



Diagnosis, genetic characterization and clinical follow up of mitochondrial fatty acid oxidation disorders in the new era of expanded newborn screening: A single centre experience

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ABSTRACT

Introduction: Mitochondrial fatty acid oxidation disorders (FAODs) are a heterogeneous group of hereditary autosomal recessive diseases included in newborn screening (NBS) program in Italy. The aim of this study was to analyse FAODs cases, identified either clinically or by NBS, for clinical and genetic characterization and to evaluate a five years' experience of NBS, in the attempt to figure out the complexity of genotype-phenotype correlation and to confirm the clinical impact of NBS in our centre experience.

Materials and methods: We analysed FAODs patients diagnosed either by NBS or clinically, followed since February 2014 to April 2019 at the Regional Screening Centre and Inherited Metabolic Diseases Unit of Verona. Diagnosis was confirmed by plasma acylcarnitines, urinary organic acids, enzymatic and genetic testing. For not clear genotypes due to the presence of variants of uncertain significance, in silico predictive tools have been used as well as enzymatic activity assays. Patients underwent clinical, nutritional and biochemical follow up.

Results: We diagnosed 30 patients with FAODs. 20 by NBS: 3 CUD, 6 SCADD, 5 MCADD, 4 VLCADD, 2 MADD. Overall incidence of FAODs diagnosed by NBS was 1:4316 newborns. No one reported complications during the follow up period. 10 patients were diagnosed clinically: 2 CUD, 2 CPT2D, 1 VLCADD, 5 MADD. Mean age at diagnosis was 29.3 years. Within this group, complications or symptoms were reported at diagnosis, but not during follow-up. 12 mutations not previously reported in literature were found, all predicted as pathogenic or likely pathogenic.

Discussion and conclusions: Our study highlighted the great phenotypic variability and molecular heterogeneity of FAODs and confirmed the importance of a tailored follow up and treatment. Despite the short duration of follow up, early identification by NBS prevented diseases related complications and resulted in normal growth and psycho-motor development as well.

Abbreviations: FAODs, fatty acid oxidation disorders; CUD, carnitine uptake defect; CPT1/2 D, carnitine palmitoyl-CoA transferase 1/2 deficiency; MCADD, medium-chain acyl-CoA dehydrogenase deficiency; VLCADD, very-long-chain acyl-CoA dehydrogenase deficiency; LCHADD, Long chain 3-hydroxyacyl-CoA dehydrogenase deficiency; TFPD, trifunctional protein deficiency; CACTD, carnitine-acylcarnitine translocase deficiency; SCADD, short chain acyl-CoA dehydrogenase deficiency; MADD, multiple acyl-CoA dehydrogenase deficiency; TMS, tandem mass spectrometry; DBS, dried blood spots; NBS, newborn screening; PCR, polymerase chain reaction; DNA, Deoxyribonucleic acid; NGS, next generation sequencing; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; CK, creatine kinase

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1. Introduction

Mitochondrial fatty acid oxidation is the major pathway for the degradation of fatty acids which are a crucial energy source for heart, skeletal muscle, and liver, especially when glucose supply is reduced as prolonged fasting, febrile illness or muscular exertion [1,2]. Mitochondrial fatty acid oxidation disorders (FAODs) are a heterogeneous group of genetic autosomal recessive diseases caused by the altered activity of the enzymes involved in the fatty acid transport and their oxidation in the mitochondria [3]. FAODs include carnitine uptake defect (CUD), carnitine palmitoyl-CoA transferase 1–2 deficiency (CPT1-2D), carnitine-acylcarnitine.

translocase deficiency (CACTD), very long chain acyl-CoA dehydrogenase deficiency (VLCADD), long chain hydroxyacyl-CoA dehydrogenase deficiency or mitochondrial trifunctional protein deficiency (LCHADD/MTP), medium chain acyl-CoA dehydrogenase deficiency (MCADD), medium/short chain hydroxyacyl-CoA dehydrogenase deficiency (M/SCHADD), short chain acyl-CoA dehydrogenase deficiency (SCADD), and multiple acyl-CoA dehydrogenase deficiency (MADD) [1–3].

These defects have a heterogeneous clinical presentation and different ages of onset related to enzyme involved, residual activity, and exposure to catabolic events throughout life [2–4]. Given the great importance of this metabolic pathway, especially in conditions of high energy demand, MCADD patients are at risk of developing severe hypoketotic hypoglycaemia which may progress to life-threatening Reye-like-Syndrome and a very poor outcome [5,6]. Moreover, VLCAD, LCHAD, CPT2, and CACT deficiencies can present cardio-muscular complications such as hypertrophic or dilated cardiomyopathy and conduction defects, myopathy with acute rhabdomyolysis or muscle weakness [1,4]. Even if the pathophysiological mechanism is still unknown, two putative pathophysiological mechanisms have been proposed for explain the muscular involvement in these patients. Both the energy shortage due to reduced ATP production and the toxic accumulation of acylcarnitines intermediates could play a role in the derangement of cellular metabolism [7,8]. Late onset forms of FAODs may present in the adolescence or adulthood and are mainly characterised by muscular signs and symptoms, such as fatigue, muscular pain, reduced exercise tolerance, and even a fatty liver disease [1,4]. Polyneuropathy and retinopathy are specific complications of LCHAD and MTP deficiencies [1].

