 mg/kg, 900 mg/kg, and 1800 mg/kg sodium-D,L-3-HB). Human doses were converted to rat doses on the basis of endogenous ketone body production rates as determined by D-(-)-3-hydroxy[4,4,4-2H3]butyrate tracer studies.[4,5] In neonates and infants, the endogenous ketone body production approximates 18.6 µmol/kg/min upon 3.5 to 8.5 hours of fasting.[5] In overnight-fasted Wistar rats, endogenous KB production rate is circa 17.9 µmol/~225g/min, i.e. 79.6 µmol/kg/min.[4] Based on these turnover rates, a conversion factor of *4.28 was calculated. This resulted in the corresponding animal doses of salt-free D,L-3-HB of 1579 mg/kg (low dose; i.e. sodium-D,L-3-HB dose of 1926 mg/kg), 3159 mg/kg (medium dose; i.e. sodium-D,L-3-HB dose of 3852 mg/kg), and 6317 mg/kg (high dose; i.e. sodium-D,L-3-HB dose of 7704 mg/kg).

Based on allometric conversion between animal and human doses, the used animal doses would be equivalent to circa 653 mg/kg, 1307 mg/kg, and 2614 mg/kg salt-free D,L-3-HB for the low, medium and high dose respectively, for an infant weighing 6.0 kg.[6]

Supplementary Data 3. The procedures for sample preparation.
Blood samples
Immediately after collection, 100 µL whole-blood from the EDTA cups was deproteinized with an equal volume of 1.0 M perchloric acid. The solution was gently mixed by finger tapping, stored on ice and centrifuged at 1700 xg for 10 minutes at 4 ˚C. The supernatant was centrifuged again under the same conditions, pipetted off and stored at -80 ˚C for 3-HB enantiomer analysis. For patients 2 and 3, plasma was obtained from EDTA and lithium-heparin cups, respectively, and stored at -80 °C during shipment and until further processing for the 3-HB enantiomer analysis. The samples for the analysis of AcAc in patient 2 were immediately deproteinized upon arrival in the laboratory, stored at -70 ˚C until shipment to the analyzing laboratory on dry ice, where they were also stored at -70 ˚C until analysis which occurred within one week..

Tissue samples
Collected tissue samples were rapidly freeze-clamped in liquid nitrogen and stored at -80 ˚C. Frozen tissue samples were crushed in liquid nitrogen. Homogenates were prepared by adding phosphate-buffered saline solution (pH 7.4) to frozen powder aliquots in 20% weight:volume

ratio. The homogenates were sonified at 11-12 W for 30 seconds (pulse time of 1 second), with the tube immersed in ice. Subsequently, the supernatants were collected after 10 minutes of centrifugation at 12800 rpm at 4 ˚C. Protein concentrations were analyzed using a Pierce BCA protein assay kit. From the remaining sample, 100 µL was deproteinized using an equal volume of 1.0 M perchloric acid. The subsequent work-up was identical as described for the blood samples. Supernatants were stored at -80 ˚C for 3-HB enantiomer analysis.

For the analysis of the concentrations of D-3-HB- and L-3-HB-carnitine in the collected tissue samples, homogenates were prepared by adding 0.9% NaCl to powder aliquots which were subsequently sonified with a tip sonicator. The protein concentration of the homogenate was measured with a Pierce BCA protein assay kit and analyzed essentially as described previously[7], and the D-3-HB and L-3-HB-carnitine levels were calculated using a C4-carnitine internal standard and expressed micromole per gram of protein.

Supplementary Data 4. Specification of the population pharmacokinetic model development.

Modeling was performed using MW/Pharm pharmacokinetic software version 3.86 (MwPharm, Zuidhorn, the Netherlands).[8] The initial models were derived by automated linear curve stripping of data from a rat in the lowest dose group with the KinStrip module. All curves could subsequently be adequately fitted in a non-linear one compartment model in the KinFit module, with estimates for the linear metabolic clearance (Cl_m), oral absorption rate constant (k_A), volume of distribution of central compartment (V_1), and absorption lag-time (t_{lag}; for total 3-HB only). Since there was no data available upon intravenous D,L-3-HB administration, the bioavailability (F) was fixed at 1. Essay error was set at the level of 0.05. The initial models were further developed into population models by non-Bayesian fitting, followed by Iterative Two-Stage Bayesian analysis. The concentration-time, dosing, and weight data were used from the low dose group only, as the medium and high dose group did not have sufficient data on both the absorption and elimination phases.

The model performance was evaluated in each developmental step on the basis of the Akaike Information Criterion (AIC), weighted sum of squares of the residuals divided by the degrees of freedom (ΣWSS/df) and visual inspection of the overall goodness-of-fit. The model performance was assessed as adequate for the low dose group only. The models were therefore unfit to estimate the distribution and elimination parameters for the medium and high dose groups.

References to Supplementary Data 1-4

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Supplementary Table 1. Observations in three patients with multiple acyl-CoA dehydrogenase deficiency after an oral dose D,L-3-hydroxybutyrate.

	Salt-free D,L-3-HB dose (mg/kg/day)	Time (min)	D-3-HB (mmol/L)	L-3-HB (mmol/L)	Total 3-HB (mmol/L)	AcAc (mmol/L)
Patient 1	1123 in 5 daily doses	-20	0.129	0.492	0.621	ND
		60	0.187	0.521	0.708	
Patient 2	736 in 8 daily doses	0	0.050[a]	0.329	0.379	0.02
		30	0.214	0.892	1.106	0.09
		60	0.145	0.846	0.991	0.09
		90	0.086	0.692	0.778	0.27
		120	0.074	0.651	0.725	0.50
		180	0.050[a]	0.400	0.450	0.10
	896 in 8 daily doses	0	0.050[a]	0.305	0.355	0.17
		40	0.289	1.666	1.955	0.40
		80	0.050[a]	1.074	1.124	0.37
Patient 3	820 in 4 daily doses	0	0.125	0.050[a]	0.175	ND
		30	0.617	0.783	1.400	
		60	1.538	2.384	3.922	
		90	1.130	2.326	3.456	
		150	0.327	1.725	2.052	
		180	0.050[a]	0.726	0.776	

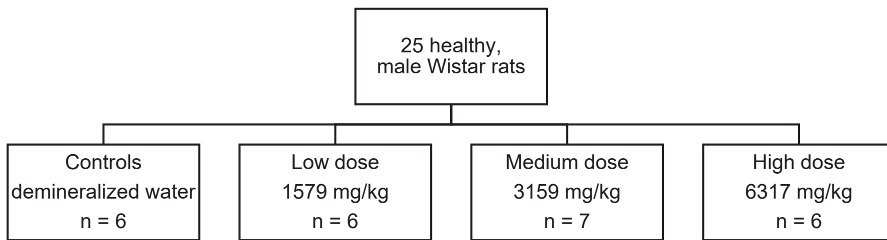
The concentrations of 3-HB, D-3-HB and L-3-HB, determined in supernatants of deproteinized blood for patient 1 and in plasma for patient 2 and 3, after an oral dose of D,L-3-HB. Acetoacetate concentrations were only measured in patient 2. [a]For measurements of <0.050, a cutoff value of 0.050 was used. Patient 1 received a salt-free D,L-3-HB dose of 1123 mg/kg/day (sodium-D,L-3-HB dose of 1370 mg/kg/day; body weight 17.3 kg; 5 years old). Patient 2 received a salt-free D,L-3-HB dose of 736 mg/kg/day (D,L-3-HB salt solution dose of 1115 mg/kg /day; body weight 4.5 kg; 3 months old), and later 896 mg/kg/day (D,L-3-HB salt solution dose 1357 mg/kg/day; body weight 5.2 kg; 4 months old). Patient 3 received 820 mg/kg/day of salt-free D,L-3-HB (sodium-D,L-3-HB dose of 1000 mg/kg/day; body weight 40.0 kg; 16 years old). Abbreviations: ND, not determined.

Supplementary Table 2. Variables of the final enantiomer-specific population pharmacokinetic models after a single, oral dose of D,L-3-hydroxybutyrate in rats.

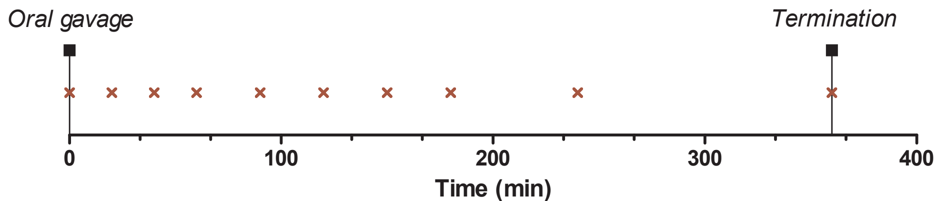
Population parameter	D-3-HB	L-3-HB	Total 3-HB
Cl_m	8.27 (3.02)	1.29 (0.13)	0.95 (0.13)
V_1	0.39 (0.16)	0.04 (0.01)	0.04 (0.01)
k_A	1.74 (1.19)	0.59 (0.05)	0.79 (0.35)
T_{lag}	-	-	0.09 (0.08)
F	100 (fixed)	100 (fixed)	100 (fixed)

Data are presented as mean (SD). Abbreviations (in alphabetical order): Cl_m, metabolic clearance (L/h), F, biological availability (%), k_A, absorption constant of oral dose (h^{-1}), T_{lag}, lagtime (h), V_1, volume of distribution of central compartment (L/kg).

A)

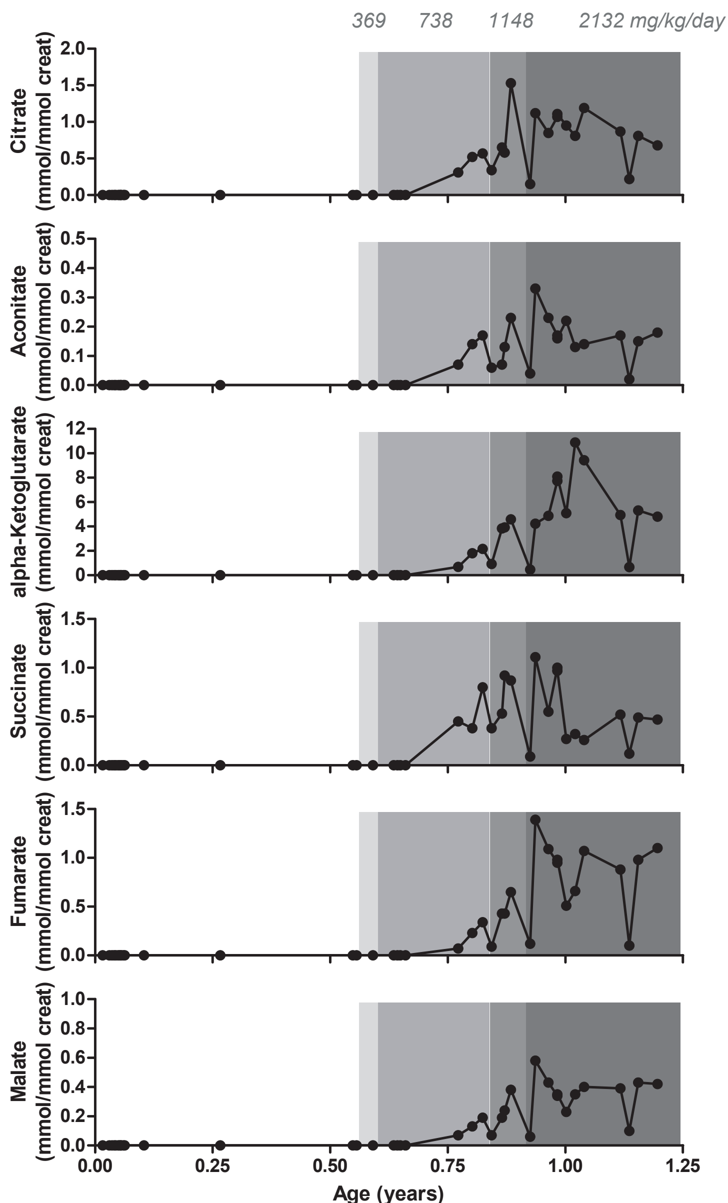


B)

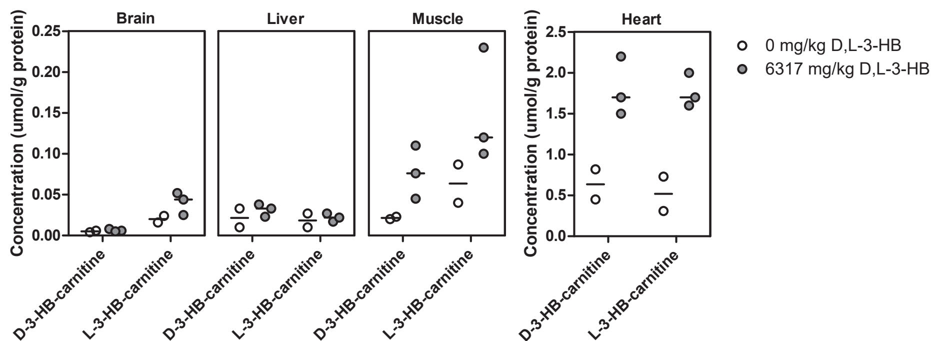


Supplementary Figure 1. Flowchart and timeline diagram of the experimental design of the animal study. The animals were randomly assigned to three experimental groups and one control group, as depicted in the flowchart (A). The timelime diagram (B) demonstrates a schematic representation of the experimental design. x = collection of blood sample via tail vein nick bleed. Upon termination, brain, heart, liver, and muscle tissue were collected in 10 to 15 minutes.

