Original Article

Mutation Spectrum of Primary Lipid Storage Myopathies

Seena Vengalil, Kiran Polavarapu, Veeramani Preethish-Kumar, Saraswati Nashi, Gautham Arunachal[1], Tanushree Chawla, Mainak Bardhan, Dhaarini Mohan[2], Rita Christopher[3], Nandeesh Bevinahalli[4], Karthik Kulanthaivelu[5], Ichizo Nishino[6], Mohammad Faruq[7], Atchayaram Nalini

Departments of Neurology, [1]Human Genetics, [2]Neurology, Department of Clinical Neurosciences, [3]Neurochemistry, [4]Neuropathology and [5]Neuroimaging and Interventional Neuroradiology, National Institute of Mental Health and Neurosciences, Bengaluru, Karnataka, India, [6]Department of Neuromuscular Research, National Centre of Neurology and Psychiatry, Kodaira, Tokyo, Japan, [7]Department of Genomics and Molecular Medicine, CSIR-Institute of Genomics and Integrative Biology, Delhi, India

Abstract

Background: Lipid storage myopathies (LSM) constitute an important group of treatable myopathies. Genetic testing is essential for confirming the diagnosis and also helps in explaining phenotypic heterogeneity. The objective of this study was to describe the clinical features and genetic spectrum of LSM seen in a quaternary referral center in India. **Methods:** Eleven cases of suspected LSM underwent clinical, biochemical, histopathological and genetic evaluation. Tandem Mass Spectrometry and clinical exome sequencing with Sanger validation were performed. **Results:** All patients had exertion induced myalgia and either progressive or episodic limb girdle muscle weakness (LGMW). The age of onset ranged 10 to 31 years (mean- 21 ± 6.7y), age at presentation- 14 to 49 years (mean- 26.5 ± 9.5y). Mutations identified: *ETFDH* = 5, *CPT2* = 3, *FLAD1* = 1, *ACADVL* = 1, *PNPLA2* = 1. Dropped head syndrome was seen in two patients with *ETFDH* mutations. Bulbar symptoms and Beevor's sign were noted in a patient with FLAD1 variant. Novel variants were identified in seven patients. **Conclusions:** This is the first report on the genetic spectrum of LSM from India. LSM should be considered in patients with exertion induced myalgias, LGMW, cranial nerve involvement or dropped head syndrome. Genetic testing is essential for identification of these treatable disorders.

Keywords: Dropped head syndrome, limb girdle weakness, lipid storage myopathy, mutation spectrum

Introduction

Disorders of lipid metabolism are a heterogeneous group of diseases with autosomal recessive inheritance. They encompass a spectrum ranging from Reye-like syndrome, seizures, mental retardation, cardiomyopathy, neuropathy to recurrent myoglobinuria and less commonly progressive lipid storage myopathy (LSM).[1] A long delay in diagnosis is not uncommon as many patients remain asymptomatic except for manifestations during fasting or prolonged exercise and routine investigations and Tandem mass spectrometry (TMS) mostly remain normal in between acute episodes.[1] Genetic testing is crucial to identify the gene defect, which help in explaining phenotypic heterogeneity.

Only a few studies describe the genetic mutations in lipid metabolism/fatty acid oxidation (FAO) disorders with myopathy and no reports are from India. In this study, we report the mutation spectrum of a series of eleven cases of LSM.

Methods

Informed consent and institutional ethics committee approval were obtained. Eleven individuals with suspected LSM, were evaluated in a quaternary referral center in South India from 2011 to 2019. The inclusion criteria consisted of: (a) patients with symptoms suggestive of LSM– myalgias or muscle weakness precipitated or worsened by exertion, fasting or stress; or (b) TMS or muscle biopsy suggestive of LSM and genetic testing confirming the same. Detailed clinical evaluation and investigations including creatine kinase (CK) were done. Free carnitine and acyl carnitines were measured using a triple and quadruple liquid chromatography tandem mass spectrometer in dried blood spot specimens (Quattro Micro API triple quadruple system, Waters Corporation, Milford, MA 01757 USA). The muscle imaging protocol consisted of axial T1 weighted, T2W, axial Short Tau Inversion Recovery (STIR)/T2W axial fat saturation sequences. The lower limbs were imaged from iliac crest to medial malleoli as three stations. The section thickness was 5 mm with interslice gap of 5 mm. Fatty infiltration was assessed using Mercuri scoring in T1W images and myoedema as per Borsato and Carlo staging on STIR images. Muscle biopsy obtained from either quadriceps or biceps was observed under Oil Red 'O' stains. Clinical exome sequencing (CES) followed by Sanger validation was performed in all patients.