Diagnosis of FAODs relies on acylcarnitines profile by tandem mass spectrometry (TMS) using plasma samples. Each defect has a specific acylcarnitine pattern. Confirmation is performed by molecular analysis or enzyme assay [9]. Testing of urine organic acids may be helpful for some FAODs and reveal a diagnostic pattern of dicarboxylic acids [10]. In the particular case of MADD, urinary organic acid analysis usually displays various combinations of increased dicarboxylic acids, glutaric acid, ethylmalonic acid, 2-hydroxyglutarate, and glycine conjugates, supporting the diagnosis [10,11].

FAODs treatment is mostly dietetic and consists in some common indications for all the defects, except for CUD (if under carnitine treatment), such as the fasting limitation, emergency regimen to apply during catabolic states, and other disease specific approaches, such as the restriction of dietary long-chain fatty acids for the long-chain fatty acid oxidation defects [4]. Carnitine, Riboflavin and coenzyme Q supplementation are indicated in specific disorders [12].

The introduction of newborn screening (NBS) had a dramatic impact in diagnosis of inborn errors of metabolism, including FAODs, allowing early detection and treatment of most cases, reducing the mortality and complications of these disorders [4,13,14]. Furthermore, due to NBS the number of diagnosis of FAODs rapidly increased, as well as the phenotypes variability of these defects. As a consequence, personalization of treatment, especially in case of mildly affected patient, become particularly challenging.

The aim of this study was to analyse FAODs cases identified either

clinically or by NBS for clinical and genetic characterization and to evaluate a five years' experience of NBS, in the attempt to figure out the complexity of genotype-phenotype correlation and to confirm the clinical impact of NBS in our centre experience.

2. Materials and methods

We retrospectively analysed FAODs patients diagnosed and followed from February 2014 to April 2019. NBS program for Inherited Metabolic diseases has been introduced from January 2014 in our Veneto Region, North East of Italy and from October 2016 in whole Italy.

Analysis of acylcarnitines and amino acids profiles were performed in the Laboratory of Regional Centre for the newborn screening, diagnosis and treatment of metabolic and endocrinological congenital diseases of Verona. For newborn screening of FAO, the biological sample used is a dried blood spot collected at 48–72 h of life. A 3,2 mm punch of blood spot sample was processed by mean of the NeoBase Non derivatized MSMS kit (Perkin Elmer, Wallac Oy) and analysed by multiple reaction monitoring (MRM) in a tandem mass spectrometer TQD detector (Waters) equipped with an ESI source positively charged. Confirmation of diagnosis was performed by the analysis of acylcarnitines profiles in plasma and eventually by genetic analysis and/or enzyme assay in lymphocytes or in fibroblasts.

Genomic deoxyribonucleic acid (DNA) was extracted from peripheral venous blood on EDTA by means of the QIAmp DNA Blood Mini kit (QIAGEN S.p.A, Milan, Italy) or with the High Pure PCR Template Preparation Kit (Roche, Mannheim Germany), following the manufacturer's instructions. All exons and part of the flanking intron regions of the *ACADM*, *ACADS*, *ETFDH*, *ETFA*, *ETFB*, and *SLC22A5* genes were amplified by polymerase chain reactions and sequenced for mutation analysis.

Since September 2016, for all FAODs patients identified by NBS, the following genes *ACADM*, *ACADVL*, *CPT1A*, *CPT2*, *ETFA*, *ETFB*, *ETFDH*, *FLAD1*, *HADHA*, *HADHB*, *SLC25A20*, *SLC25A32*, *SLC52A1*, *SLC52A2*, *SLC52A3* were analysed using a custom-designed Agilent Haloplex HS Panel, in the Medical Genetics Laboratory University of Padua, Italy. Libraries were prepared according to the manufacturer's protocol and run in an Illumina MiSeq sequencer. Results were analysed using the Agilent SureCall software. Copy number analysis was performed with the same software using the “Default Haloplex Copy-Number Method”. Variants were confirmed by Sanger Sequencing.

For each variant identified, the following databases available online have been used to verify if mutations were already reported in literature and likely to be pathogenic, such as 1000 genomes, dbSNP, ExAC, ClinVar, Leiden Open Variation Database (LOVD), Genome Variant Database for Human Diseases (GVDHD). If not previously reported in literature, in silico prediction analysis were performed for novel missense and splicing variants using the following softwares: PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>), SIFT (<http://sift.jcvi.org/>), Mutation Taster (<http://www.mutationtaster.org/>), Alternative Splice Site Predictor (ASSP) (<http://wangcomputing.com/assp/>) and human splicing finder (<http://www.umd.be/HSF/>).