6



Supplementary Figure 2. Urinary excretion of tricarboxylic acid cycle intermediates upon treatment with D,L-3-hydroxybutyrate in a patient with multiple acyl-CoA dehydrogenase deficiency. Concentrations of tricarboxylic acid cycle intermediates in urine during the course of D,L-3-HB treatment in patient 1. Shaded areas represent the salt-free D,L-3-HB doses between 369 and 2132 mg/kg/day (i.e. sodium-D,L-3-HB dose of 450 to 2600 mg/kg/day).



Supplementary Figure 3. The enantiomeric-specific tissue distribution of 3-hydroxybutyrylcarnitine after a single, oral dose of D,L-3-hydroxybutyrate in rats. The concentrations of D-3-HB- and L-3-HB-carnitine in brain, liver, muscle and heart of control animals (n = 2) and after oral sodium-D,L-3-HB at a salt-free dose of 6317 mg/kg (n = 3). Data are presented as scatter dot plots including median values.

7

General discussion and future perspectives

Multiple acyl-CoA dehydrogenase deficiency (MADD; also known as glutaric aciduria type II; online mendelian inheritance in man #231680) is currently not included in the Dutch newborn blood spot screening (NBS) program due to a lack of documented health gain upon early detection.[1,2] Several other NBS programs screen for MADD, but limited data is available on the outcome of patients identified by NBS, and none include the perspective of treatment with D,L-3-hydroxybutyrate (D,L-3-HB, a ketone body) salts.[3-6] MADD is also an exemplary inborn error of metabolism (IEM) that can escape identification due to nonspecific symptoms and unexpected childhood death. The main objectives of this thesis were twofold:

1. *to study which IEMs are associated with unexpected death in early childhood, and how their detection through acylcarnitine profile analysis can be improved*; and,
2. *to provide evidence to re-evaluate the possibility of population NBS for MADD in The Netherlands*.

The impact of this thesis' outcomes and the perspectives for future research are discussed here. Part I focuses on the metabolic differential diagnosis of unexpected childhood death within the context of treatability and detectability by tandem mass spectrometry (MS/MS), and discusses parameters that can affect acylcarnitine profiles. In part II, the eligibility of NBS for MADD in The Netherlands is explored according to three main principles of early disease detection as described by Wilson and Jungner: the disease, the treatment and the test.[7]

PART I – Inborn errors of metabolism and unexpected childhood death

The metabolic differential diagnosis of unexpected death in early childhood

In **chapter 2**, we reported 43 IEMs that are associated with a risk of unexpected death in infancy, of which 26 can already present during the neonatal period. Treatment is available for at least 32 of the IEMs, and a minimum of 26 are detectable by MS/MS analysis of metabolites in DBS. The identified IEMs mostly concern disorders of mitochondrial fatty acid oxidation (FAO), urea cycle defects and organic acidurias. At least ten IEMs were identified that are currently not included in the Dutch NBS program, despite treatment options being available and detectability by MS/MS analysis in DBS (i.e. 3-hydroxyacyl-CoA dehydrogenase deficiency (also known as short chain 3-hydroxyacyl-CoA dehydrogenase deficiency), alpha-methylacetoacetic aciduria (also known as beta-ketothiolase deficiency), argininosuccinic aciduria, carbamoyl phosphate synthetase 1 deficiency, carnitine-acylcarnitine translocase deficiency, carnitine palmitoyltransferase 2 (CPT2) deficiency, citrullinemia type 1,

glycogen storage disease type 2 (also known as Pompe disease), 3-methylglutaconic aciduria type 1, ornithine transcarbamylase deficiency). A neonatal presentation has been reported in at least six of these IEMs. Besides, supported by the evidence described in **chapter 5** and based on the concept of treatability adhered in the most recent recommendation of the Health Council of The Netherlands1 (see part II of this discussion), MADD may be added to this list. This information has important implications for the improvement of population NBS programs and postmortem diagnostic protocols. Our findings likely also contribute to an increased awareness among neonatologists and pediatricians that IEMs can cause unexpected death in early childhood.

It remains difficult to quantify the risk of unexpected childhood death due to IEMs. A recent meta-analysis estimated that IEMs account for 0.4% of all child deaths.[8] As stated in the general introduction of this thesis, IEMs can remain undiagnosed because of nonspecific clinical presentations combined with the respective disorder not being included in the population NBS program. Extensive metabolite screening, including acylcarnitine and amino acid analysis, therefore forms an important part of diagnostic protocols upon unexpected childhood death, in addition to next generation sequencing and, preferentially, obtainment of material such as skin fibroblasts for functional studies. The combination of clinical and biochemical phenotyping and DNA-sequencing techniques may harbor the best diagnostic yield.[9] In collaboration with the National Institute for Public Health and the Environment (i.e. 'Rijksinstituut voor Volksgezondheid en Milieu' (RIVM) in Dutch), we are currently performing a national, retrospective cohort study to determine the prevalence of (treatable) IEMs in children who died in early childhood. To this aim, we measured acylcarnitine and amino acid profiles in stored DBS of 1,569 deceased children (birth cohorts 2013 to 2017). Preliminary results concern several metabolite profiles that are suspect for IEMs including carnitine transporter deficiency, citrullinemia, glutaric aciduria type I, methylmalonic or propionic aciduria, peroxisomal disorders, and tyrosinemia.

In some IEMs, symptoms and signs including death may already occur before the NBS test results become available or even before the blood required for testing has been drawn. This is especially relevant in areas where the NBS sample is collected relatively late in life, such as in The Netherlands, where this is often combined with the newborn hearing screening (i.e. 96-168 hours after birth).[10] This causes a timeframe of at least four days for the potential clinical manifestations or even death before the NBS sample is obtained. In fact, in 2018, 371 neonates died before they could participate in the Dutch NBS program.[11] Since IEMs are mostly autosomal recessive disorders, there is a recurrence risk within families of at least 25%. We advocate that every child deserves participation in a population NBS

program, even after death, using a peri- or postmortem sample. Prompt metabolite analysis combined with the obtainment of appropriate specimens for further diagnostics[12], may in some cases perhaps even avoid the need for an autopsy.

The timing of the Dutch NBS program might warrant a second, earlier screening moment. In fact, many other population NBS programs in Europe and the United States of America aim to obtain the NBS sample already between 48-96 hours[13] and 24-48 hours[14] after birth, respectively; considerably earlier than in the Netherlands. Of course, in this respect, other aspects of NBS programs, such as the interval between sampling and analysis, and the timeliness referral to a metabolic pediatrician, also need to be considered.[13] Timely screening is particularly relevant for children at an increased risk of IEMs. To this aim, we reported our perinatal protocol for neonates at risk of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, which involves instructions regarding monitoring of the newborn and a feeding regimen combined with recommendations for metabolite analysis in DBS from cord blood and DBS obtained on the first and second day of life prior to feeding.[15] Although not formally studied, parents reported to us that the protocol importantly reduces the stress about the diagnosis and feeding regimen while awaiting the results of the national NBS program. Several other case studies have reported the use of cord blood for the detection of IEMs in high-risk neonates (e.g. 3-methylcrotonyl-CoA carboxylase deficiency[16], CPT2 deficiency[17,18], holocarboxylase synthetase deficiency[19], isovaleric acidemia[20], long-chain 3-hydroxyacyl-CoA dehydrogenase/mitochondrial trifunctional protein deficiency[21], and MCAD deficiency[16,20]). Contrarily, IEMs can also be misinterpreted because the associated metabolites are not yet elevated in cord blood samples (e.g. 3-hydroxy-3-methylglutaryl-CoA lyase deficiency, argininosuccinic aciduria, maple syrup disease, methylmalonic aciduria, phenylketonuria), or may be influenced by maternal (nutritional and/or disease) status (e.g. 3-methylcrotonyl-CoA carboxylase, carnitine transporter deficiency).[16,22] Use of (sequential) acylcarnitine ratio analysis or postanalytical tools may bypass these problems. Prospective studies should focus on the sensitivity and specificity of this technique to provide an early indication of IEMs.

Metabolic stress and long-term storage can affect acylcarnitine profiles

It is important to realize that metabolite profiles are influenced by various factors including the feeding state, endogenous metabolism, sample type and (pre-) analytical conditions. In **chapter 3a** and **3b**, we reported that metabolic stress and long-term storage at room temperature can have a significant impact on acylcarnitine concentrations. This applies to more analytes used in population NBS, such as amino acids, endocrine- and enzyme markers.[23,24] Our results emphasize the importance

of obtaining background information on samples. This improves the interpretation of potential deviations and can reveal subtle changes that would otherwise not have been discovered. To improve sample integrity upon long-term storage, DBS should preferably be stored at low humidity (< 30%) and under frozen condition (≤ -20 C°).[23-25]

In view of metabolite analysis in DBS of deceased infants and children, it is important to consider the impact of peri- or postmortem sample collection on the metabolite profiles. Postmortem DBS are characterized by an increase in free carnitine, short-chain- and hydroxybutyryl-carnitines, and many amino acids.[26] Reference ranges have been established for acylcarnitine concentrations, whereas for amino acids the relative changes can indicate an IEM.[26,27] This enabled the presumptive diagnosis of mitochondrial FAO and organic acidurias in several cases.[26,28,29]

PART II – Toward population newborn screening for multiple acyl-CoA dehydrogenase deficiency

THE DISEASE

The natural history

The natural history of a disease can be defined as follows: "*The course a disease takes in the absence of intervention in individuals with the disease, from the disease's onset until either the disease's resolution or the individual's death.*"[30(p2)] It aims to establish factors that correlate with disease progression and outcome, and includes data on genotypes, phenotypes, morbidity, mortality, and biomarkers.[30] **Chapter 4** adds important knowledge about the natural history of MADD. Some additional data is featured below. As stated in the general introduction, MADD patients are historically classified into three subtypes: neonatal onset patients with or without congenital anomalies (type I and II, respectively) and patients with a later, relatively milder onset (type III).[31] **Figure 1** summarizes genetic variants associated with MADD, classified to the historical subtypes. Genetic variants expected to have a large effect on protein function (e.g. nonsense and stop-loss variants, deletions, insertions, duplications, and splicing defects) predispose for neonatal onset MADD.

Many neonatal onset patients develop symptoms within the first week of life. **Figure 2A** shows that the age at onset in type III MADD extends beyond 65 years, although half of these patients display first symptoms before the age of 20

years. The organ system involvement differs by MADD subtype, as demonstrated in **Figure 2B**. Severe symptoms as leukodystrophy and cardiomyopathy are not limited only to type I and II MADD. **Figure 2C** shows that cardiomyopathy seems to develop relatively early in life, whereas the onset of cardiac arrhythmias and leukodystrophy can extend to adulthood. The minimal case fatality rate of MADD ranges from approximately 90% and 78% in type I and type II MADD, respectively, to 3% in type III MADD, as depicted in **Figure 2D**. The case fatality rate in type II MADD decreased to 38% upon treatment with D,L-3-HB.

Given the phenotypical overlap between the historical subtypes, it can instead be considered to classify the spectrum of MADD patients according to a severe (neonatal onset; multi-organ involvement), intermediate (infantile- or early childhood onset; mainly hepatic presentation, sometimes combined with other organ systems) and myopathic form (school age- or adult onset, sometimes combined with hepatic dysfunction), similar as described for other long-chain FAO disorders[32], and as previously proposed for MADD by Yamada et al.[33]. Some patients may remain asymptomatic because they have a mild defect or because they have not been exposed to the required amount of metabolic stress.[32] This complicates the determination of an overall treatment and monitoring strategy, as both overtreatment and undertreatment should be avoided.

Disease severity prediction

The spectrum of MADD patients challenges phenotype prediction based solely on genotypes or age at onset. Prognostic markers that distinguish patient subtypes and provide guidance for treatment and monitoring regimens, are of utmost importance, especially in view of population NBS. In **chapter 4**, we found that functional studies in fibroblasts differentiate neonatal from later onset MADD. Our results suggest that a low FAO flux, combined with particularly high [U-^{13}C]C16-carnitine and low [U-^{13}C] medium- and [U-^{13}C]short-chain acylcarnitine concentrations upon palmitate loading, indicating an almost complete block of FAO, are associated with the development of severe symptoms as leukodystrophy and cardiomyopathy. Additionally, the high concentration of (unlabeled) C5-carnitine in neonatal onset patients suggests a profound deficiency of isovaleryl-CoA dehydrogenase. Although FAO flux activities varied from normal to mildly decreased combined with normal to (mildly) increased acylcarnitine concentrations of variable chain lengths in later onset MADD patients, these measurements may be temperature sensitive.[34] It can be hypothesized that an increased body temperature, for example during intercurrent illness, causes a drop in FAO flux activity which induces a risk of symptom development.