Address for correspondence: Dr. Atchayaram Nalini, Professor of Neurology, Neuromuscular Specialist, Department of Neurology, Neuroscience Faculty Center, National Institute of Mental Health and Neurosciences, Bengaluru - 560 029, Karnataka, India.
E-mail: atchayaramnalini@yahoo.co.in

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Results

11 patients were confirmed to have LSM by mutation studies. Nine were males. The mean age at onset was 21.3 ± 6.7 years (10-31) and at presentation was 26.5 ± 9.53 years (14 – 49). The mean duration of illness was 69 ± 59 months, of weakness was 33.8 ± 7.13 months and of myalgias was 62.6 ± 29 months. The time to diagnosis ranged from 3 months to 22 years. All patients had exertion induced myalgia with limb girdle muscle weakness (LGMW) except for Patient 8 who had episodic weakness precipitated by exertion. Genetic testing revealed mutations in Electron Transport Flavoprotein Dehydrogenase *(ETFDH)* in 5, Carnitine Palmitoyl Transferase II (CPT2) deficiency in 3, Flavin Adenine Dinucleotide Synthetase1 *(FLAD1),* Very Long Chain Acyl CoA Dehydrogenase *(ACADVL*) and Patatin Like Phospholipase Domain Containing 2 (*PNPLA2)* gene in one each. All patients had recessive pathogenic or likely pathogenic variants as per American college of medical genetics (ACMG) Richards classification,[2] except P7 who had a single heterozygous CPT 2 missense variant of uncertain significance. All 3 patients with CPT 2gene mutations and the single patient with *ACADVL* mutation had recurrent myoglobinuria precipitated by physical exertion. Head drop was seen in 2 patients with *ETFDH* mutations [Figure 1 a, b]. *ETFDH* patients also had symptoms of abdominal pain, recurrent vomiting, hypophonia of speech and weight loss. Beevor's sign was noted in *FLAD1 case* [Figure 1c]. Table 1 summarizes the clinical features of the patients.

Creatine kinase (CK) values ranged from normal to 10 fold elevation (mean - 1161.3 ± 804 IU/L). TMS was done in 9/11 patients and abnormalities were demonstrated in four. Muscle biopsy, performed in 8 cases, confirmed the diagnosis of LSM in 5 cases with presence of Oil Red 'O' positive vacuolated fibers [Figure 2a-i]. Magnetic resonance imaging of muscle was done in four patients which demonstrated fatty changes in gluteus maximus in all while two had early fatty infiltration in anterior and posterior thigh and leg muscles. Myoedema was seen in gluteus maximus, quadriceps and hamstrings in one patient and posterior leg muscles in two patients [Figure 3]. Table 2 summarizes the biochemical, imaging and histopathological findings.

The genetic mutations in each category are shown in Table 3.

Patients were advised to avoid precipitating factors and to consume high carbohydrate, low fat diet. Patients with *ETFDH* mutations showed good response to 300 mg/day of oral riboflavin. Exertional myalgia and limb girdle weakness improved in all.

Discussion

Disorders of lipid metabolism are a heterogenous group of disorders. In the current report we describe the genetic diversity of 11 cases of LSM. For ease and clarity, the discussion is based on specific categories.