The measurement of VLCAD and MCAD enzyme residual activity was performed in lymphocytes by assaying the oxidation rate of palmitoyl-CoA [15] and the oxidation rate of octanoyl-CoA [16], respectively, (Laboratory of Clinical Biochemistry and Metabolism, Centre for Paediatrics and Adolescent Medicine, Medical Centre, University of Freiburg, Germany). The residual activity of the patients was expressed as percent of the mean value of all controls.

The measurement of MAD residual activity in two patients was performed in cultured fibroblasts by assaying the oxidation rate of palmitoyl-CoA (Laboratory Genetic Metabolic Diseases, Amsterdam, Netherlands).

Three adult patients were referred to the Department of Neurology in Verona to investigate for myopathy. They underwent biopsy of vastus

lateralis muscle which showed lipid storage picture. Acylcarnitine profile and urinary organic acid analysis were performed. They haven't undergone enzyme assay.

Patients were started on dietary regimens and data on nutritional intake, maximal fasting time, medical food supplementation and dietary changes during illness and sports were collected. The nutritional treatment of LcFAODs patients is mainly based on the European consensus recommendations [17,18], while the indications for the other defects are based on case reports and on the published experience of other metabolic centres [19,20].

After the diagnosis some of the aspects of the diet like LCT restriction/MCT supplementation and maximal fasting time, were tailored to the patients taking into account clinical, biochemical presentation and follow up data. VLCADD and CPT2 patients follow a low LCT diet, supplemented with MCT and essential fatty acids. MCADD patients were restricted only in MCT from natural and medical food and the amount of LCT of the diet was not limited. MADD or GA2 patients were prescribed a moderately low fat diet and low protein diet. CUD and SCADD patients were not on special diet.

Emergency dietary regimen consists in immediate supplementation of readily absorbed carbohydrates (maltodextrines) in different concentrations according to age (<http://www.bimdg.org.uk/site/guidelines.asp>) and reduction of individual fasting time. If the patient is unable to drink or eat or is in critical clinical conditions hospital admission for parenteral glucose infusion is recommended.

All the FAODs patients received an emergency letter with these indications, except for CUD patients (if taking well oral carnitine) and the 3 patients with a very late onset MADD. No one patient needed the nocturnal tube feeding as the parents prefer to wake up and give the night feeds. All our FAODs patients received the indication to limit fasting time, except for CUD patients and clinically diagnosed adults with the other deficiencies. Maximal nocturnal fasting time was defined 2–3 h for neonates and infants and gradually increased from 6 months of age adding 1–2 h every year, based on general and metabolic biochemistry, in healthy clinical conditions. CUD patients were supplemented with carnitine and MADD subjects with carnitine, riboflavin and coenzyme Q [4,12].

Patients have been evaluated by metabolic specialist paediatrician, dieticians, geneticist, cardiologists and neurologists.

FAODs patients identified by NBS generally underwent a metabolic evaluation at diagnosis, at 6 months for weaning, at one year of age and

then every year. Clinical evaluations were changed as needed, especially for the beginning of weaning or in case of therapy changes.

For patients identified by NBS with defects at risk for cardiac involvement (e.g. VLCAD, CUD, CPT2 and MADD) we performed a baseline cardiological evaluation at diagnosis, at one year and then every 2 years. For patients identified clinically, we performed a cardiological evaluation at diagnosis, and then, if normal, every 2 years, otherwise as suggested by cardiologist.

The following data were evaluated: nutritional and growth assessments, symptoms and complications, number of acute decompensations and hospitalizations, use of emergency diet, blood chemistry values and acylcarnitine profiles. The psychomotor development wasn't assessed with standardized specific test, but it was part of a pediatric evaluation checking standard developmental milestone for age.

Hypoglycaemia was defined as blood glucose concentrations < 45 mg/dL (< 2,5 mmol/L); ammonia reference values were: newborn < 150 umol/L; infant-child < 70 umol/L, adult < 45 umol/L; metabolic acidosis was defined as pH < 7,35 and hypertransaminasemia when liver enzymes alanine and aspartate aminotransferase (ALT, AST) > 2 standard deviation from reference values.

Normal Plasma creatine kinase (CK) concentration between 20 and 180 U/L and acute rhabdomyolysis was defined as a plasma CK concentration > 1000 U/L [21].

3. Results

3.1. Clinical characterization and follow up

We diagnosed 30 patients with FAODs. 20 patients by NBS: 3 CUD, 6 SCADD, 5 MCADD, 4 VLCADD, and 2 MADD. In addition, we also identified 4 patients with 2 heterozygous mutations in 2 different genes of FAO metabolic pathway: 3 patients with *ACADM/ETFDH* mutations (two NBS detected siblings and the father with the same genotype) and 1 by NBS with *ACADVL/HADHA* genotype. Overall incidence of FAODs diagnosed by NBS was 1 in 4316 newborns. Female-to-male ratio was 11/20 (55%). None of above patients reported any complication during follow up period. They all showed normal growth and psycho-motor development. In addition, all patients except for MCADD and SCADD, had a complete routine cardiac evaluation including electrocardiography and echocardiography, without reports of cardiac abnormalities. MCADD and MADD required frequently emergency

Table 1
Clinical follow up in NBS and no-NBS patients.