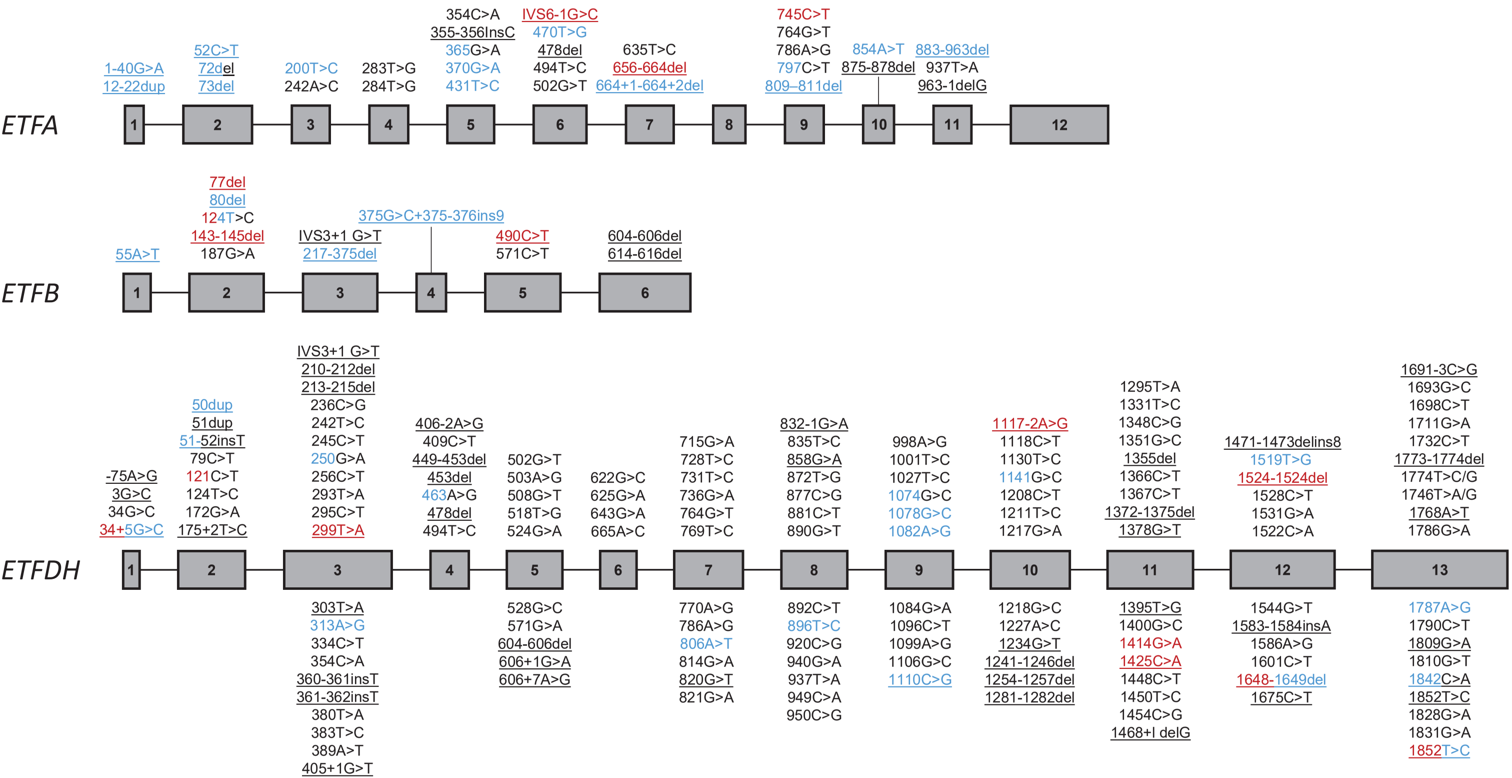


Figure 1. Genotypes of patients with multiple acyl-CoA dehydrogenase deficiency according to their historical subtypes. The data was collected from the findings described in chapter 4 and 5. Historical subtypes are depicted (in part) in red (i.e. MADD type I), blue (i.e. MADD type II), and black (i.e. MADD type III). Genetic variants expected to have a large effect on protein function are underlined.

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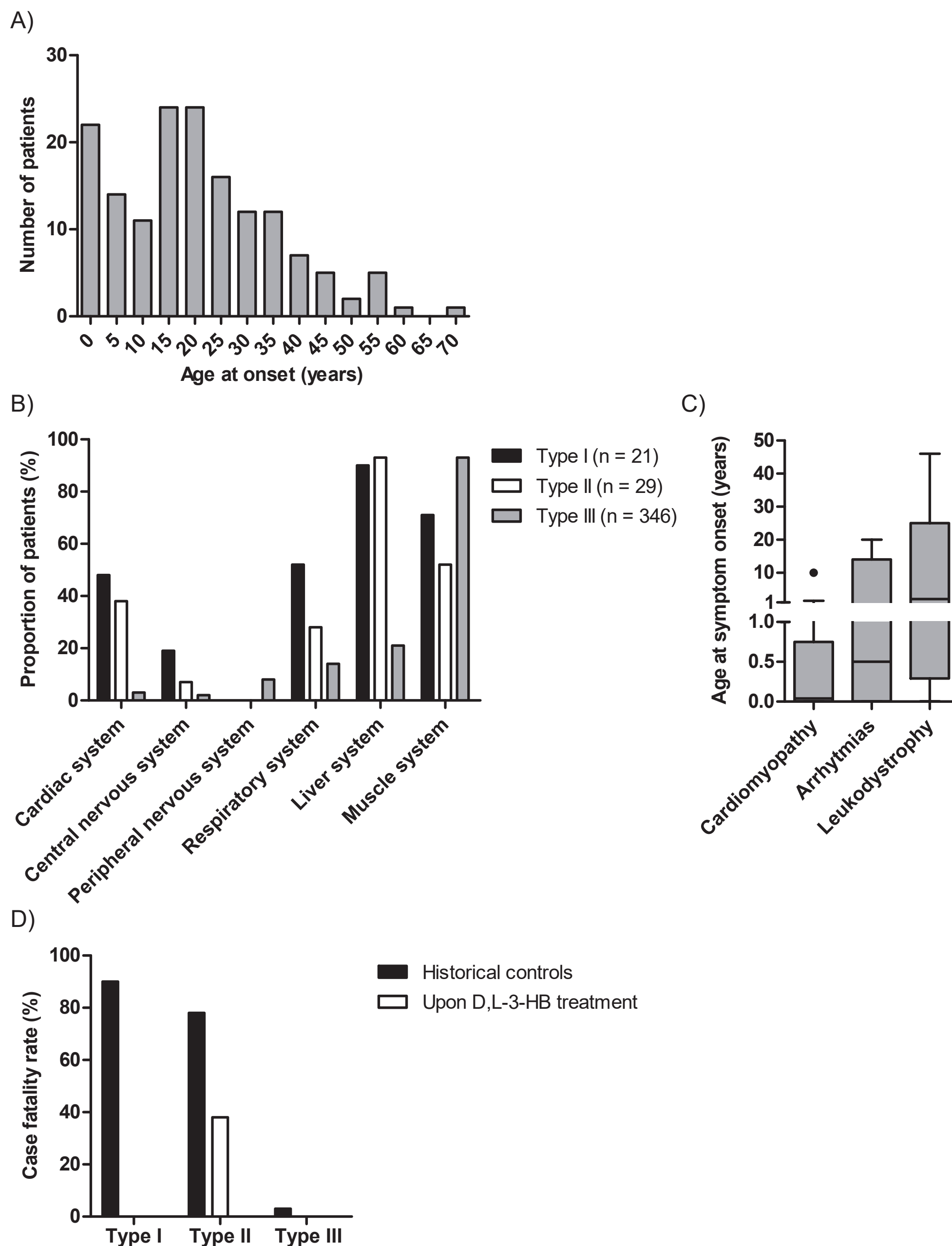

Figure 2. Phenotypes of patients with multiple acyl-CoA dehydrogenase deficiency. The data was reanalyzed, as previously collected in the literature meta-analysis described in chapter 4. A) The age at onset in type III MADD patients (n = 156). B) The organ system involvement according to MADD subtype. C) The age at onset of cardiomyopathy (n = 17), arrhythmias (n = 7) and leukodystrophy (n = 7). D) The case fatality rate -defined as the number of deaths compared to the total number patients- in historical controls not reported to have been treated with D,L-3-HB (type I: 19/21; type II: 21/27; and type III: 10/344) compared to the case fatality rate in MADD patients treated with D,L-3-HB (type II: 3/8; type III: 0/11) described in chapter 5.

Figure 3 presents a flowchart that can provide a starting point for phenotype prediction within the context of the proposed classification of severe, intermediate and myopathic MADD. Similar as for very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, the predicted phenotype can be used as a basis for an individualized treatment strategy for (newly identified) MADD-patients, as will be discussed in the final section of this general discussion.[35]

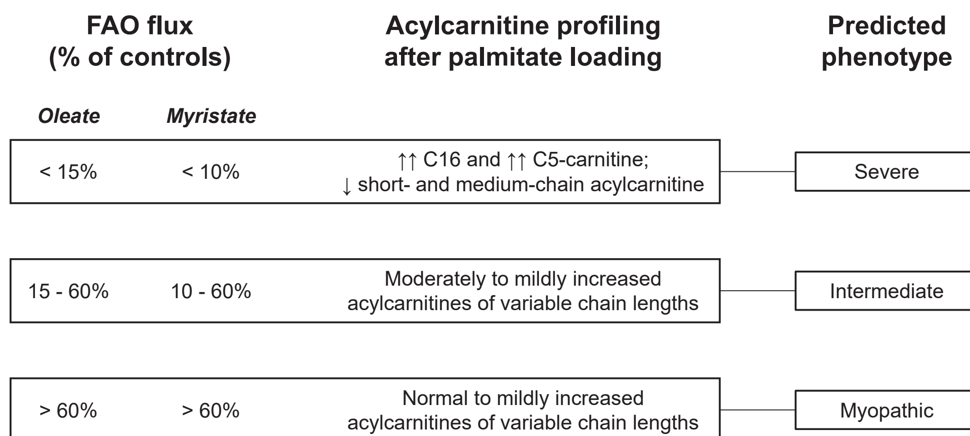

Figure 3. Proposed flowchart for phenotype prediction in multiple acyl-CoA dehydrogenase deficiency based on functional studies in fibroblasts. The proposed cut-off values are based on the data described in chapter 4. The outcomes of acylcarnitine profiling after palmitate loading concern [U-^{13}C]-labeled acylcarnitines and unlabeled C3- and C5-acylcarnitine, as reported in chapter 4. Abbreviation: FAO, fatty acid oxidation.

Drawbacks of these fibroblast assays involve the need for an invasive skin biopsy and the concomitant turnaround time of at least four weeks due to fibroblast culturing conditions. Future studies should investigate whether our results can be replicated in more readily available samples such as isolated lymphocytes[36], peripheral blood cells[37,38], or whole blood[39-42]. It would also be interesting to examine the activities of flavin adenine dinucleotide-dependent acyl-CoA dehydrogenases. Besides, the MADD phenotype discriminating capacities of [U-^{13}C]C16-, C5-, and [U-^{13}C]C2-carnitine upon palmitate loading in fibroblasts may be extrapolated to acylcarnitine profiles in plasma and DBS, although the amount of metabolic stress experienced at the time of blood sample obtainment may form a confounding variable.[32 33,43] Samples from different laboratories can be compared using Z-scores to overcome interlaboratory, analytical differences.[44] Additionally, in vivo stable isotope-labeled tracer studies can provide an interesting approach to evaluate a patient's FAO and amino acid metabolism.

7

Monitoring of disease progression

Monitoring strategies vary widely between centers and may depend on the availability of techniques. Metabolite studies are regularly used during follow-up, but it is questioned whether these metabolite profiles correlate with a patient's health status. To facilitate uniform, clinical description of disease severity, we developed an MADD-disease severity scoring system (MADD-DS3) in chapter 4. The MADD-DS3 does not require assessments beyond standard patient care and includes the patient's/parent's perception on disease burden, an approach that is increasingly recognized within the field of IEMs. Upon validation, for example using the Clinical Global Impressions scale[45,46], it may provide a standardized method to observe a patient's (organ-specific) clinical trend. The strictness and extent of the monitoring can be adapted to the individual patient based on the predicted phenotype. Nonetheless, establishment of a baseline health status can be of importance to any patient.

THE TREATMENT

In the most recent recommendation of the Health Council of The Netherlands, the concept of treatability is described as follows: "*A condition is considered untreatable when the scientific literature does not allow reliable conclusions about an anticipated favorable effect of medical intervention of a relevant size on clinical outcome measures, meaning mortality, morbidity or quality of life.*"[1(p24)] The available regular treatment options are generally sufficient for intermediate to myopathic MADD.[43,47,48] However, the lack of systematic evidence for the health benefit of D,L-3-HB in severely affected MADD patients was a major complicating factor for the inclusion of MADD in the Dutch NBS program. This also limited the clinical application, availability, and reimbursement of D,L-3-HB treatment in MADD.