ETFDH

ETFDH mutations cause Multiple Acyl CoA Dehydrogenase deficiency type III (MADD-III/RR-MADD) and constituted around 50% of our cases. In a study from Iran,[3] 65% cases with suspected LSM had *ETFDH* mutations. In cases of MADD with chronic symptoms, exercise intolerance, myalgias and muscle weakness are predominant.[4] All our patients had exertion induced myalgia and LGMW. Two patients had an unusual finding of head drop, which has been documented in few case reports.[5,6] The mean age of onset and presentation of our patients are similar to previous report.[3] None of our patients had breathing difficulty in contrast to the Iranian study where 78.9% had respiratory insufficiency.[3] Two of our patients had abdominal pain and weight loss, which have also been reported by Liang *et al*.[7] Recurrent vomiting was reported by one of our patients whereas 6 in the Iranian cohort had nausea and vomiting.[3]

CK values ranged from 195 to1394 IU/L which is in concordance with other studies.[8,9] Tandem mass spectrometry done in 3 of the 5 patients showed abnormality in two and was normal in one. This highlights the need for genetic confirmation. Muscle biopsy was diagnostic of lipid storage myopathy in two cases, unlike in previous studies where all the suspected cases showed lipid accumulation.[8] Muscle MRI done in two of our cases showed fatty changes in gluteus maximus and quadriceps, soleus in one, while myoedema was observed in soleus and gastrocnemius in one. Hong *et al*.,[9] have demonstrated a dis-similar pattern with edema and fatty

Figure 1: (a and b) Dropped head syndrome (ETFDH) (c) Patient of *FLAD1* with Beevor's sign

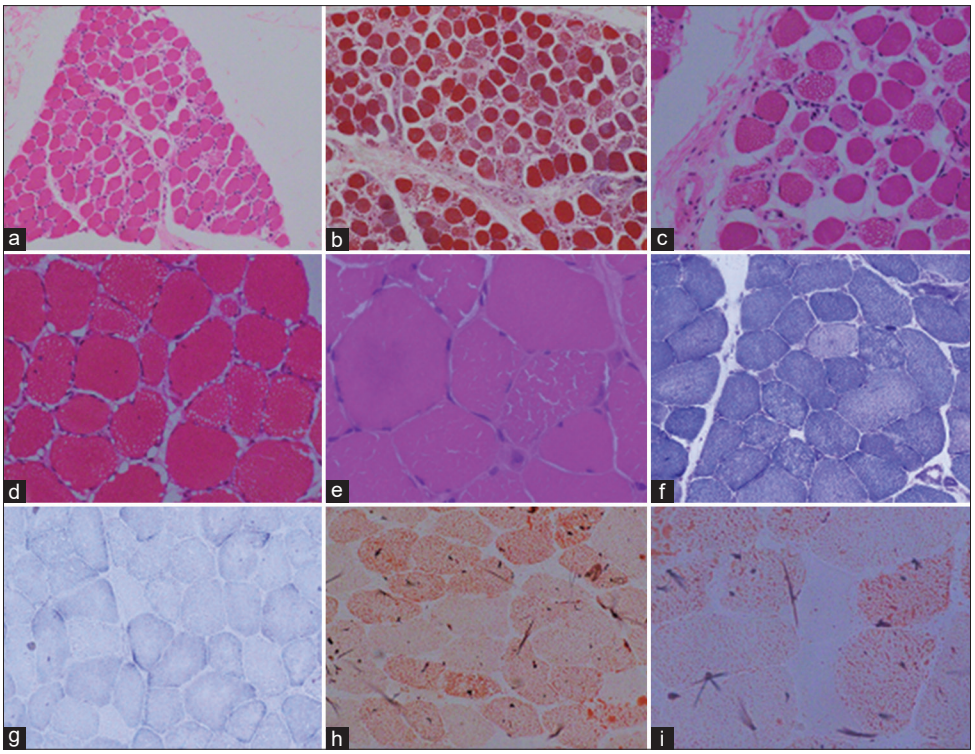

Figure 2: (a-i) Microphotograph showing transverse sections of the muscle with preserved architecture and showing vacuolar change in scattered muscle fibers. (a) H & E X 100. (b) Masson Trichrome stain X 100.- Vacuolar changes seen in few muscle fibers (c, d) Prominent vacuolar change in scattered muscle fibers. H & E X 200. (e) Variation in fiber size and vacuolar change in scattered muscle fibers. H & E X 400. (f, g) vacuoles seen on oxidative enzyme stains. F- NADH, G – SDH, X200. (h, i) The vacuoles show Oil Red O stain deposits. Oil Red O stain; H -X 200, I – X 400