Defect	Number of patients		Mean age at diagnosis (years)		Mean follow up duration		Symptoms or complications at diagnosis (n. of pts)		Complications during follow up (n. of pts)		Mean number of emergency treatments		Mean number of admissions to hospital	
					(years)	(months)								
NBS or not	No-NBS	NBS	No-NBS	NBS	No-NBS	NBS	No-NBS	NBS	No-NBS	NBS	No-NBS	NBS	No-NBS	NBS
CUD	2	3	18,5(20.5)	0	8(1.4)	18(18.6)	2	0	0	0	0	0	0	0
CPTIID	2	–	4.25(1.1)	–	5.5(3.5)	–	2	–	0	–	2(2.8)	–	1.5(2.1)	–
SCADD	–	6	–	0	–	37(22.0)	–	0	–	0	–	0	–	0,3(0.5)
MCADD	–	5	–	0	–	35(9.6)	–	0	–	0	–	3.6(4.3)	–	3(3.4)
VLCADD	1	4	20	0	23	25(16.0)	1	0	0	0	1	0.75(1.0)	1	0.75(1)
MADD	5	2	45.4(27.5)	0	8(10.2)	58(6.4)	5	0	0	0	0.2(0.4)	4.5(0.7)	0.2(0.4)	3.5(0.7)
MCADD/ MADD	1	2	44	0	1	32(18.4)	1	0	1	0	0	0	0	0
VLCADD/ LCHADD	–	1	–	0	–	15	–	0	–	0	–	0	–	0
Total count	11	23	30.6(25.5)	0	8.3(8.5)	32(18.1)	11	0	1	0				

The number of newborn screening and no-screening patients, the average age at diagnosis (in years) and the average follow-up duration (in months for NBS patients and in years for no-NBS patients) are described for each defect and expressed as mean (deviation standard). Numbers in the columns of symptoms and/or complications at diagnosis and during follow-up refer to the number of patients presenting symptoms or complications. For emergency treatment column, the number refers to the mean number of emergency protocol use for each group and column for admissions to hospital refer to the mean number of admissions for acute decompensation or to prevent acute decompensation during intercurrent diseases for each group, expressed as mean (deviation standard).

therapies and hospitalization to prevent acute decompensation, compared to other defects. The 5 patients with MCADD had a mean of 1 hospital admissions per year and 1.2 emergency treatment use per year. Respectively, the 2 patients with MADD had a mean of 0.7 admission to hospital per year and a mean of 0.9 of use of emergency treatment per year (Table 1). All recovered completely. No patients died or were lost at follow-up.

10 patients with clinical onset were diagnosed by our centre: 2 CUD, 2 CPT2D, 1 VLCADD, 5 MADD. Female-to-male ratio was 6/10 (60%). Diseases more frequently identified were SCADD for NBS and MADD for “no-NBS” group.

In “no-NBS” group symptoms and complications were reported at diagnosis, but during follow up clinical stabilization and remission were observed.

Symptomatic forms showed a late onset with myopathic picture that occurred in adulthood and a mean age at diagnosis of 29.3 years (± 26.5 standard deviation). MADD patients had a late onset and a late diagnosis with a mean age at diagnosis 45.4 years (± 27.5 SD). 4/5 presented with muscular weakness, except for one patient who was diagnosed at 14 years with recurrent vomiting with weight loss of about 20% of body weight, paraesthesia of the left inferior limb, metabolic acidosis, high liver transaminases. He also had bilateral hypoacusia, mild mental retardation and later developed a type 1 diabetes mellitus.

The two CPT2D patients had an earlier onset and they were diagnosed in their first years of life (mean age at diagnosis: 4.25 years) for recurrent exercise-induced rhabdomyolysis episodes. The patient with VLCADD was diagnosed for recurrent episodes of rhabdomyolysis at age 20.

The two CUD patients presented with muscular weakness and exercise intolerance (33 yrs), and dilated cardiomyopathy (4 yrs). Treatment with carnitine was effective for both (Tables 1 and 2).

3.2. Molecular analysis

The mutation analysis was performed in 34 patients (20/20 of the NBS group and 10/10 of the no-NBS group and, also in the 4 subjects with double heterozygosity). In 1/20 of the NBS group and in 1/10 of the no-NBS group analysis is still in progress. In 1 patient of the no-NBS group with MADD, the molecular analysis of the genes *ETFDH*, *ETFA*, *ETFB* didn't detected any variants. In 2 patients with MADD referred to the Department of Adult Neurology for suspected myopathy in their seventies, the molecular analysis showed only a single mutation in *ETFDH* gene and these cases have been already extensively described by Macchione et al. [22].