The efficacy and safety of D,L-3-hydroxybutyrate

In **chapter 5**, we summarized data on the clinical presentation, treatment method and physician reported outcome in 23 MADD(-like) patients upon treatment with D,L-3-HB salt. Compared to data from historical controls, there was a considerably improved survival upon D,L-3-HB treatment, as also depicted in Figure 2D. Clinical improvement was reported in 70% of the patients for cardiomyopathy, leukodystrophy, liver symptoms, muscle symptoms, and respiratory failure.

D,L-3-HB may target the pathophysiology and clinical manifestations of MADD through several mechanisms. Insufficient levels of endogenous KB render patients vulnerable to energy deficiency and the accumulation of toxic metabolites,

and also impair cholesterol synthesis, required for myelination.[49] Besides, intracellular signaling function can modulate gene expression levels, reduce lipolysis, and decrease glutamate excitotoxicity and the generation of reactive oxygen species.[50-52] Administration of exogenous D,L-3-HB may bypass the disrupted ketogenesis. The potential scope of D,L-3-HB indication extends to all IEMs in which ketogenesis is disturbed, as described in **chapter 5**, and as an additive for patients using a ketogenic diet. The magnitude of the exogenous KB requirement in individual patients likely depends on the severity of the metabolic block and may vary throughout development.[53,54]

Upon D,L-3-HB administration, side effects occurred in 35% of the MADD(-like)-patients, and included abdominal pain, constipation, dehydration, diarrhea, and vomiting or nausea. The risk of side effects appeared to increase with dose. Nonetheless, they never formed a reason to discontinue D,L-3-HB in case of reported efficacy. It was also complicated to discriminate side effects from the natural course of the disorder and from intercurrent illness. However, similar symptoms have been reported upon D,L-3-HB administration in healthy adults.[55,56] It remains to be investigated whether these side effects are actually caused by the D,L-3-HB itself, or the associated cation load. Besides, future studies are warranted to examine the impact on fluid-, electrolyte-, and acid–base homeostasis.

The mutagenicity, carcinogenicity and reproduction toxicity of D,L-3-HB salts have not yet been investigated in traditional in vivo animal studies. However, the outcomes of such studies for other ketogenic compounds may provide indirect evidence. A two-year feeding study of 1,3-butanediol (isomers not specified) did not demonstrate adverse effects or carcinogenicity.[57] Some in vitro culture studies using isolated mouse embryos exposed to D,L-3-HB-containing medium, suggest that D,L-3-HB may negatively affect embryogenesis with respect to preimplantation growth, cardiovascular development and the induction of malformations including neural tube defects, similarly as the congenital defects observed in offspring of mothers with poorly controlled (pregestational) diabetes mellitus.[58,59] However, in vivo developmental toxicity studies using 1,3-butanediol (isomers not specified) and a D-3-hydroxybutyl-D-3-HB ester did not demonstrate teratogenic effects, although the development of fetal skeletal tissue may be slightly delayed.[60,61] There was no evidence for mutagenic and cytogenic toxicity.[60]

The absorption and distribution of D,L-3-hydroxybutyrate

It is important to realize that 3-HB is a chiral molecule with a D- and L-enantiomer, which can have different pharmacokinetic and pharmacodynamic properties.[62] In **chapter 6**, we investigated the enantiomer-specific pharmacokinetics of D,L-3-HB

using reversed phase ultra-performance liquid chromatography-MS/MS. Enteral D,L-3-HB administration induced substantially higher L-3-HB concentrations compared to D-3-HB. Moreover, we provided information on the distribution of both 3-HB enantiomers in major organ systems involved in severe MADD, after one single, oral dose. In MADD patients, we found that concentrations of D-3-HB and L-3-HB returned toward baseline concentrations after about three hours. We therefore recommend a dosing schedule of six to eight daily administrations upon initiation of D,L-3-HB in MADD, which can subsequently be tailored to the individual patient.

The enantiomer-specific pharmacokinetics implies differential metabolic fates of D-3-HB and L-3-HB. A schematic representation of the proposed utilization routes of both 3-HB enantiomers is depicted in **chapter 6**. Follow-up studies using ^{13}C-labeled D-3-HB and L-3-HB would allow for a more detailed study of the enantiomer-specific metabolism.

The organ-specific effects of D-3-HB and L-3-HB remain to be elucidated. D-3-HB oxidation could mainly be responsible for the beneficial actions in peripheral tissues, whereas L-3-HB may predominantly act as a substrate for sterol and fatty acid synthesis in the central nervous system.[63,64] The increased L-3-HB concentrations in brain tissue after D,L-3-HB administration indicate that this compound can cross the blood brain barrier and reach the brain, which would be essential to target leukodystrophy. Currently, MADD patients are mostly supplemented with racemic D,L-3-HB salts. In theory, the most effective D-3-HB:L-3-HB ratio may however depend on the (organ-specific) treatment indication. For example, it can be hypothesized that an excess of L-3-HB may be applied for leukodystrophy, whereas increasing the D-3-HB content would be indicated for symptoms that are expected to be caused by an impaired energy homeostasis such as cardiomyopathy, liver symptoms, and muscle symptoms. Repetition of the study protocol in a preclinical model for MADD can illustrate the effects of D,L-3-HB administration (in various ratio compositions) on clinical outcome. Although a knock-in mice model for riboflavin-responsive MADD has been described[65], no genetic mammalian model is available for severe MADD unresponsive to riboflavin. Perhaps this could be created using CRISPR-Cas9 technology. Otherwise, models of 'acquired MADD' that mimic the biochemical and metabolic status, such as dietary riboflavin deficiency[66-68] and hypoglycin A intoxication[69-72], may form useful alternatives.

Alternative ketogenic compounds

Aside from D,L-3-HB salts, one or both enantiomers can also be administered in the form of a polymer, or a 1,3-butanediol- or glycerol-derived ester.[73-77] The intestinal hydrolysis required to liberate the D,L-3-HB could induce prolonged increments.

This would also provide an opportunity to circumvent the salt load that currently accompanies D,L-3-HB treatment in MADD. On the other hand, a potential strong first-pass effect for D-3-HB would limit the use of D,L-3-HB polymers or -esters since the slowed absorption could prevent the high peak doses required for it to reach the circulation. Besides, the conversion of the 1,3-butanediol ester by alcohol- and aldehyde dehydrogenases is rate-limiting. The potential enzyme saturation upon chronic administration may therefore cause a risk similar to that of chronic hepatic ethanol exposure.[74] In contrast, the backbone of the glycerol-derived ketone ester would be phosphorylated and incorporated into triglycerides and glucose.[76] Nevertheless, the existence of multiple ketogenic compounds in a rapidly emerging field of applications, warrants careful description of the compound and the enantiomers used, along with the salt-free dose, to ensure correct data interpretation.

Toward equal and sustainable access to D,L-3-HB

The story of D,L-3-HB for MADD forms an excellent example of the complex journey towards sustainable and available treatment for patients with an ultra-rare disease. Treatment with D,L-3-HB lies at the interface of a food supplement and a drug therapy, with both approaches having fundamental differences regarding the applicable laws and regulations. In The Netherlands, D,L-3-HB is unlicensed and prescribed as magistral formula prepared by a pharmacist. In the European Union, orphan drug regulation has been in force since 2000, as incentive to encourage treatment development for rare diseases.[78] Designated orphan drugs are required to apply for centralized marketing authorisation to ensure international availability. Pharmaceutical companies usually act as leading sponsors in these processes. Decisions about pricing and reimbursement follow at the level of each Member State. Extremely high costs of orphan drugs are increasingly common, despite the absence of transparent development expenditures.[79,80] Moreover, even though the successful (re)development of orphan drugs often strongly depended on publicly funded research and patient contributions.[79-81] FAIR (i.e. Findable, Accessible, Interoperable, Reusable)[82] and transparant strategies are essential to establish sustainable orphan drugs.[80,83] This is substantiated by the International Committee of Medical Journal Editors that considers data sharing an ethical obligation for clinical trials.[84] The nature of an ultra-rare, heterogenous disease as MADD emphasizes this need, as the phenotypic variation requires detailed patient data for adequate interpretation. Hence, we included individual patient data as well as information on the preparation and pricing of D,L-3-HB in our open-access paper (**chapter 5**).

To date, the scientific evidence underpinning D,L-3-HB treatment in

MADD has been generated by clinicians along with the challenges of patient care. Paradoxically, the aim of publishing our data as detailed as possible, in order to improve access to D,L-3-HB for patients worldwide, simultaneously induced a risk of so-called ‘daylight robbery’[85], which could potentially hamper the general availability for MADD patients over time. An academic-based application for orphan drug designation and registration of D,L-3-HB, in attempt to stimulate equal and continuous access, is yet an untrodden path. The experience and resources of pharmaceutical companies puts them at an advantage. Therefore, the supporting initiatives by the Dutch Medicines Evaluation Board (MEB; i.e. ‘College ter Beoordeling van Geneesmiddelen’ (CBG) in Dutch) and the European Medicines Agency (EMA), to provide regulatory, financial and protocol guidance for academic groups and small companies, are very welcome. An even more active role, for example by participating in the completion of the required forms in order to reduce the burden of the complex paperwork, would likely further increase the chances of a successful application from academic groups. There is a shared responsibility for clinicians, researchers, pharmacists, policy makers, investors, hospitals, pharmaceutical companies, health insurances, regulatory organisations, and governments, preferably in collaboration with patients, to facilitate the development of, and access to novel treatments for rare diseases. These parties should join forces and develop collective strategies, and thereby prevent the occurrence of diffused responsibility, which could ultimately cause a risk of inaction rather than decisiveness. Centers of expertise, unified in so-called European Reference Networks (e.g. ‘MetabERN’); multidisciplinary collaborations such as United for Metabolic Diseases (UMD); and foundations such as Fair Medicine, which introduces a coalition model that involves all stakeholders in a transparent development process of pharmaceuticals; can perhaps play a guiding role. The lessons learned from the hurdles encountered in the pursuit toward equal and sustainable access to D,L-3-HB, can provide a blue print for comparable, unlicensed agents.

The challenges of evidence-based treatments for rare diseases

In treatment development, randomized placebo-controlled patient trials are considered the gold standard. The rarity and heterogeneity of MADD, combined with the potential small window of therapeutic opportunity in a life-threatening disease, causes implementation issues and ethical concerns.[86] It is often complicated to obtain a power-based, sufficient sample size and it appears unethical to allocate severely affected MADD patients to standard care in the light of the recent findings. Instead, comparison with natural history data can offer an adequate approach to provide supportive evidence.[30,86]

The success rate for orphan drug designation and registration increases upon a clearly defined, specific indication.[87] The MEB adviced us to first focus on the subtype of patients in whom the health benefit can be objectively assessed through major clinical endpoints (i.e. survival in neonatal onset MADD). The indication for D,L-3-HB treatment could thereafter be extended to later onset MADD and MADD-like diseases through additional applications. Although this can be understood from a regulatory perspective, one may wonder whether this categorization is justifiable given the phenotypical overlap between the historical subtypes. The proposed classification of severe, intermediate and myopathic MADD may therefore be more appropriate. Besides, in **chapter 5**, we also reported severely affected, later onset patients in whom D,L-3-HB treatment resulted in substantial clinical improvement of myopathy and exercise intolerance. These symptoms are likely based on a similar pathophysiology as the debilitating myopathy in neonatal onset MADD, but where the clinical consequences depend on the severity of the metabolic block. It can therefore be hypothesized that the working mechanism of D,L-3-HB is the same. In one later onset patient suffering from exercise-induced myopathy, the symptoms relapsed after the D,L-3-HB had to be discontinued because of the associated costs, which were borne by the family. Nonetheless, we re-analyzed our D,L-3-HB efficacy data to asses the outcome in neonatal onset, classic MADD (i.e. genetic defect in *ETFA*, *ETFB*, *ETFDH*), and compared it to data of historical controls, as presented in **Figure 4** and **Table 1**. Three patients died despite D,L-3-HB treatment, of whom the detailed information is included in **Table 2**. In patient A and B, the prescribed D,L-3-HB doses appear relatively low, whereas in patient C, the window of opportunity for D,L-3-HB may have had already surpassed.

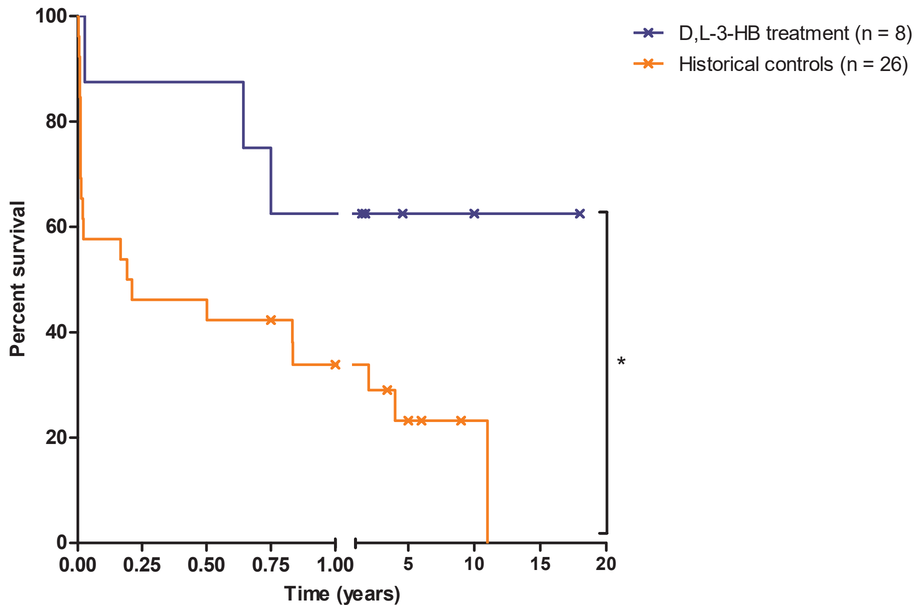

Figure 4. Improved survival in neonatal onset multiple acyl-CoA dehydrogenase deficiency patients upon D,L-3-hydroxybutyrate treatment. The data was reanalyzed from the findings described in chapter 4 and 5. Kaplan–Meier curve of the survival in neonatal onset MADD patients treated with D,L-3-HB (n = 8) compared to the survival in historical controls not reported to have been treated with D,L-3-HB (n = 26). There is a significant difference between both curves, as demonstrated by the log-rank test ($p < 0.05$). x = censored patient.