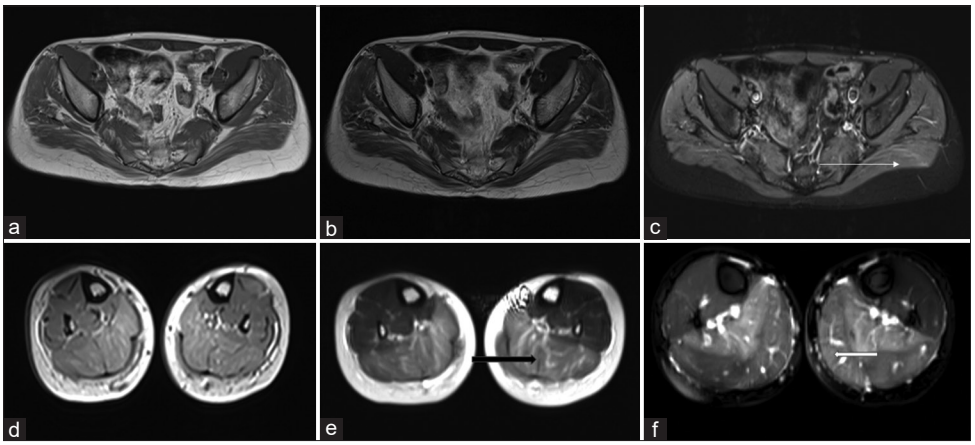

Figure 3: (a-c) (Patient 1- *ETFDH*): Axial T1 (a), T2 (b), PD fat saturated (c) images of the pelvis shows mild atrophy of the gluteus maximus with no significant edema (white arrow). (d-f) (Patient 4- *ETFDH*): Axial T1 (d), T2 (e), PD fat saturated (f) images of the legs show moderate edema in (Grade 3) in bilateral soleus and gastrocnemius (solid arrows)

infiltration more in biceps femoris longus, semimembranosus and adductor magnus in thigh muscles and in soleus, tibialis anterior and tibialis posterior in leg muscles.

Three of our patients had homozygous missense mutations and two had compound heterozygous mutations. More than 640 patients with MADD III have been reported worldwide with molecular analysis performed for 523 patients, 187 mutations identified so far.[10] However, the variants identified in our patients have not been recorded in genetic databases except for the mutation, c.152G >A (p.Arg51Gln)- in exon2 in P5.[11] The majority of mutations reported worldwide are missense followed by frameshift, splice site variations and nonsense mutations.[10] All patients having MADD carry at least one missense mutation[10] and our patients had a similar finding. Authors from China have reported a large series of ETFDH mutations, with c.250G >A (p.Ala84Thr) identified as founder mutation in 28% of cases.[12] We did not find this variant. Other common variants reported are c.770A >G, c.1227A >C (8.9%).[12] c.1130T >C[12] and c. 1130T >C[3]

Table 1: Salient clinical features in the 11 patients

Patient Number	Sex	Consanguinity	Family History	Age at onset (yr)	Age at presentation (yr)	Initial symptom	Exertional myalgia	Limb Girdle weakness	Myoglobinuria	Head Drop	Systemic symptoms
P1 (ETFDH)	M	No	No	24.5	25	Lower limb proximal weakness	Yes	Yes	No	No	Hypophonia , Weight loss
P2 (ETFDH)	F	Yes	No	16	17	Proximal limb weakness and exertional myalgia	Yes	Yes	No	No	Nil
P3 (ETFDH)	M	No	Yes	10	14	Exertional myalgia	Yes	Yes	No	Yes	Recurrent abdominal pain, vomiting, weight loss
P4 (sibling of P3)(ETFDH)	M	No	Yes	17	23	Exertional myalgia	Yes	Yes	No	Yes	Nil
P5 (ETFDH)	M	No	No	26.5	29	Exertional myalgia	Yes	Yes	No	No	
P6 (CPT2)	M	Yes	No	29	33	Exertional myalgia and proximal limb weakness	Yes	Yes	Yes	No	Nil
P7 (CPT2)	M	No	No	27	49	Exertional myalgia	Yes	Yes	Yes	No	Nil
P8 (CPT2)	M	No	No	26	26	Exertional myalgia, rhabdomyolysis	Yes	Yes (episodic)	Yes	No	Nil
P9 (FLAD1)	M	No	No	31	36	Exertional myalgia	Yes	Yes	No	No	Bulbar symptoms
P10 (ACADVL)	F	Yes	No	11	18	Exertional myalgia	Yes	Yes	Yes	No	Nil
P11 (PNPLA2)	M	No	Yes	21	29	Asymmetrical proximal upper and lower limb weakness	Yes	Yes	No	No	Nil