We identified 42 different mutations in 7 genes: 1 in *HADHA*, 5 in

ACADVL, 8 in *ACADM*, 8 in *ETFDH*, 9 in *ACADS*, 10 in *SCL22A5* and 1 in *CPT2* genes, (Table 3). 29 mutations were already reported in literature: 5 in *ACADVL*, 2 in *ACADM*, 5 in *ETFDH*, 9 in *ACADS*, 6 in *SCL22A5*, 1 in *CPT2*, and 1 in *HADHA* gene. On the other hand, 13 mutations were not previously reported in literature: 10 in the NBS group, 2 in the no-NBS group, and 1 in the familial case with the *ACADM/ETFDH* genotype. 11 predicted pathogenic or likely pathogenic by variants databases and prediction software's tools and 2 probably affecting a splicing site.

3.3. Enzymatic assay

We performed enzymatic assay in 14 patients: 5 VLCADD, 3 MCADD, 2 MADD, and, also in the 4 subjects with the genotype characterised by two heterozygous mutations in different genes of the FAO metabolic pathway (Table 4). In the no-NBS group the activity of patient with VLCADD was 5%. In NBS group enzymatic activity was tested in 4 VLCADD, 3 MCADD, and 2 MADD. All VLCADD showed activities between 10%–20%. The 3 MCADD showed a low activity (below 5%).

4. Discussion

Our study describes the clinical and the genetic features of 30 patients with FAODs, 20 identified by newborn screening and 10 on clinical grounds. The results support the statement that introduction of NBS revolutionized the diagnostic and therapeutic approach to FAODs. It made possible an early diagnosis with implementation of dietetic and therapeutic strategies to avoid the life threatening and chronic complications and it helped to identify the broad spectrum of FAODs.

None of our 20 patients identified by NBS developed metabolic complications during the follow up period, not even the cases with MCADD and VLCADD associated with significant low enzymatic activity ($< 10\%$). Growth and psychomotor development were normal. MCADD and MADD patients required more emergency therapies and hospitalizations to prevent acute decompensation during febrile illness or difficult feeding, compared to the others.

As regarding SCADD, we reported 6 patients identified only by NBS. They didn't develop any clinical symptom, nor required medical interventions. Currently there is a growing consensus on considering SCADD as a benign biochemical phenotype, rather than a disease. Meanwhile, further studies and long term follow up are needed to clarify the clinical relevance of SCADD phenotype and hence its inclusion in ENS programs [23].

Implementation of NBS had significantly reduced morbidity and mortality of FAODs, but also identified a great number of mildly

Table 2
Biochemical and clinical complications in NBS and no-NBS patients.

Defects	Number of patients		Hypoglycemia		High-CK		High-ALT/AST		Metabolic Acidosis		Cardiomyopathy/Arrhythmia		Myopathy/Rhabdomyolysis		Neuropathy	
	No-NBS	NBS	No-NBS	NBS	No-NBS	NBS	No-NBS	NBS	No-NBS	NBS	No-NBS	NBS	No-NBS	NBS	No-NBS	NBS
CUD	2	3	0	0	1	1	0	1	0	0	1	0	1	0	0	0
CPTII	2	–	0	–	1	–	1	–	0	–	0	–	2	–	0	–
SCADD	–	6	–	0	–	0	–	0	–	0	–	0	–	0	–	0
MCADD	–	5	–	2	–	0	–	2	–	1	–	0	–	0	–	0
VLCADD	1	4	0	0	1	2	0	2	0	0	0	0	1	0	0	0
MADD	5	2	0	1	2	1	2	0	1	0	0	0	4	0	0	0
MCADD/MADD	1	2	0	0	1	0	1	0	0	0	0	0	0	0	0	0
VLCADD/LCHADD	–	1	–	0	–	0	–	0	–	0	–	0	–	0	–	0

Numbers refer to the number of patients presenting at least once the biochemical and clinical complication reported in columns, at diagnosis (in the case of no-NBS subjects) or during follow up (NBS subjects).

Table 3
Mutation and molecular classification of all variants identified.

HADHA	Molecular consequence	Reported citation or pathogenic classification	ACADVL	Molecular consequence	Reported citation or pathogenic classification	ACADM	Molecular consequence	Reported citation or pathogenic classification	ETFDH	Molecular consequence
c.1528G > C p.Glu510-Gln	missense	Piekutowska-Abramczuk et al. 2010 [40]	c.1269 + 1G > A	splicing donor	Hoffmann et al. 2012 [26]	c.817_829del p.Ala273Leufs*7	nonsense	Likely pathogenic, ExAC 0.00001	c.524G > A p.Arg175His	missense
			c.1096C > T p.Arg366Cys	missense	Miller et al. 2015 [29]	c.388-14A > G	splicing	Likely pathogenic, ExAC 0.00008	c.1387G > C p.Gly463Arg	missense
			c.604C > G p.Leu202Val	missense	Miller et al. 2015 [29]	c.244insT	frameshift	Andresen et al. 2001 [42]	c.1448C > T p.Pro483Leu	missense
			c.848 T > C p.Val283Ala	missense	Goetzman et al. 2007 [41]	c.978G > A p.Met326Ile	missense	likely pathogenic, ExAC -	c.814G > A p.Gly272Arg	missense
			c.1500_1502del p.Leu502del	deletion	Bouvier et al. 2016 [28]	c.985A > G p.Lys329Glu	missense	Gramer et al. 2015 [30]	c.940G > A, p.Glu314Lys	missense
						c.30 + 1G > T	splicing	ExAC -, alteration of splicing site by Alternative Site Splicing Predictor and Human Splicing Finder softwares	c.521 T > C, p.Val174Ala	missense
						c.387del p.Gln130Lysfs*20	frameshift	likely pathogenic, ExAC -	c.1531G > A p.Asp511Asn	missense
						c.31-1G > A	splicing	ExAC -, alteration of splicing site by Alternative Site Splicing Predictor and Human Splicing Finder softwares	c.560C > T p.Ala187Val	missense

