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

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Summary
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. 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. 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-13C]C2-, C5-, and [U-13C]C16-acylcarnitines were identified as key variables that discriminated neonatal from later onset patients (all P < .05) and strongly correlated to MADD-DS3 scores.

Abbreviations: DS3, disease severity scoring system; ETF, electron transfer flavoprotein; FAO, fatty acid oxidation; IEM, inborn error of metabolism; MADD, multiple acyl-CoA dehydrogenase deficiency; NBS, newborn bloodspot screening.

Willemijn J. van Rijt and Sacha Ferdinandusse should be considered joint first author.
oleate: $r = -0.86$; myristate: $r = -0.91$; [U-13C]C2-acylcarnitine: $r = -0.96$; C5-acylcarnitine: $r = 0.97$; [U-13C]C16-acylcarnitine: $r = 0.98$, all $P < .01$). 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) and follow-up treatment appropriately. This is especially relevant in view of the inclusion of MADD in population newborn screening programs.

**KEYWORDS**
disease severity scoring system, fatty acid oxidation, functional fibroblast studies, multiple acyl-CoA dehydrogenase deficiency, prognostic marker

1 | INTRODUCTION

Multiple acyl-CoA dehydrogenase deficiency (MADD, or glutaric aciduria type II; MIM #231680) is an ultra-rare (ie, <1:50 000)1 mitochondrial fatty acid oxidation (FAO) disorder caused by pathogenic variants in the genes encoding the electron transfer flavoproteins (ETFs; ETFA or ETFB) or ETF dehydrogenase (ETFDH). The disrupted transfer of reduced flavin adenine dinucleotides toward 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 with/without congenital anomalies (type I/II) or 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 fibroblasts12,13 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.

2 | METHODS

2.1 | 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.

2.2 | 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 “PRI-SMA-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). 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.18 Contribution of disease domains and symptoms to the total MADD-DS3 score was weighed using their relation to MADD morbidity and mortality.

2.3 | 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 [9,10-3H]oleic acid and [9,10-3H]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-13C]palmitate and 0.4 mM L-carnitine at 37°C, 5% CO2, 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.

2.4 | Statistical analysis

Data analysis was performed using GraphPad Prism v7.02 (GraphPad Software, La Jolla, California) 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 <.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

3 | RESULTS

3.1 | 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 1.

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 (ie, ≥1 increased metabolite indicative of MADD). The glutaric aciduria type II-index, as defined by the New England Newborn Screening Program,7 could be calculated in four neonatal onset patients who all
<table>
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<th>Arrhythmias</th>
<th>Leukodystrophy</th>
<th>Other brain defects</th>
<th>Extra pyramidal symptoms</th>
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<th>Muscle weakness/hypotonia or $\geq 2$ PRO$^b$</th>
<th>Muscle insufficiency requiring mechanical ventilation</th>
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Note: $^a$Sibling pairs; $^b$hypospadia; $^c$obduction demonstrated periportal hepatic steatosis; $^d$including hyperammonemia, hyperbilirubinemia, hypoalbuminemia, coagulation disorders, and encephalopathy; $^e$including muscle weakness, myalgia, exercise intolerance, and fatigue.

Abbreviations: FS, family screening; NBS, newborn screening; PRO, patient-reported outcome; SUD, sudden unexpected death.
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.

### 3.2 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 Supporting Information Data S1. 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 ETFD (neonatal onset patients: 33% vs later onset patients: 3%, P < .0001) and ETFB (18% vs 1%, P < .0001). In contrast, ETFD variants were more frequently identified in later onset patients (48% vs 96%, P < .0001). The occurrence of two genetic variants expected to have a large effect on protein function (eg, nonsense and stop-loss variants, deletions, insertions, duplications, and splicing defects) was increased in neonatal compared to later onset patients (45% vs 1%, P < .0001). This was also significantly related to the incidence of congenital anomalies (85% vs 20%, P = .0004). In contrast, compound heterozygous missense variants were more frequently identified in later onset patients (30% vs 82%, P < .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% vs 3%, P < .0001; ie, cardiomyopathy, arrhythmias), central nervous system (12% vs 2%, P = .0041; ie, leukodystrophy), hepatic (92% vs 21%, P < .0001; ie, hypoglycemia, liver dysfunction/failure), and respiratory problems (38% vs 14%, P = .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% vs 93%, P < .0001), except for hypotonia which was reported more often in neonatal onset patients, as described in Supporting Information Data S1.

Nine health care professionals participated in our survey. Supporting Information Data S2 presents the data on the prioritization and selection of disease domains and symptoms to be included in the MADD-DS3. This resulted in (a) addition of the domains “congenital anomalies,” “patient reported,” and “age at onset,” and the symptom “cognitive impairment,” and (b) 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 each 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 Supporting Information Data S2.

The lifetime MADD-DS3 score of the MADD-patients included in the retrospective cohort ranged from 0 to 29, as presented in Table 1. 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) vs 4 (2-7), P = .0043).