Table 2: Salient features in biochemical, muscle MR imaging and histopathology

Parameters	P1 (ETFDH)	P2 (ETFDH)	P3 (ETFDH)	P4 (ETFDH)	P5 (ETFDH)	P6 (CPT2)	P7 (CPT2)	P8 (CPT2)	P9 (FLAD1)	P10 (ACADVL)	P11 (PNPLA2)
CK (IU/L)	1079	1394	195	1233	1385	873	449	2559	2691	144	772
MRI T1 - Fatty infiltration (grade)											
Gluteus maximus	1	-	-	1	-	-	1	-	2a	-	-
Quadriceps	0	-	-	1	-	-	2a	-	2a	-	-
Hamstrings	0	-	-	0	-	-	2b	-	2a	-	-
Adductors	0	-	-	0	-	-	2a	-	2a	-	-
Anterior leg	0	-	-	0	-	-	0	-	2a	-	-
Posterior leg	0	-	-	1	-	-	1	-	2a	-	-
STIR -Myo-edema											
Gluteus Maximus	0	-	-	0	-	-	0	-	2a	-	-
Quadriceps	0	-	-	0	-	-	2b	-	0	-	-
Hamstrings	0	-	-	0	-	-	2b	-	0	-	-
Adductors	0	-	-	0	-	-	2a	-	0	-	-
Anterior leg	0	-	-	0	-	-	0	-	0	-	-
Posterior leg	0	-	-	3	-	-	1	-	0	-	-
TMS	Short and medium chain acyl carnitine elevated	NA	Increase in long chain acyl carnitines	Normal	NA	Normal	Normal	Normal	Short, medium and long chain acyl carnitine elevated	Tetradocenoyl carnitine elevated	Normal
Muscle biopsy											
Fibre size variation	Yes	NA	Yes	NA	-	Yes	Yes	NA	-	No abnormality	-
Positive Oil Red 'O'staining	-	NA	Yes	NA	Yes	-	Yes	NA	Yes	-	Yes

- Means absent or normal, NA means not available

Table 3: Mutation spectrum of 11 cases of LSM

Gene	Mutations identified	ACMG Classification
ETFDH (ENST00000511912.1)	P1- c.818T>C (p.Ile273Thr) -homozygous	LP
	P2- c.829dupG (p.Glu277GlyfsTer6), c.853A>G (p.Asn285Asp)- compound heterozygous	P, LP
	P3 and P4- c.587C>A (p.Pro196His)- homozygous	LP
	P5- c.1083C>G (p.Tyr361Ter); c.152 G>A (p.Arg51Gln)- compound heterozygous	P, LP (Reported)
CPT 2	P6- c.631C>T (p.Pro211Ser) -homozygous	LP
(ENST00000371486.3)	P7- c.1234G>T (p.Val412 Leu)- heterozygous	VUS
	P8- c.631C>T (p.Pro211Ser)-homozygous	LP
FLAD1	P9- c.1588C>T (p.Arg530Cys)-homozygous	LP (Reported)
(ENST00000292180.3)		
ACADVL	P10- c.520G>A (p.Val174Met), c.1181A>T (p.Glu394Val)- compound heterozygous	LP (Reported), LP
(ENST00000356839.5)		
PNPLA2	P11- c.873del (p.Gly292GlufsTer28)-homozygous	P
(ENST00000336615.4)		

*LP – Likely Pathogenic; P- Pathogenic

CPT2

CPT 2 deficiency is characterized by myalgia, cramps, transient weakness and episodes of myoglobinuria precipitated by exertion, stress, fever, high fat diet or fasting.[10] Our three patients had a similar presentation and two had severe episodes with acute renal failure requiring hemodialysis. A meta-analysis has shown myoglobinuria in 97% of patients,[13] however myoglobinuria was absent in 14% in a Spanish study and 21% in a German report.[14,15] Two of our patients are normal between attacks while P7 with a heterozygous CPT 2 variant, has progressive proximal lower limb weakness in his 5th decade. Fixed weakness is unusual and has been reported only in a few cases.[16,17] Age at onset of our patients ranged from 26 to 29 years. The myopathic form usually presents in early adulthood, however onset in infancy and early childhood or late presentations at 61 years of age are known.[15,18,19] Time to diagnosis in our patients ranged from 3 months to 22 years, as in another case series where delay of few months to 40 years was observed.[15]