HADHA	Reported citation or pathogenic classification	ACADS	Molecular consequence	Reported citation or pathogenic classification	SCL22A5	Molecular consequence	Reported citation or pathogenic classification	CPT2	Molecular consequence	Reported citation or pathogenic classification
c.1528G > C p.Glu510-Gln	Yotsumoto et al. 2008 [37]	c.625G > A p.Gly209Ser	missense	common variant known to confer susceptibility to develop SCADD, Gallant et al. 2012 [35]	c.820_821del p.Trp274Valfs*5	frameshift	Likely pathogenic, Exac -	c.338C > T p.Ser113Leu	missense	Fanin et al. 2012 [55]
	likely pathogenic, ExAC -	c.1054G > A p.Ala352Thr	missense	Tonin et al. 2016 [43]	c.1048_1052 + 5-del CTGTGGTATG	deletion causes frameshift	Likely pathogenic, Exac -			
	Olsen et al. 2007 [33]	c.531G > A p.Trp177*	nonsense	Tonin et al. 2016 [43]	c.254_264dup GGCTCGCCACC	duplication causes frameshift	Shibbani et al. 2014 [48]			
	Chen et al. 2018 [34]	c.765G > T p.Gly255Gly	synonymous	Tonin et al. 2016 [43]	p.Ile89GlyfsTer45 c.1396A > G p.Ser466Gly	missense	Likely pathogenic, Exac -			

(continued on next page)

Table 3 (continued)

HADHA	Reported citation or pathogenic classification	ACADS	Molecular consequence	Reported citation or pathogenic classification	SCL22A5	Molecular consequence	Reported citation or pathogenic classification	CPT2	Molecular consequence	Reported citation or pathogenic classification
	likely pathogenic, ExAC	c.529 T > C p.Trp177Arg	missense	Pedersen et al. 2008 [44]	c.136C > T p.Pro46Ser	missense	Filippo et al. 2011 [49]; Rasmussen et al. 2014 [50]			
	likely pathogenic, ExAC	c.988C > T p.Arg330Cys	missense	van Maldegem et al. 2006 [45]	c.394-16 T > A	splicing	Rose et al. 2012 [51]			
	Macchione et al. 2019 [22]; Macchione et al. 2019 [22]	c.815G > A p.Arg272His c.1156C > T p.Arg386Cys	missense	van Maldegem et al. 2006 [45]; Merinero et al. 2006 [46]	c.667_69del TTC p.Phr23del c.254_264dup p.Ile89GlyfsX45	deletion duplication cause premature stop codon	Lamhomwah et al. 2002 [52]; Wang et al. 2001 [53]			
		c.136C > T p.Arg46Trp	missense	Pedersen et al. 2003 [47]	c.400C > G p.Leu134Val	missense	likely pathogenic, ExAC			
					c.338G > A p.Cys113Tyr	missense	Wang et al. 2014 [54]			

Molecular consequences (synonymous, missense, nonsense, splicing) are described; for each variant the citation, if the variant was already reported in literature, or the pathogenic classification by in silico prediction analysis with Exome Aggregation Consortium (ExAC) Browser allele frequency are reported.

affected patients who might never had developed clinical symptoms throughout life, increasing the prevalence and the clinical heterogeneity of these defects [4,14,24]. In literature an incidence of 1:9000 newborns was described [14], even if a few recent studies have reported higher incidence [24,25]. This trend was confirmed by our study with 1 newborn in 4316.

Regarding the no-NBS group, all patients showed at diagnosis a clinical phenotype. Following treatment, symptoms remission and clinical stabilization in all patients was noted. Particularly, in the two CUD patients the treatment with carnitine not only resolved symptoms, but also reverted the complications, such as the cardiomyopathy found at the diagnosis in one case. In the 5 symptomatic MADD adults, riboflavin and L-carnitine treatment improved their clinical picture and their biochemical profile.