Table 1. Neonatal onset multiple acyl-CoA dehydrogenase deficiency patients treated with D,L-3-hydroxybutyrate compared to historical controls.

	Treated with D,L-3-HB (n = 8)	Historical controls (n = 27)
Alive	5 (63%)	6 (22%)
Age at last follow-up	4.5 y (8.5 y)	4 y (4 y)
Death	3 (38%)	21 (78%)
Age at death	8 m (4 m)	8 d (7 m)[a]
Age at onset	1 d (1 d)	0 d (1 d)
Positive newborn screening	7 (88%)	3 (11%)
Genetic analysis	8 (100%)	23 (85%)[b]
ETFA	6[c]	8[d]
ETFB	-	3
ETFDH	2	10
Enzyme assay	2 (25%)	11 (41%)
ETF deficiency	1	9
ETF-QO deficiency	1	2
D,L-3-HB indication (efficacy if applicable)		
Cardiomyopathy	2 (CI in 2/2)	4
Leukodystrophy	1 (CI in 1/1)	-
Liver symptoms	4 (CI in 3/4)	25
Muscle symptoms	6 (CI in 5/6)	14
Respiratory failure	1 (CI in 2/1)	4
Preventive	3	NA
D,L-3-HB treatment		NA
Age at start	6 m (6 m)	-
Minimum D,L-3-HB salt dose	275 (180) mg/kg/day	-
Maximum D,L-3-HB salt dose	825 (1275) mg/kg/day	-
Maximum number of gifts/day	6 (2)[e]	-
D,L-3-HB discontinuation	4 (50%)	NA
Age at discontinuation	8 m (5 m)	-
Cause of discontinuation	Death (n = 3); SE (n = 1)	-
D,L-3-HB treatment duration	10 m (4.5 y)	NA

The data was reanalyzed from the findings described in chapter 5. Values are presented as number of patients or median (interquartile range). aCalculated from available data of 20 patients. bIn two patients the diagnosis was genetically confirmed, but no mutations were reported. cIn one patient, DNA analysis also demonstrated compound heterozygous variants in ETFB (c.217–4G>T and c.438+20C>T), which were classified as variant of uncertain significance and likely benign, respectively. dIn one patient, only one mutation was identified; the diagnosis was confirmed through enzyme analysis. eContinuous nocturnal administration (n = 1) and continuous 24-hour administration (n = 1). Abbreviations (in alphabetical order): CI, clinical improvement; NA, not applicable; SE, side effects.

Table 2. Neonatal onset multiple acyl-CoA dehydrogenase deficiency patients whom deceased during D,L-3-hydroxybutyrate treatment.

	Patient A	Patient B	Patient C
Age at onset	1 d	3 d	1 d
Age at death	9 m	8 m	10 d
Positive NBS	Yes	Yes	Yes
Genetic analysis	Yes	Yes	Yes
ETFA	Homozygous mutation c.370G>A (p.A124T)	-	Homozygous mutation c.797C>T (p.T266M)
ETFB	-	-	-
ETFDH	-	Homozygous mutation c.1141G>C (p.G381R)	-
Enzyme assay	No	Yes	No
ETF deficiency	-	-	-
ETF-QO deficiency	-	0.05 nmol/min/mg protein (C: 0.22±0.09)	-
D,L-3-HB indication (efficacy if applicable)			
Cardiomyopathy	-	-	-
Leukodystrophy	-	-	-
Liver symptoms	X (+)[a]	X (?)	-
Muscle symptoms	X (+)[b]	X (?)	-
Respiratory failure	X (+)	-	-
Preventive	-	X (?)	X (-)
D,L-3-HB treatment			
Age at start	1 m	2 m	9 d
Minimum D,L-3-HB salt dose	300 mg/kg/day	355 mg/kg/day	900 mg/kg/day
Maximum D,L-3-HB salt dose	600 mg/kg/day	395 mg/kg/day	1800 mg/kg/day

Maximum number of gifts/day	6	6	Continuous 24-hour
D,L-3-HB treatment duration	8 m	6 m	1 d
Further details by treating physician	Child arrested and died. Difficult to attribute cause of death.	D,L-3-HB was started as metabolic treatment to prevent complications. Patient seemed more stable during D,L-3-HB.	Progressive neurological deterioration with central apnea and bradycardia, loss of consciousness, opisthotonos, epileptic fits, eventually resulting in death.

The data was reanalyzed from the findings described in chapter 5. Presented values concern the individual patient data with present (+), questionable (?), or absent (-) clinical improvement. aLiver symptoms included hypoglycemia and metabolic acidosis. bMuscle symptoms included hypotonia and myopathy.

7

Together, our findings provide evidence of the proof of target engagement (i.e. "*the drug is reaching the intended organ and binding to its intended target*"[86(p762)]), biological activity (i.e. "*its intended mechanism of action*"[86(p762)]) and concept (i.e. "*the downstream effects of the drug beyond its immediate action, for example changes in secondary pathophysiology, organ function, or clinical effects*"[86(p762)]) of D,L-3-HB treatment in MADD. However, efficacy does not always coincide with real-world effectiveness.[88] The lack of treatment protocols and standardized outcome parameters complicated detailed assessment of efficacy in the retrospective study. Prospective (long-term follow-up) studies with predefined clinical endpoints (e.g. survival, improvement in morbidity or functional performance, and quality of life) and (bio)markers (e.g. laboratory measures, imaging findings) are warranted to investigate the actual therapeutic effect, dose-response relation and assess the benefit-risk profile.[86] These studies can include extensive, independent patient registries[80,89] as well as alternative clinical trial designs (e.g. n-of-1, cross-over, enriched enrollment, randomized placebo-phase, randomized withdrawal)[90], of which the value must not be underestimated, particularly within the context of (ultra-)rare diseases. In this respect, upon validation, the MADD-DS3 can be used as composite endpoint.[86] Another innovative biomarker could involve in vivo phosphorous magnetic resonance spectroscopy (31P-MRS) measurements as unique, non-invasive insights into tissue-specific energy metabolism[91,92], although the correlation with clinically meaningful outcome would have to be documented first.

Meanwhile, the unmet medical need in severe to intermediate MADD, combined with the significantly improved survival, may qualify D,L-3-HB for the EMA's PRIority MEdicines scheme (i.e. PRIME scheme), possibly even in conjunction with conditional marketing authorization, in order to speed up patient access. Comprehensive post-marketing surveillance through abovementioned study designs, and with clear clinical endpoints, can subsequently be used to validate its real-world value.[88,93] Finally, the currently available evidence calls for a reconsideration of the reimbursement of D,L-3-HB by health insurances.

THE TEST

MADD patients can be identified through acylcarnitine analysis in DBS (i.e. increased C4-, **C5-**, **C5-DC-**, C6-, **C8-**, *C10-*, C12:1-, C12-, C14:2-, **C14:1-**, C14-, *C16-*, and/or C18:1-carnitine and several molar ratios; in **bold**: the acylcarnitine species related to the detection of MADD that are already reported by the Dutch NBS program; in *italic*: the acylcarnitine species related to the detection of MADD that are already reported

by the Dutch NBS program as part of a molar ratio).[32,94] Therefore, no additional NBS test assay is required for the inclusion of MADD in the Dutch NBS program.

GeneReviews states that population NBS programs primarily use C4-, C5-, and C8-carnitine as screening parameters for MADD, sometimes combined with additional acylcarnitine species.[48] For many acylcarnitine markers, there is an overlap with the detection of other IEMs, inducing a risk of incidental findings. Currently, C6-, C10-, C12:1-, C14:2-carnitine can additionally be reported in the Dutch NBS program without the risk of so-called 'bycatch' of other IEMs that are not a target disorder.[2,94] The sensitivity and specificity of (the best combination of) acylcarnitine species to detect MADD should be evaluated in a combined retrospective and prospective pilot study. Especially in myopathic MADD, acylcarnitine profiles can be (close to) normal in the absence of metabolic stress.[33,43] Studies on the experiences with NBS for MADD did not report any false negative NBS test results, whereas false positive NBS test results have been described.[3,95-97] Ratio testing may help to discriminate between true- and false-positive NBS test results and to prevent missed diagnoses. To this aim, postanalytical tools such as the Collaborative Laboratory Integrated Reports (CLIR, formerly known as Region 4 Stork (R4S)), which estimates a disease risk using probability calculation that involves multiple disease-related analytes[98-100], and machine learning approaches such as decision tree-based random forest analysis[101], could also be helpful.

The potential role of next-generation sequencing techniques in population NBS remains to be discussed, but may currently rather supplement abnormal or equivocal metabolite screening results instead of replacing them.[102] This is substantiated by a recent study that found a lower overall sensitivity and specificity of NBS by whole-exome sequencing alone when compared to the results of NBS by MS/MS, whereas it could reduce false-positive results as second tier test.[103]

Finally, a proposed approach toward cases with positive NBS test results for MADD is discussed in the next section.

THE RE-EVALUATION

The aim of population NBS is to prevent or limit health damage through early disease detection.[1] From a family perspective, potential benefits involve narrowing of a diagnostic odyssey, preparedness for the onset of (severe) symptoms, and possible considerations regarding subsequent pregnancies.[1,104] Although MADD can be detected via acylcarnitine analysis in DBS, the disorder has been classified as 'category 3 condition' due to a lack of evidence for sufficient health gain through

detection by NBS, and is therefore not included in the Dutch NBS program.[1] Yet, evaluations may shift upon new observations and developments. This thesis adds substantial proof to the required Dutch NBS criteria for knowledge about the natural history, disease severity prediction, and monitoring of MADD. We also provided considerable, systematic evidence of the clinical benefits of D,L-3-HB treatment. Based on these advancements, it may be concluded that the eligibility of MADD for inclusion in the Dutch NBS program could be reconsidered. The rationale for this evaluation will be explained in the following paragraphs.

For decision-making, lessons can be learned from previous experiences and comparable assessments for other IEMs, particularly mitochondrial FAO disorders:

- Population NBS has resulted in a substantial reduction of the mortality and severe adverse events in children with MCAD deficiency and VLCAD deficiency.[105,106] However, this coincided with an increased detection of asymptomatic cases. Some of these concern patients who can manifest symptoms, for example, upon metabolic stress, whereas in others the abnormal metabolite profile may be caused by benign variants, whereby it can even be debated whether the term 'patient' applies.[107,108] Risk stratification is important to predict if patients are likely to develop symptoms and to optimize individual treatment strategies, while minimizing the risk of overtreatment.[34,35,107]
- In their most recent advice, the Health Council of The Netherlands considered population NBS eligible for carnitine-acylcarnitine translocase deficiency and CPT2 deficiency, two mitochondrial FAO disorders with a heterogenous phenotype that can be similar to MADD.[1] The health benefits of early disease detection were regarded as sufficient for myopathic and late-intermediate patients because of the available dietary treatment options, possibly supplemented with carnitine. The potential health benefits for patients categorized as severe and early-intermediate were only based on limited, small case studies.[2]
- Propionic aciduria and methylmalonic aciduria have been included in the Dutch NBS program since 2019. However, a recent Dutch retrospective cohort study suggested that the health gain of NBS in overall outcome may be limited, by comparing the outcomes of index siblings with the outcomes of patients identified by family testing.[109] Although it can be debated whether the diagnosis of a child in the setting of a known family can substitute the impact of NBS, this study does emphasize the importance of careful consideration of (expected) NBS efficacy, prior and post the implementation of new IEMs to the NBS panel, combined with the introduction of continuation and stop

criteria based on clinical outcome parameters.[109]

The results of **chapter 4** and **5** can be synthesized into a potential plan of action with regard to positive NBS test results for MADD. Upon identification by NBS, functional studies in fibroblasts can facilitate phenotype prediction in MADD patients and provide guidance on treatment and monitoring regimens, similarly as has been described for VLCAD deficiency.[35] **Figure 5** presents a proposed approach to neonates with a suspect diagnosis of MADD and for before outcomes of functional studies in fibroblasts are known. Based on the (predicted) phenotype, an individual treatment strategy can be suggested, as presented in **Figure 6**. This approach also allows risk assessment for the development of symptoms in patients who are asymptomatic upon identification through NBS. Subsequent monitoring based on the (predicted) phenotype, for example according to the MADD-DS3, may reveal the development of symptoms as cardiomyopathy and leukodystrophy early on.