CK values were two to tenfold elevated in between attacks in our patients, while during the acute attacks the values were more than 100 fold. CK levels may be normal in between attacks[14] or persistently elevated[20] and may rise further during periods of metabolic decompensation. TMS shows elevation of C16:0 and C18:1 acylcarnitines while acetyl carnitine C2 is not elevated. The ratio of C16:0 + C18:1/C2 was found to be elevated in all nine patients of CPT 2 deficiency in a study.[21] Curiously, our patients had a normal TMS as in a case report from Turkey.[22]

Muscle biopsy in one of our patients showed lipid accumulation, while in two patients it showed non-specific changes. Deschauer *et al*.,[17] have shown lipid accumulation in half of their 23 muscle biopsies, the remaining being non-specific.

Muscle MRI in one of our cases showed involvement of quadriceps, especially vastus medialis, adductors of thigh, hamstrings and posterior leg muscles. Whole body MRI in two siblings had revealed dis-similar features with mild fatty changes of thoracic extensors, tensor fascia latae and gluteus maximus with one also showing mild involvement of sartorius and soleus and marked atrophy of lateral gastrocnemius.[23]

Two of our patients had novel variants with homozygous missense variant in exon 4 while one had a heterozygous missense variant in exon 4. The three common variants accounting for 75% of CPTII deficiency are c.338C >T (p.Ser113Leu), c.149C >A (p.Pro50His) and a complex variant c.[1238_1239del; 1342T >C] (p.[Lys414ThrfsTer7;Phe448Leu]). More than 95% of patients of CPTII deficiency harbor S113L variant in at least one allele.[17,24] Mild missense variants in both alleles cause milder myopathy while biallelic truncating variants cause lethal neonatal forms. Patients with truncating variant in one allele and missense variant in the other may have episodes of myoglobinuria precipitated by fasting while those with missense variants in both alleles are at lower risk.[17] Heterozygous carriers of CPT 2 variants can be occasionally symptomatic with mild stress induced myopathy comparable with patients having recessive variants due to possible dominant-negative effect.[25] We suspect our patient P7 may be one such symptomatic heterozygous carrier or he may be carrying an unidentified CPT2 variant in the second allele.

FLAD1

Mutations in *FLAD1* encoding Flavin Adenine Dinucleotide Synthase (FADS) cause MADD like phenotype.[26] The typical phenotypes described previously are hypotonia and bulbar symptoms, with few having scoliosis, respiratory compromise, cardiac arrhythmias and cardiomyopathy.[26-28] The majority of these patients presented in infancy and four died in infancy and childhood. In contrast, our single patient had onset in the 3rd decade and had LGMW and bulbar symptoms. One case described by Olsen *et al*.,[26] had onset at 20 years of age. She had worsening of myopathy during pregnancy[29] and had substantial improvement with riboflavin supplementation. Our patient also has good response to riboflavin but has residual proximal weakness. Interestingly, he also had positive Beevor's sign and exaggerated tendon reflexes.

CK value of our patient was 2691 IU/L. Previous reports show 5 to10 fold elevation with profound elevations during stress.[29] TMS and muscle biopsy aided diagnosis, as reported before.[26] Muscle MRI in our case showed fatty infiltration of anterior and posterior compartment of thigh and leg muscles while MRI of a patient reported by Auranen *et al*.,[29] showed fatty infiltration in soleus and medial head of gastrocnemius with sparing of thigh muscles.

Our patient had homozygous missense variant in c.1588C >T (pArg530Cys), affecting the FADS domain. This has been reported in a compound heterozygous state in two patients with onset in adulthood,[26] with a mild course. This feature might be due to impaired, yet detectable FADS activity. *FLAD1* codes for a protein consisting of MPTb and FADS domains. There are isoforms of *FLAD1* which encode only for FADS domain (isoform 5 and 6). Patients with biallelic frameshift variants may still have detectable FADS activity due to the presence of isoforms 5 and 6.