4.1. Molecular analysis, enzymatic essays and genotype-phenotype correlation

The NBS not only has modified the diagnosis of FAODs increasing the clinical heterogeneity, but also has increased the molecular heterogeneity of these defects. This added further complexity to the genotype-phenotype correlation in FAODs. Predict the severity and the age of the onset of a phenotype is pivotal to design a personalized follow up and treatment. This represents a big challenge for FAODs, also because the age of onset and the clinical phenotype are widely conditioned by the exposure to catabolic stresses throughout life. Correlation between residual enzyme activity and clinical presentations of MCAD and VLCAD deficiencies has been shown, providing a further understanding and a better classification of the patients, especially those identified by NBS [26,27]. Patients with a residual activity < 10% have clinical symptoms. Patients with a residual activity > 20% might never manifest severe symptoms, just a mild biochemical phenotype. Finally, for patients with a residual activity between 10%–20% there's still not enough evidence about the effect of catabolic stress during intercurrent illness as trigger to possible severe symptoms. Considering the residual enzymatic activity in 4 VLCADD patient in our NBS group, which resulted between 10%–20%, there is no sufficient evidence and follow up and treatment as well are currently recommended [27]. These values of residual activity could be indicative of a late onset phenotype with a prevalent muscular expression.

On the other hand, in the case of symptomatic VLCADD patient, the homozygous mutation c. 1500_1502del (p.Leu502del) was already reported in literature in a patient of 9 years with exercise intolerance with cramps and myoglobinuria triggered by fasting and cold and residual activity in fibroblast of VLCAD of 7% [28]. Our patient with the same mutation and a clinical history of recurrent episodes of rhabdomyolysis in his first two decades of life, had residual activity of 5%, supporting this genotype-phenotype correlation.

The 3 MCADD patients identified by NBS showed activity below 5%, the lowest enzyme activity between the whole group of FAODs tested. According to literature [26], activity < 10% correlates with a subject certainly symptomatic if not treated and indeed, in our experience, MCADD patients required the largest number of preventive admissions to the hospital.

We identified 3 mutations in ACADVL, ACADM and CPT2 genes frequently reported in literature. Particularly, the c.848 T > C (p.V283A) variant of ACADVL gene was detected in homozygosis in 2 out of 4 NBS -VLCADD patients. Miller et al. reported that at least one copy was found in ~10% of all individuals with a positive NBS. This mutation in literature correlate with a late onset mild phenotype with mild muscular symptomatology, which responds well to standard treatment and present rare hypoglycaemic events [29]. The residual enzyme activity of 12% found in our two patients confirmed this statement and correlated with previously reported enzymatic studies using lymphocytes, which found 11% and 12% residual activity in two individuals homozygous for the same p.V283A variant [27].

Table 4
Mutation analysis and residual enzymatic activity in patients with MCADD, VLCADD and MADD.

ID Patient	NBS or No-NBS	Disease	Gene	Mutation 1	Mutation 2	Enzymatic activity
Pt 1	NBS	VLCADD/LCHADD	ACADVL	c.1269 + 1G > A	–	45%
Pt 2	NBS	VLCADD	ACADVL	IN PROGRESS	IN PROGRESS	10%
Pt 3	NBS	VLCADD	ACADVL	c.1096C > T p.Arg366Cys	c.604C > G p.Leu202Val	18%
Pt 4	NBS	VLCADD	ACADVL	c.848 T > C p.Val283Ala	c.848 T > C p.Val283Ala	12%
Pt 5	NBS	VLCADD	ACADVL	c.848 T > C p.Val283Ala	c.848 T > C p.Val283Ala	12%
Pt 6	NBS	MCADD	ACADM	c.817_829del p.Ala273Leufs*7	c.388-14A > G	4%
Pt 7	NBS	MCADD	ACADM	c.244insT	c.978G > A p.Met326Ile	0%
Pt 8	NBS	MCADD	ACADM	c.985 A > G p.Lys329Glu	c.985 A > G p.Lys329Glu	0%
Pt 11	NBS	MCADD/MADD	ACADM	c.31-1G > A	–	35%
Pt 12	NBS	MCADD/MADD	ACADM	c.31-1G > A	–	45%
Pt 13	NBS	MADD	ETFDH	c.1387G > C p.Gly463Arg	c.1387G > C p.Gly463Arg	C10,C12,C14,C16
Pt 14	NBS	MADD	ETFDH	c.1448C > T p.Pro483Leu	c.814G > A p.Gly272Arg	C5,C12,C14
Pt 24	No-NBS	VLCADD	ACADVL	c.1500_1502del p.(Leu502del)	c.1500_1502del p.(Leu502del)	5%
Pt 27	No-NBS	MCADD/MADD	ACADM	c.31-1G > A	–	49%

The measurement of VLCAD and MCAD enzyme residual activity was performed in lymphocytes by assaying the oxidation rate of palmitoyl-CoA and the oxidation rate of octanoyl-CoA, respectively. The residual activity of the patients was expressed as percent of the mean value of all controls. The measurement of MAD residual activity was achieved in fibroblasts by assaying the oxidation rate of palmitoyl-CoA.