### 3.3 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 = .0357; 1% (0-7%) vs 70% (57-108%), P = .0357, respectively). Acylcarnitine profiling in fibroblasts loaded with [U-13C]palmitate demonstrated significantly increased C5- and [U-13C]C16-acylcarnitine 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 hours, P = .0357; 18.6 (16.5-30.1) vs 1.6 (1.1-3.9) nmol/mg protein/96 hours, P = .0357, respectively). [U-13C]C2-, [U-13C]C4-, [U-13C]C6-, and [U-13C]C8-acylcarnitine 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 hours, P = .0357; 0.0 (0.0-0.2) vs 0.5 (0.4-1.7) nmol/mg protein/96 hours, P = .0179; 0.1 (0.0-0.1) vs 0.5 (0.4-1.5) nmol/mg protein/96 hours, P = .0357; and 0.1 (0.0-0.3) vs 1.1 (0.5-4.0) nmol/mg protein/96 hours, P = .0357, respectively). The principal component analysis model identified FAO flux activities, [U-13C]C2-, [U-13C]C4-, and [U-13C]C16-acylcarnitine 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.

### 3.4 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
<table>
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<th>Mutation allele 2</th>
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<td>2a</td>
<td>ETFA</td>
<td>c.797C&gt;T</td>
<td>p.T266M</td>
<td>c.73delA</td>
<td>p.Ile25X</td>
<td>11%</td>
</tr>
<tr>
<td>3a</td>
<td>ETFA</td>
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<tr>
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</tr>
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<tr>
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<td>c.242A&gt;C</td>
<td>p.H81P</td>
<td>c.242A&gt;C</td>
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</tr>
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<td>p.A63T</td>
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<tr>
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<td>94%</td>
</tr>
<tr>
<td>9</td>
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<td>c.1414G&gt;A</td>
<td>p.G472R</td>
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<tr>
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<td>p.P27S</td>
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<tr>
<td>11c</td>
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<td>c.1842G&gt;A</td>
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</tr>
<tr>
<td>13</td>
<td>ETFDH</td>
<td>c.881C&gt;T</td>
<td>p.T294I</td>
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<td>p.K590X</td>
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<tr>
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<td>p.A18Cifs</td>
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<td></td>
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</tr>
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<td>c.51dupT</td>
<td>p.A18Cifs</td>
<td></td>
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<td>103%</td>
</tr>
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**Note:** 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-acylcarnitine. Control values of acylcarnitine profiling in fibroblasts are presented in Supporting Information Data S3. Biochemical profiles indicative of MADD are indicated with a + sign. a,b,cSibling pairs; d molecular studies only performed in sibling; the glutaric aciduria type II-index, as defined by the New England Newborn Screening Program, was above 0.005 or below 0.005.

**Abbreviations:** AC, acylcarnitines; FAO, fatty acid oxidation; NR, not reported; PT, patient; UOA, urinary organic acid.
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,C. MADD-DS3 scores were also strongly associated with [U-13C]C2-, C5-, and [U-13C]C16-acylcarnitine (Pearson $r = -0.96; P = .0002$; Pearson $r = .97; P < .0001$; and Pearson $r = .98; P < .0001$, respectively). Oleate and myristate flux activity strongly correlated to [U-13C]C2- (Pearson $r = .82; P = .0121$; and Pearson $r = .93; P = .0009$, respectively), C5- (Pearson $r = -.88; P = .0044$; and Pearson $r = -.93; P = .0009$, respectively), and [U-13C]C16-acylcarnitine (Pearson $r = -.88; P = .0042$; and Pearson $r = .86; P = .0058$, respectively).

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-13C]C16-acylcarnitine levels and low medium- and short-chain acylcarnitines 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-acylcarnitine 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. 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. 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 toward 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

**FIGURE 3** Correlation between disease severity and fatty acid oxidation flux activity. Correlation between [9,10-3H]oleate and [9,10-3H]myristate FAO flux activities (A), and correlation between disease severity as defined by the MADD-DS3 scores and FAO flux activities measured with [9,10-3H]oleate (B), or [9,10-3H]myristate (C) in fibroblasts of three neonatal onset (○) and five later onset MADD-patients (●). r, Pearson correlation coefficient.
stress. 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. The used average scoring method eliminates biased estimates in case of missing items when completing the score. 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. 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 inferential 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. 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.

5 | 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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COMPETING INTERESTS

Willemijn J. van Rijt, Sacha Ferdinandusse, Panagiotis Giannopoulos, Jos P.N. Ruiter, Lonneke de Boer, Annet M. Bosch, Hidde H. Huidekoper, M. Estela Rubio-Gozalbo, Gepke Visser, Monique Williams, Ronald J.A. Wanders, and Terry G.J. Derks declare that they have no conflict of interest relevant to this article to disclose.

AUTHOR CONTRIBUTIONS

W.J.v.R. contributed to the design of the study, the data collection, data analysis and interpretation, drafted the initial manuscript, and critically revised the manuscript. S.F. contributed to the design of the study, the data collection, data analysis and interpretation, and critically reviewed and revised the manuscript. P.G. contributed to the data collection, data analysis and interpretation, and critically reviewed the manuscript. J.P.N.R. contributed to the data collection, and critically reviewed the manuscript. L.d.B., A.M.B., H.H.H., E.R.-G., G.V., Monique Williams contributed to the data collection, and critically reviewed the manuscript. R.J.A.W. contributed to the design of the study, the data collection, and critically reviewed the manuscript. Terry G.J. D. contributed to the design of the study, the data collection, data analysis and interpretation, drafted the initial manuscript, and critically revised the manuscript. All authors approved the final manuscript as submitted.

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DETAILS OF ETHICS APPROVAL AND PATIENT CONSENT STATEMENT

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). The study was approved.
for waived consent as it concerned retrospective, anonymous data. 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).

ANIMAL RIGHTS
This article does not contain any studies with animal subjects performed by any of the authors.

REFERENCES


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