ACADVL

ACADVL mutations cause Very Long Chain Acyl CoA Dehydrogenase (VLCAD) deficiency. VLCAD can present as severe infantile form with cardiomyopathy, milder form with hypoketotic hypoglycemia[30] or late onset forms with myalgia/myoglobinuria precipitated by exertion/fasting.[31-33] In a series of 55 patients,[31] 8 patients had mild, late onset form beginning after 13 years of age with episodic myoglobinuria. Our patient was aged 11 years at symptom onset, had episodic rhabdomyolysis and weakness precipitated by exertion and fasting. TMS was diagnostic with increase in long chain acyl carnitines. However, muscle biopsy was non-diagnostic. Ohashi *et al*.,[34] have demonstrated reduced to absent immune-reactivity to VLCAD in 13 patients whose muscle biopsy was non-diagnostic.

Our case had two compound heterozygous missense variants in exon 3 of *ACADVL* gene. The mutation spectrum is wide, with 58 different variants identified in 55 patients by Andresen *et al*.[31] Those with null variants have absent VLCAD activity and present with multi-system involvement, while those with missense variants have residual enzyme activity and develop symptoms only during increased demand.[31]

PNPLA2

Patatin-like phospholipase domain-containing 2 *[PNPLA2]/* Adipose Triglyceride Lipase *(ATGL)* mutations or co-activator comparative gene identification-58 [*CGI-58*]/alpha/beta-hydrolase domain-containing protein 5 (*ABHD5*) mutations cause Neutral Lipid Storage Disease (NLSD).[10] ATGL is required for hydrolysis of triacylglycerols to diacylglycerols and fatty acids.[10] *ABHDF5* promotes activation of ATGL.

NLSD comprises of two autosomal recessive disorders, i.e., Neutral lipid storage disease with myopathy (NLSDM) and Neutral lipid storage disease with ichthyosis (NLSDI).[35] In NLSD, lipids accumulate in several tissues like skin, skeletal muscle, liver, heart, thyroid, pancreas, central nervous system, and leukocytes. PNPLA2 mutations cause NLSDM characterized by myopathy and cardiomyopathy while CGI58 mutations cause NLSDI with ichthyosis, myopathy and severe hepatic symptoms without cardiac involvement.[35]

A review of patients with NLSDM showed onset ranging from 1 to 64 years.[36] All had asymmetric upper limb predominant weakness with atrophy, axial and distal muscle weakness, exercise intolerance, myalgias, hypertrophic cardiomyopathy and hepatic steatosis in a few.[36] Our patient had a similar phenotype with onset at 21 years of age with asymmetric shoulder girdle involvement, myalgia and proximal lower limb weakness. His sibling was similarly affected with onset in the third decade. CK value is usually elevated 1.5 to 8 fold[36] as in our patient. Profile of acyl carnitine is typically normal.

A novel homozygous single base pair deletion in exon 7 of the PNPLA2 gene, resulting in a frameshift and premature truncation of the protein was detected in our case. Most of the variants reported previously are missense, while a few are nonsense and frameshift variants.

Limitations of the study

4 of our patients had compound heterozygous variants. Segregation analysis in parents was not done and hence cis or trans nature of these variants could not be ascertained. We considered the mutations as likely pathogenic due to the strong phenotype correlation.

Conclusion

LSM are phenotypically and genotypically heterogenous disorders. LSM should be considered in patients with exertion induced myalgias, LGMW, cranial nerve involvement or dropped head syndrome. High index of suspicion and knowledge of presenting features and precipitating factors is essential for a prompt diagnosis. Additional features like cardiomyopathy or bulbar symptoms may help in further subgrouping. Though TMS and muscle biopsy may aid in the diagnosis, a negative test does not rule out FAO disorder. Genetic testing is essential for accurate diagnosis and genotype-phenotype correlation. Functional studies may be needed to explain the clinical heterogeneity within the same group of disorders. Early identification and prompt treatment are needed for alleviation of symptoms of these potentially treatable and life-threatening disorders.

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Conflicts of interest

There are no conflicts of interest.

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