The most common *ACADM* mutation of our case study was the c.985A > G (p.Lys329Glu) that was found in homozygosity in 1 patient and in heterozygosity in another one, both identified by NBS. This is the most common mutation in patients with MCADD reported in literature and occurs at a frequency up to 90% of disease alleles in symptomatic MCADD patients of European origin [30,31].

The two CPT2D patients clinically identified showed in homozygosity the missense variant c.338C > T (p.Ser113Leu), the most frequent *CPT2* pathogenic mutation reported in Caucasians, found in up to 90% of symptomatic patients. Around 60% - 70% of the patients with CPT2D are homozygous for the c.338C > T mutation [32].

The c.1448C > T (p.Pro483Leu) and c.814G > A p.Gly272Arg mutations of the *ETFDH* gene, found in compound heterozygosity in one of the two MADD patients identified by NBS, were already described in literature as responsive to riboflavin therapy [33,34].

Among the 6 SCADD patients identified by NBS, 3 of them have the susceptibility variant c.625G > A p.Gly209Ser [35].

We identified 13 mutations not previously reported in literature, all predicted pathogenic or likely pathogenic by variants database and prediction tools.

The molecular analysis alone may be inconclusive in cases of novel mutations or genetic variants for which no information on functional and clinical relevance is given [15]. In these cases, the enzymatic assays might be pivotal in predict the phenotype and the severity of the symptomatology, especially in subjects identified by NBS. Indeed, in the two NBS cases with MCADD with the mutations c.978G > A (p.Met326Ile), c.388-14A > G and c.817_829del (p.Ala273Leufs*7) not previously reported in literature but predicted likely pathogenic, the enzymatic assay of MCAD resulted 0%–4% confirming the predictions of molecular pathogenicity.

4.2. Synergistic heterozygosity

Usually the FAODs are autosomal recessive defects and the heterozygous subjects don't manifest the disease symptomatology. Patients with a manifested phenotype and/or with an altered biochemical pattern with a monoallelic mutation and without a complete functional deficiency of the expected enzyme were described in literature [36,37]. These patients presented concomitant monoallelic mutations in different genes encoding for different enzymes of the same metabolic pathway. This phenomenon was defined synergistic heterozygosity [38]. In our case study we identified 4 patients with a genotype characterised by 2 mutations in heterozygosity in 2 different genes of the same metabolic pathway that could be considered as cases of synergistic heterozygosity. A familiar case with two heterozygous

mutations, one in *ACADM* and another in *ETFDH* gene, composed by two siblings found by NBS and the father with a very similar biochemical pattern. The father was 44 years old at the diagnosis and never referred symptoms with the exception of the evidence of a fatty liver at the ultrasound performed after diagnosis, in absence of other known causes of steatosis. The acylcarnitine profile showed an increase in C8, C10, and C14:1 with no alteration of organic acids in the urine analysis (Supplementary Tables). The genotype was characterised by the single mutation c.31-1G > A in the gene *ACADM* (not previously reported in literature and predicted to affect the splicing site with high probability) and the pathogenic mutation c.524G > A (p.Arg175His) of the gene *ETFDH*, already reported in literature [39]. We performed in these subjects the enzymatic activity of MCAD that resulted between 35% and 49%, confirming the state of heterozygosity of the gene *ACADM* and therefore, the pathogenicity of the c.31-1G > A variant.

The fourth case that could be considered a synergistic heterozygosity is a patient diagnosed by NBS with a VLCADD/LCHADD biochemical phenotype. He presented the monoallelic splicing mutation c.1269 + 1G > A of the gene *ACADVL* and the missense mutation c.1528G > C (p.Glu510Gln) of the *HADHA* gene, both already reported in literature likely pathogenic for their respective diseases [27,40].

The acylcarnitines profile of neonatal DBS showed an increase in C14:1, C14-OH, and other long chain acylcarnitines, in particular C16-OH and C18-OH (Supplementary Tables), supporting a synergistic heterozygosity biochemical phenotype.

The three patients identified by NBS didn't developed any metabolic complications during the follow up period, neither necessitated of emergency therapy or hospitalization (Tables 1 and 2). To date, literature available data are very scanty about these newly genotypes and their clinical relevance. Further investigations are necessary to clarify the role of synergistic heterozygosity in FAODs.

5. Conclusion

NBS had started a new era in the field of mitochondrial FAODs. A true change in the course of many of these diseases became possible, improving morbidity and mortality by implementing prevention strategies. Moreover, NBS has enabled the detection of patients with milder phenotype, increasing the prevalence and the clinical heterogeneity of these defects. Achieving an adequate follow up and treatment for these patients is challenging and pivotal in the context of an ever more extended newborn screening program. The follow up period in our current study was short and life-long follow up data are required for understanding the incidence of long-term complications. Only sharing

databases and information experiences at an international level will provide a better characterization of these defects and a deeper understanding of the genotype-phenotype correlation in order to guarantee an early stratification of the risk and a personalized follow up and treatment.

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Ethical standards

Written informed consent was obtained from the patients.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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