Planned evaluation studies are required to assess the real-life benefits of NBS for MADD, including the perspectives of child and families. However, the potential health gain by early identification of MADD through population NBS can be estimated within the context of several patient scenarios. In general, timely instituted (dietary) treatment options combined with the use of emergency protocols, can prevent substantial catabolic-related morbidity and mortality. For intermediate and myopathic MADD, and possibly some severe MADD patients with a late-neonatal presentation, early detection by NBS is likely to improve or even prevent an adverse outcome of the first hepatic manifestation, as has also been reported for other mitochondrial FAO disorders. For riboflavin-responsive patients (i.e. the majority of late-onset MADD, particularly caused by genetic variants in ETFDH), excellent outcome has been reported for muscle symptoms upon initiation of riboflavin therapy, possibly in conjunction with coenzyme Q10 in case of secondary coenzyme Q10 deficiency.[43,112,113]

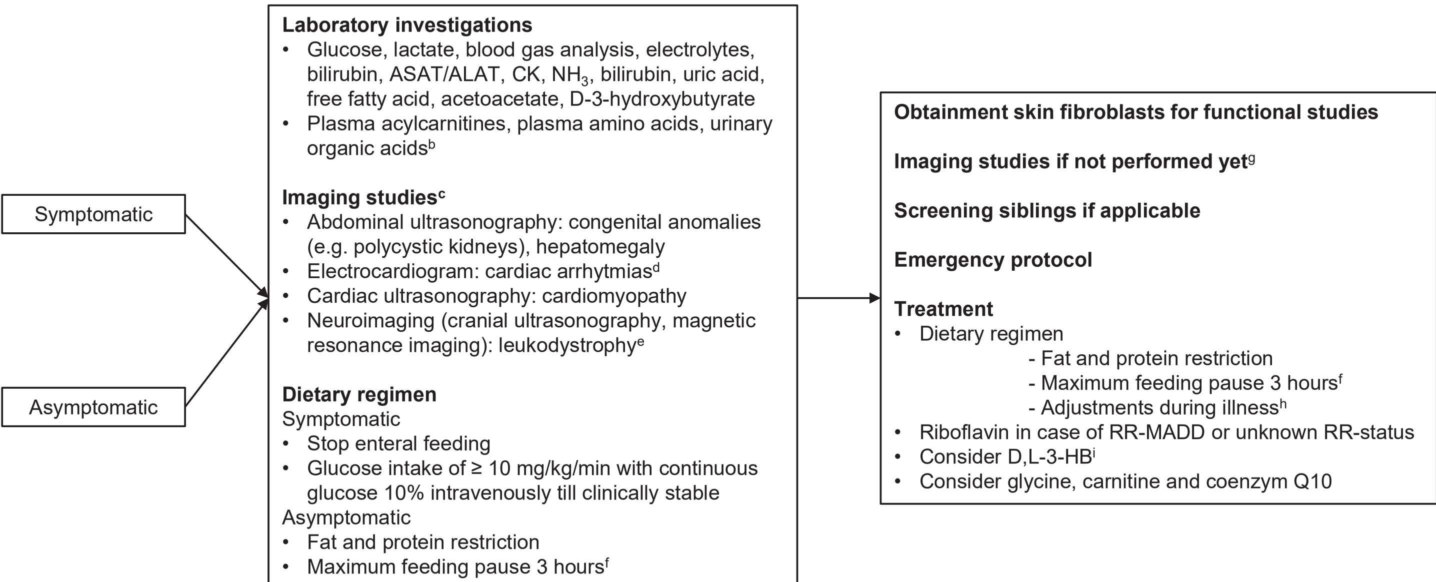

Figure 5. Proposed approach upon suspect diagnosis of multiple acyl-CoA dehydrogenase deficiency by newborn screening. Adapted from [35]. The functional studies in fibroblasts include oleate- and myristate flux assays and acylcarnitine profiling after palmitate loading, as described in chapter 2. The proposed starting dose for D,L-3-HB salt is 900 mg/kg/day (salt-free dose: 738 mg/kg/day) in six to eight doses. [a]Diagnosis by biochemical or DNA analysis. [b]In case of symptomatic patients, metabolite analyzes should preferably be performed in crisis samples. [c]Prompt imaging workup is specifically important for symptomatic patients. [d]Consider electrocardiogram-holter monitoring, especially in symptomatic patients. [e]Although magnetic resonance imaging is more sensitive for detection of leukodystrophy, cranial ultrasound can provide early (bedside) detection of cysts, calcifications, structural brain abnormalities, and white matter echogenicity.[110] [f]Consider continuous (night) feeding. [g]Consider complete imaging workup for baseline characteristics, also in asymptomatic patients. [h]During illness, adequate energy intake should be ensured, for example according to the steps of an emergency protocol.[111] [i]Consider (preventive) D,L-3-HB in ill patients or patients with a positive family history for the severe phenotype of MADD. Abbreviations (in alphabetical order): ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CK, creatine kinase; D,L-3-HB, D,L-3-hydroxybutyrate; FAO, fatty acid oxidation; NH3, ammonia; RR, riboflavin-responsive(ness).

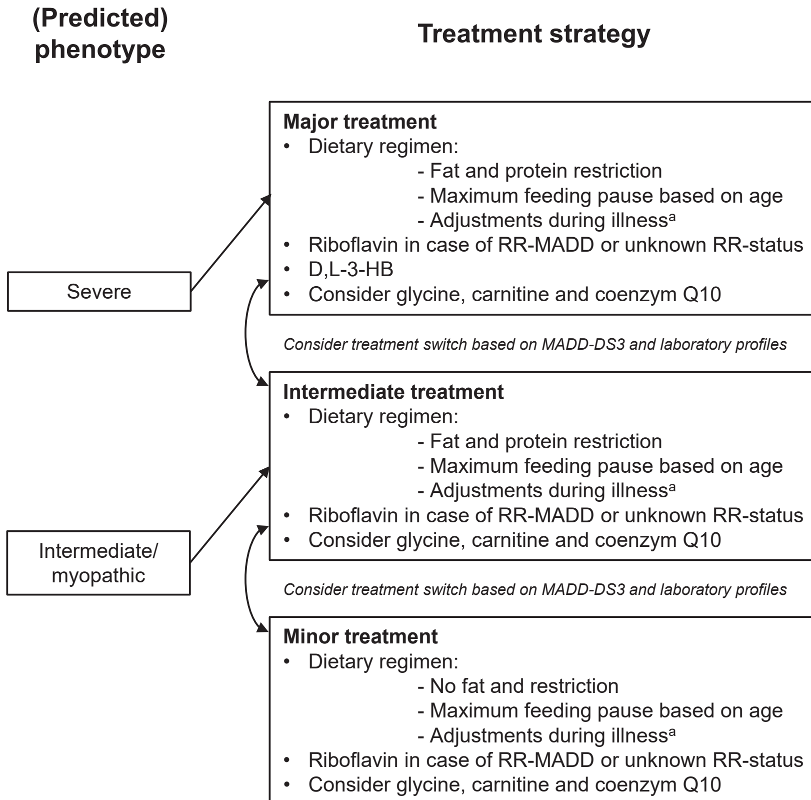

Figure 6. Proposed treatment strategy for multiple acyl-CoA dehydrogenase deficiency patients identified by newborn screening. Adapted from [35]. Phenotype prediction based on functional studies in fibroblasts including oleate- and myristate flux assays and acylcarnitine profiling after palmitate loading, as described in chapter 2. The patient should be monitored structurally, with treatment adaptations considered based on (progressing/ declining) symptoms and signs. For example, in a patient with an intermediate, hepatic phenotype that progresses to severe symptoms such as leukodystrophy, cardiomyopathy or myopathy under "intermediate treatment", initiation of D,L-3-HB treatment likely to be indicated (i.e. "major treatment"). The proposed starting dose for D,L-3-HB salt is 900 mg/kg/ day (salt-free dose: 738 mg/kg/day) in six to eight doses. Especially for severe to intermediate (predicted) phenotypes continuous (night) feeding or, after infancy, late-night feeding with for example uncooked cornstarch can be considered. [a]During illness, adequate energy intake should be ensured, for example according to the steps of an emergency protocol.[111] Abbreviations (in alphabetical order): D,L-3-HB, D,L-3-hydroxybutyrate; MADD-DS3, multiple acyl-CoA dehydrogenase deficiency-disease severity scoring system; RR-MADD, riboflavin-responsive multiple acyl-CoA dehydrogenase deficiency.

Many severely affected MADD patients that are unresponsive to riboflavin already develop (non-specific) life-threatening symptoms during the first days of life. Population NBS can still cause relevant health gain through acceleration of the diagnostic process. This could allow for an earlier start of D,L-3-HB treatment, which is relevant because of the potential small window of therapeutic opportunity. The efficacy and safety of D,L-3-HB treatment have been described in an international cohort. The health benefit is most clearly reflected in the significantly improved survival of neonatal onset MADD patients without congenital anomalies. Timely initiation of D,L-3-HB may in some cases even further improve the beneficial effects on clinical outcome. Moreover, it can be proposed to start with D,L-3-HB preventively in asymptomatic patients with a positive family history for the severe phenotype of MADD. Based on the predicted phenotype by functional studies in fibroblasts, it could eventually be decided whether or not the D,L-3-HB treatment should be continued. Whether population NBS and D,L-3-HB treatment also results in a reduced morbidity and mortality for severely affected MADD patients with congenital anomalies remains to be elucidated. However, the similar dilemmas apply for VLCAD deficiency and CPT2 deficiency.[2,106,108]

The sensitivity and specificity, and thresholds for the potential (second tier) MADD screening parameters have yet to be determined. Yet, this also applies to carnitine-acylcarnitine translocase deficiency and CPT2 deficiency, for which the inclusion is scheduled for 2021, pending validation studies.[114] One could argue that for MADD, acylcarnitine profiles are more difficult to interpreted and that there is a risk of missing patients, especially those with a myopathic phenotype.[43] However, similar risks also exists for other mitochondrial FAO disorders, including mild deficiencies of VLCAD and isolated long-chain 3-hydroxylacyl-CoA dehydrogenase/mitochondrial trifunctional protein during anabolism[32], and CPT2 deficiency screening in DBS[115].

To conclude, by combining the findings of this thesis with existing knowledge and experience, it can be advocated that MADD meets the Dutch criteria for population NBS, and therefore could be reconsidered for inclusion in the Dutch NBS program.

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CONNECTING THE DOTS

This thesis demonstrates that health care and research are intimately connected, especially for patients with (ultra-)rare diseases. Close collaboration between patients, clinicians, researchers and all other stakeholders is fundamental for innovation and developments.(Augustine et al., 2017) We combined reviews of the literature, with experimental research, with studies on clinical outcome in patients with multiple acyl-CoA dehydrogenase deficiency (MADD; also known as glutaric aciduria type II). An important and valuable aspect of translational research, is to evaluate the actual impact on the clinical and public health practice. Based on our results, it can be advocated that MADD could be reconsidered for inclusion in the Dutch newborn blood spot screening (NBS) program. We also recommend that every child participates in a population NBS program, even after death. Our data on phenotype prediction and D,L-3-hydroxybutyrate treatment can guide clinicians in their care of MADD patients and their families. It can also support decision-makers in their aims to improve NBS programs and facilitate access to novel treatments for (ultra-)rare diseases. Finally, we were able to find answers to questions that arose directly from the clinical setting, and which can be used for an improved substantiation of patient care. Hence, we connected the dots from bed to bench and back again.

Appendices

Summary
Samenvatting
Abbreviations
Dankwoord
About the author
Contribution to the field

SUMMARY

Inborn errors of metabolism (IEM) can be defined as conditions that lead to a disruption of a metabolic pathway, including a deficient transporter or enzyme, or a cofactor involved. This can result in the accumulation of (toxic) metabolites prior, and deficiencies of metabolites after the metabolic block. Population newborn blood spot screening (NBS) programs can identify patients suffering from IEMs, mostly via tandem mass spectrometry (MS/MS) analysis of metabolites in dried blood spots (DBS), including acylcarnitines and amino acids.

Multiple acyl-CoA dehydrogenase deficiency (MADD; also known as glutaric aciduria type II) is an ultra-rare (i.e. <1 : 50,000) disorder of mitochondrial fatty acid oxidation and amino acid metabolism with a heterogenous phenotype. The disorder is not included in the Dutch NBS program due to a lack of evidence for sufficient health gain upon early detection. Complicating factors concern the limited knowledge on the natural history, disease severity prediction and monitoring of the spectrum of MADD patients, and the absence of (sufficient, systematic evidence on) an effective treatment for severely affected patients. MADD is also an exemplary IEM that can escape identification due to nonspecific symptoms and unexpected childhood death. Presumably, many other IEMs are associated with a similar risk. Increased knowledge is important, because it improves timely recognition, and by that may lead to prompt initiation of treatment in order to minimize the risk on life-threatening events and death.

This thesis can be categorized into two parts that are closely intertwined. In the first part, we studied the IEMs that are associated with unexpected death in early childhood, and how their detection through acylcarnitine profile analysis can be improved. In the second part we aimed to provide evidence to re-evaluate the possibility of NBS for MADD in the Netherlands, based on three main principles of early disease detection as described by Wilson and Jungner: the disease, the treatment and the test.

Part I – Inborn errors of metabolism and unexpected childhood death

The metabolic differential diagnosis of unexpected death in early childhood

In **chapter 2**, we reported 43 IEMs that are associated with a risk of unexpected death in infancy, of which 26 can already present during the neonatal period. Treatment is available for at least 32 of the IEMs, and a minimum of 26 are detectable by MS/MS analysis of metabolites in DBS. The identified IEMs mostly concern disorders

of mitochondrial fatty acid oxidation, urea cycle defects and organic acidurias. At least ten IEMs were identified that are currently not included in the Dutch NBS program, despite treatment options being available and detectability by MS/MS analysis in DBS. A neonatal presentation has been reported in at least six of these IEMs. This information has implications for the improvement of population NBS programs and postmortem diagnostic protocols upon unexpected childhood death, of which the latter preferably includes extensive metabolite screening in addition to next generation sequencing and the obtainment of material such as skin fibroblasts for functional studies. Our findings likely also contribute to the awareness among neonatologists and pediatricians that IEMs can cause unexpected childhood death.

In some IEMs, symptoms and signs including death may already occur before the NBS test results become available or even before the blood required for testing has been drawn. Since IEMs are mostly autosomal recessive disorders, there is a recurrence risk within families of at least 25%. We advocate that every child deserves participation in a population NBS program, even after death, using a peri- or postmortem sample.

Metabolic stress and long-term storage can affect acylcarnitine profiles

It is important to realize that metabolite profiles are influenced by various factors including the feeding state, endogenous metabolism, sample type and (pre-)analytical conditions. In **chapter 3a**, we demonstrated that fasting-induced counter-regulatory mechanisms to maintain energy homeostasis are age-dependent. This affects the changes in basic metabolic parameters and acylcarnitine profiles. In **chapter 3b**, we reported that acylcarnitine profiles in DBS stored at room temperature are subject to metabolite instability. Our results emphasize the importance of obtaining background information on samples. This improves the interpretation of potential deviations and can reveal subtle changes that would otherwise not have been discovered.

<u>Part II – Toward population newborn screening for multiple acyl-CoA dehydrogenase deficiency</u>

THE DISEASE

Chapter 4 adds important knowledge about the natural history of MADD. We described the characteristics of a cohort of 18 patients, and developed an MADD-disease severity scoring system (MADD-DS3) in order to facilitate uniform, clinical description of disease severity. Finally, we found that functional studies in fibroblasts can be used for phenotype prediction in order to start treatment and follow-

up appropriately. This is especially relevant in view of the inclusion of MADD in population NBS programs.

THE TREATMENT

In **chapter 5**, we pooled international data on the clinical presentation, treatment method and physician reported outcome in 23 MADD(-like) patients upon treatment with D,L-3-hydroxybutyrate (D,L-3-HB, a ketone body) salt. Compared to data from historical controls, there was a considerably improved survival upon D,L-3-HB treatment. Clinical improvement was reported in 70% of the patients for cardiomyopathy, leukodystrophy, liver symptoms, muscle symptoms, and respiratory failure. Reported side effects included abdominal pain, constipation, dehydration, diarrhea, and vomiting or nausea. The risk of side effects appeared to increase with dose. Nonetheless, they never formed a reason to discontinue D,L-3-HB in case of reported efficacy. The potential scope of D,L-3-HB indication extends to all IEMs in which ketogenesis is disturbed and as an additive for patients using a ketogenic diet.

It is important to realize that 3-HB is a chiral molecule with a D- and L-enantiomer. In **chapter 6**, we investigated the enantiomer-specific pharmacokinetics of D,L-3-HB. We found that D,L-3-HB administration induced substantially higher L-3-HB concentrations compared to D-3-HB. Moreover, we provided evidence of the distribution of both 3-HB enantiomers in major organ systems involved in severe MADD. The enantiomer-specific pharmacokinetics imply differential metabolic fates of D-3-HB and L-3-HB.

In MADD patients, we recommend a dosing schedule of six to eight daily administrations upon initiation of D,L-3-HB, which can subsequently be tailored to the individual patient.

THE TEST

MADD patients can be identified through acylcarnitine analysis in DBS. For inclusion of MADD in the Dutch NBS program, no additional NBS test assay is required. Other population NBS programs primarily use C4-, C5-, and C8-carnitine as screening parameters for MADD, sometimes combined with additional acylcarnitine species.

THE RE-EVALUATION

The aim of population NBS is to prevent or limit health damage through early disease detection. MADD is not included in the Dutch NBS program due to a lack of evidence for sufficient health gain through detection by NBS. Yet, evaluations may shift upon new observations and developments. This thesis adds substantial proof to the required Dutch NBS criteria for knowledge about the natural history, disease severity

prediction, and monitoring of MADD. We also provided considerable, systematic evidence on the clinical benefits of D,L-3-HB treatment. The results of **chapter 4** and **5** can be synthesized into a potential plan of action with regard to positive NBS test results for MADD. Upon identification by NBS, functional studies in fibroblasts can facilitate phenotype prediction. An individual treatment and monitoring strategy can be proposed based on the (predicted) phenotype. Through several patient scenarios, it can be estimated that timely instituted (dietary) treatment options combined with the use of emergency protocols can prevent substantial morbidity and mortality. Based on these advancements, it can be advocated that MADD could be reconsidered for inclusion in the Dutch NBS program.

SAMENVATTING

Stofwisselingsziekten kunnen worden gedefinieerd als aandoeningen die leiden tot een verstoring van een metabole route, zoals een deficiëntie van een transporter of enzym, of een betrokken cofactor. Dit kan leiden tot ophoping van (toxische) metabolieten vóór, en tekorten van metabolieten na de metabole blokkade. Bevolkingsonderzoek middels de neonatale hielprikscreening kan patiënten met stofwisselingsziekten identificeren met behulp van massaspectrometrie analyse van metabolieten in bloedspots, waaronder acylcarnitines en aminozuren.

Multiple acyl-CoA dehydrogenase deficiëntie (MADD) is een ultra-zeldzame (i.e. < 1 : 50.000) aandoening van de mitochondriële vetzuuroxidatie en het aminozuur metabolisme, met een heterogeen fenotype. De aandoening is niet geïncludeerd in de Nederlandse hielprikscreening door een gebrek aan voldoende bewijs voor gezondheidswinst door vroege detectie. Complicerende factoren betreffen de beperkte kennis over het natuurlijk beloop, voorspelling van de ziekte ernst en monitoring van het spectrum van MADD patiënten, en de afwezigheid van (toereikend, systematisch bewijs voor) voor een effectieve behandeling van ernstig aangedane patiënten. MADD is ook een exemplarische stofwisselingsziekte die kan ontsnappen aan identificatie door aspecifieke symptomen en onverwacht overlijden op de kinderleeftijd. Waarschijnlijk zijn veel andere stofwisselingsziekten geassocieerd met eenzelfde risico. Toegenomen kennis over dit onderwerp is belangrijk, omdat het zorgt voor meer tijdige herkenning, en daardoor kan leiden tot prompte behandeling om zo het risico op levensbedreigende gebeurtenissen en overlijden te minimaliseren.

Dit proefschrift bestaat uit twee delen die nauw met elkaar verweven zijn. In het eerste deel hebben we onderzocht welke stofwisselingsziekten geassocieerd zijn met onverwacht overlijden op de jonge kinderleeftijd, en hoe de detectie van deze aandoeningen op basis van acylcarnitine profiel analyse kan worden verbeterd. In het tweede deel hebben we bewijs verzameld om de mogelijkheid van inclusie van MADD in de Nederlandse hielprikscreening te heroverwegen, gebaseerd op drie hoofd principes van vroege ziekte detectie zoals beschreven door Wilson en Jungner: de ziekte, de behandeling en de test.

Deel I – Stofwisselingsziekten en onverwacht overlijden van kinderen

De metabole differentiaal diagnose van onverwacht overlijden op de jonge kinderleeftijd

In **hoofdstuk 2** hebben we 43 stofwisselingsziekten gerapporteerd die zijn geassocieerd met een risico op onverwacht overlijden op de jonge kinderleeftijd, waarvan 26 zich al kunnen presenteren tijdens de neonatale periode. Behandeling is beschikbaar voor ten minste 32 stofwisselingsziekten, en minimaal 26 zijn detecteerbaar middels massaspectrometrie analyse van metabolieten in bloedspots. De geïdentificeerde stofwisselingsziekten betreffen voornamelijk vetzuuroxidatiestoornissen, ureum cyclus defecten en organische acidurieën. Er zijn ten minste tien stofwisselingsziekten geïdentificeerd die momenteel niet zijn opgenomen in de Nederlandse hielprikscreening, ondanks het feit dat behandeling beschikbaar is en ze detecteerbaar zijn middels massaspectrometrie analyse. Een neonatale presentatie is beschreven bij ten minste zes van deze aandoeningen. Deze informatie heeft consequenties voor het verbeteren van hielprikscreening programma's en protocollen voor postmortale diagnostiek na onverwacht overlijden op de kinderleeftijd, waarvan het laatste bij voorkeur uitgebreide metabolietenscreening omvat naast zogenaamde 'next generation sequencing' en het verkrijgen van materiaal zoals huidfibroblasten voor functionele studies. Onze bevindingen dragen waarschijnlijk ook bij aan het bewustzijn onder neonatologen en kinderartsen over stofwisselingsziekten als oorzaak van onverwacht overlijden op de kinderleeftijd.

Bij sommige stofwisselingsziekten kunnen klinische symptomen, waaronder overlijden, al optreden voordat de uitslag van de hielprikscreening beschikbaar is of zelfs voordat het benodigde bloedmonster is afgenomen. Aangezien stofwisselingsziekten over het algemeen autosomaal recessieve aandoeningen zijn, is er een herhalingsrisico binnen families van ten minste 25%. Daarom pleiten wij ervoor dat elk kind deelname verdient aan een hielprikscreening programma, ook na overlijden, met behulp van een peri- of postmortaal bloedmonster.

Metabole stress en langdurige opslag kunnen acylcarnitine profielen beïnvloeden

Het is belangrijk om te beseffen dat metabolieten profielen worden beïnvloed door verschillende factoren, waaronder de voedingstoestand, het endogeen metabolisme, monstertype en (pre-) analytische omstandigheden. In **hoofdstuk 3a** hebben we aangetoond dat de door vasten geïnduceerde contra regulerende mechanismen om de energiehomeostase in stand te houden, leeftijdsafhankelijk zijn. Dit heeft invloed op de veranderingen in basale metabole parameters en acylcarnitine profielen. In

hoofdstuk 3b hebben we gerapporteerd dat acylcarnitine profielen in bloedspots bewaard bij kamertemperatuur onderhevig zijn aan metabolieten instabiliteit. Onze resultaten benadrukken het belang van het verkrijgen van achtergrondinformatie over monstermateriaal. Dit verbetert de interpretatie van mogelijke afwijkingen en kan subtiele veranderingen aan het licht brengen die anders niet zouden zijn ontdekt.

Deel II - Op weg naar bevolkingsonderzoek bij pasgeborenen voor multiple acyl-CoA dehydrogenase deficiëntie

DE ZIEKTE

Hoofdstuk 4 voegt belangrijke kennis toe over het natuurlijke beloop van MADD. We hebben de kenmerken van een cohort van 18 patiënten beschreven en een zogenaamde 'MADD-disease severity scoring system' (MADD-DS3) ontwikkeld om uniforme, klinische beschrijving van de ziekte ernst te faciliteren. Ten slotte hebben we aangetoond dat functionele studies in fibroblasten kunnen worden gebruikt voor het voorspellen van fenotypes, zodat behandeling en follow-up adequaat kunnen worden gestart. Dit is in het bijzonder relevant met betrekking tot de inclusie van MADD in hielprikscreening programma's.

DE BEHANDELING

In **hoofdstuk 5** hebben we internationale gegevens verzameld over de klinische presentatie, behandelmethode en door de arts gerapporteerde uitkomsten bij 23 MADD(-like) patiënten na behandeling met D,L-3-hydroxybutyraat (D,L-3-HB, een ketonlichaam) zout. Vergeleken met gegevens van historische controles was er een aanzienlijke verbetering van de overleving na behandeling met D,L-3-HB. Klinische verbetering werd gemeld bij 70% van de patiënten voor cardiomyopathie, leukodystrofie, leversymptomen, spiersymptomen en respiratoire insufficiëntie. Gemelde bijwerkingen betroffen onder meer buikpijn, obstipatie, uitdroging, diarree, en braken of misselijkheid. Het risico op bijwerkingen leek toe te nemen bij hogere doseringen. Desalniettemin vormden bijwerkingen nooit een reden om D,L-3-HB te staken in geval van gerapporteerde werkzaamheid. De potentiële reikwijdte van het indicatiegebied van D,L-3-HB strekt zich uit tot alle stofwisselingsziekten met een verstoorde ketogenese en als additief voor patiënten die een ketogeen dieet volgen.

Het is belangrijk om te beseffen dat 3-HB een chiraal molecuul is met een D- en L-enantiomeer. In **hoofdstuk 6** hebben we de enantiomeer-specifieke farmacokinetiek van D,L-3-HB onderzocht. We hebben aangetoond dat toediening van D,L-3-HB leidt tot aanzienlijk hogere concentraties van L-3-HB in vergelijking met

D-3-HB. Bovendien hebben we bewijs geleverd van de distributie van beide 3-HB enantiomeren in de belangrijkste orgaansystemen die betrokken zijn bij ernstige MADD. De enantiomeer-specifieke farmacokinetiek impliceert dat het metabolisme van D-3-HB en L-3-HB van elkaar verschilt.

In MADD patiënten, adviseren we bij het starten met D,L-3-HB behandeling een doseringsschema van zes tot acht dagelijkse toedieningen, dat vervolgens kan worden aangepast aan de individuele patiënt.

DE TEST

MADD patiënten kunnen worden geïdentificeerd door middel van acylcarnitine analyse in bloedspots. Voor inclusie van MADD in de Nederlandse hielprikscreening is geen additionele test-assay nodig. Andere hielprikscreening programma's gebruiken primair C4-, C5- en C8-carnitine als screeningparameters voor MADD, soms gecombineerd met additionele acylcarnitines.

DE HEROVERWEGING

Het doel van de neonatale hielprikscreening is om gezondheidsschade te voorkomen of te beperken door vroege ziektedetectie. MADD is niet opgenomen in de Nederlandse hielprikscreening vanwege een gebrek aan bewijs voor voldoende gezondheidswinst door detectie middels neonatale screening. Toch kunnen evaluaties verschuiven naargelang van nieuwe waarnemingen en ontwikkelingen. Dit proefschrift voegt substantieel bewijs toe aan de vereiste Nederlandse criteria voor neonatale screening omtrent kennis over het natuurlijke beloop, voorspelling van de ziekte ernst en monitoring van MADD. We hebben ook aanzienlijk, systematisch bewijs geleverd over de klinische voordelen van behandeling met D,L-3-HB. De resultaten van **hoofdstuk 4** en **5** kunnen worden samengevoegd in een potentieel plan van aanpak met betrekking tot positieve testresultaten voor MADD bij de neonatale screening. Na identificatie door de neonatale screening kunnen functionele onderzoeken in fibroblasten een voorspelling van de ziekte ernst faciliteren. Op basis van het (voorspelde) fenotype kan een individuele behandelings- en monitoringstrategie worden voorgesteld. Aan de hand van verschillende patiënt scenario's kan worden aangenomen dat tijdig ingevoerde (dieet) behandelingsopties gecombineerd met het gebruik van noodprotocollen, kan leiden tot het voorkomen van substantiële morbiditeit en mortaliteit. Op basis van deze vooruitgang kan worden beredeneerd dat een heroverweging om MADD toe te voegen aan het Nederlandse hielprikscreening programma is geïndiceerd.

ABBREVIATIONS

3-HB	3-hydroxybutyrate
AcAc	acetoacetate
AIC	Akaike Information Criterion
AUC	area under the curve
BDH_1	D-3-hydroxybutyrate dehydrogenase
Cl_m	metabolic clearance
C_{max}	maximum concentration
CoA	coenzyme A
CPT2	carnitine palmitoyltransferase 2
DBS	dried blood spot
D,L-3-HB	D,L-3-hydroxybutyrate
DS3	disease severity scoring system
EMA	European Medicines Agency
ETF	electron transfer flavoprotein
ETFDH	electron transfer flavoprotein dehydrogenase
EU	European Union
FAD	flavin adenine dinucleotide
FAO	fatty acid oxidation
FFA	free fatty acid
IEM	inborn error of metabolism
IKH	idiopathic ketotic hypoglycemia
IQR	interquartile range
k_A	oral absorption rate constant
KB	ketone body
MADD	multiple acyl-CoA dehydrogenase deficiency
MCAD	medium-chain acyl-CoA dehydrogenase
MEB	Medicines Evaluation Board (i.e. ‘College ter Beoordeling van Geneesmiddelen’ in *Dutch*)
MCT	monocarboxylate transporters
MeSH	medical subject heading
MS/MS	tandem mass spectrometry
NBS	newborn blood spot screening
OMIM	online mendelian inheritance in man
PD	pharmacodynamic
PK	pharmacokinetic

PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RFVT	riboflavin transport
RIVM	*Dutch abbreviation* for the National Institute for Public Health and the Environment
RS	Reye syndrome
SID	sudden infant death
TCA	tricarboxylic acid
$t_{1/2}$	elimination half-life
t_{max}	time to reach the maximum concentration
UPLC	ultra-performance liquid chromatography
VLCAD	very long-chain acyl-CoA dehydrogenase
V_1	volume of distribution to the central compartment
WONHS	*Dutch abbreviation* for the Dutch Research Committee on Neonatal Screening
WSS	Weighed Sum of Squares

DANKWOORD

Promotieonderzoek is te vergelijken met een beklimming op de racefiets. Na een voorbereidende trainingsperiode, start je fris en vol goede moed beneden in het dal; je hebt zin om naar boven te ‘knallen’. Goed klimmen betekent echter je inspanning doseren. Een beklimming duurt immers vaak langer dan je denkt. En alhoewel de binnenbocht soms aantrekkelijk lijkt, is de buitenbocht meestal net wat minder steil. Wanneer je dan toch buiten adem raakt, is het verstandig om een tandje terug te schakelen en het juiste ritme weer te vinden. Uiteindelijk is het namelijk ook maar een kwestie van ‘blijven trappen’ tot de top. Vooral op je eigen tempo, want te lang boven je omslagpunt leidt tot verzuring. En onderweg natuurlijk niet vergeten om je heen te kijken en te genieten; in de afdaling flitst alles voorbij. Juist als de top dan eindelijk in zicht komt, blijkt dat er nog enkele heuvels te overwinnen zijn. Dan komt het aan op doorzettingsvermogen. Eenmaal boven, maken het uitzicht en de voldoening van een overwonnen klim alle inspanning meer dan waard.

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Willemijn van Rijt was born on October 12, 1992 in Zwolle, The Netherlands. She is the daughter of Han van Rijt and José Geerling and grew up in Zwolle together with her two brothers and sister. In 2011, she completed her secondary education at the Gymnasium Celeanum.

Willemijn then moved to Groningen in order to study medicine. During her bachelor, she successfully participated in the bachelor's honors program. In 2012, Willemijn started with her first pilot project at the Section of Metabolic Diseases, under supervision of dr. T.G.J. Derks & em. prof. dr. D.J. Reijngoud. This triggered her interest in the field of inborn errors of metabolism. Several subsequent pilot projects finally resulted in a PhD research proposal on multiple acyl-CoA dehydrogenase deficiency and population newborn screening. Willemijn successfully applied for the MD/PhD program of the Junior Scientific Masterclass at the University Medical Center Groningen, which allowed her to combine the Master phase of her study medicine with obtaining a PhD degree. She started her PhD studies in April 2016 under supervision of dr. T.G.J. Derks, prof. dr. F.J. van Spronsen, prof. dr. J.L.K. Van Hove and dr. M.R. Heiner-Fokkema. Upon completion of her clinical internships at the Treant Scheper Hospital in Emmen and her differentiation clerkships at the Beatrix Children's Hospital of the University Medical Center Groningen, Willemijn obtained her medical degree in December 2019.

In addition to her medical studies and research activities, Willemijn took place in the Workgroup 'Research Internships' of the International Federation of Medical Students' Associations and participated in the organization of the International Student Congress Of (bio)Medical Sciences (ISCOMS) during two years, including one year as Executive Board Member 'Scientific Program'.

Willemijn is currently living in Groningen, together with Albert Koller. In her free time, Willemijn likes to go cycling and enjoys cooking, photography, and traveling.

Foto: Caroline van der Schaaf, Foto Met Karakter

Scientific manuscripts

- **van Rijt WJ**, Heiner-Fokkema MR, du Marchie Sarvaas GJ, Waterham HR, Blokpoel RG, van Spronsen FJ, Derks TG. Favorable outcome after physiologic dose of sodium-D,L-3-hydroxybutyrate in severe MADD. Pediatrics. 2014;134(4):e1224-8.
- **van Rijt WJ**, Koolhaas GD, Bekhof J, Heiner Fokkema MR, de Koning TJ, Visser G, Schielen PC, van Spronsen FJ, Derks TG. Inborn errors of metabolism that cause sudden infant death: A systematic review with implications for population neonatal screening programmes. Neonatology. 2016;109(4):297-302.
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- van Eunen K, Volker-Touw CM, Gerding A, Bleeker A, Wolters JC, **van Rijt WJ**, Martines AM, Niezen-Koning KE, Heiner RM, Permentier H, Groen AK, Reijngoud DJ, Derks TG, Bakker BM. Living on the edge: Substrate competition explains loss of robustness in mitochondrial fatty-acid oxidation disorders. BMC Biol. 2016;14(1):107-016-0327-5.
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- **van Rijt WJ**, Ferdinandusse S, Giannopoulos P, Ruiter JPN, de Boer L, Bosch AM, Huidekoper HH, Rubio-Gozalbo ME, Visser G, Williams M, Wanders RJA, Derks TGJ. Prediction of disease severity in multiple acyl-CoA dehydrogenase deficiency: A retrospective and laboratory cohort study. J Inherit Metab Dis. 2019;42(5):878-889.
- **van Rijt WJ**, Jager EA, Allersma DP, Aktuglu Zeybek AC, Bhattacharya K, Debray FG, Ellaway CJ, Gautschi M, Geraghty MT, Gil-Ortega D, Larson AA, Moore F, Morava E, Morris AA, Oishi K, Schiff M, Scholl-Burgi S, Tchan MC, Vockley J, Witters P, Wortmann SB, van Spronsen F, Van Hove JLK, Derks TGJ. Efficacy and safety of D,L-3-hydroxybutyrate (D,L-3-HB) treatment in multiple acyl-CoA dehydrogenase deficiency. Genet Med. 2020;22(5):908-916.

- **van Rijt WJ**, Schielen PCJI, Özer Y, Bijsterveld K, van der Sluijs FH, Derks TGJ, Heiner-Fokkema MR. Instability of Acylcarnitines in Stored Dried Blood Spots: The Impact on Retrospective Analysis of Biomarkers for Inborn Errors of Metabolism. Int J Neonatal Screen. 2020;6(4):E83.

Invited speaker

- *D,L-3-HB in MADD - Novel steps towards a generally available treatment*. 'Erfelijke Stofwisselingsziekten Nederland' (ESN) Najaarssymposium 2019, Utrecht, the Netherlands

Oral presentations

- *Gaining insight in D,L-3-HB treatment in MADD - From bed to bench and back again*. AMGRO-meeting 2017, Lemmer, the Netherlands
- *Efficacy and safety of D,L-3-HB treatment in MADD*. INFORM Symposium 2017, Rio de Janeiro, Brazil
- *Efficacy and safety of D,L-3-HB treatment in MADD*. SSIEM Annual Symposium 2018, Athens, Greece
- *The pharmacokinetics and tissue deposition of sodium-D,L-3-HB*. AMGRO-meeting 2019, Lemmer, the Netherlands

Presentations at patient meetings

- *'MADD; op weg naar inclusie in de neonatale hielprikscreening?'*. MADD 2017, 'Volwassenen, Kinderen en Stofwisselingsziekten' (VKS), Groningen, the Netherlands
- *'D,L-3-hydroxybutyraat behandeling bij MADD - Nieuwe stappen richting een algemeen beschikbare behandeling'*. MADD/MCADD 2019, 'Volwassenen, Kinderen en Stofwisselingsziekten', Groningen, the Netherlands

Poster presentations

- *Favorable outcome after physiological dose of sodium-D,L-3-HB in severe MADD*. SSIEM Annual Symposium 2014, Innsbruck, Austria
- *Pediatric reference values of plasma acylcarnitines in supervised clinical fasting studies*. SSIEM Annual Symposium 2014, Innsbruck, Austria
- *Inborn errors of metabolism causing sudden infant death*. SSIEM Annual Symposium 2015, Lyon, France & INFORM Symposium 2015, Lyon, France
- *Toward an MADD - disease severity scoring system*. INFORM Symposium 2017, Rio de Janeiro, Brazil

- *Changes in pediatric plasma acylcarnitines upon fasting: improved interpretation of stress samples*. SSIEM Annual Symposium 2018, Athens, Greece
- *Clinical and functional description of disease severity in MADD*. INFORM Symposium 2019, Amsterdam, the Netherlands & SSIEM Annual Symposium 2019, Rotterdam, the Netherlands

Parameters of esteem

- Travel Grant INFORM Symposium 2015, Lyon, France
- Junior Scientific Masterclass - Ubbo Emmius Fonds Talent Grant 2017
- Travel Grant INFORM Symposium 2017, Rio de Janeiro, Brazil
- 'Erfelijke Stofwisselingsziekten Nederland' Incentive Grant 2017
- Poster Presentation Award & Travel Grant, INFORM Symposium 2019, Amsterdam, the Netherlands