Table 1. Clinical summary of 47 patients with LSM

Pt	Age	Sex	Clinical feature	Weakness	Hypotonia	Muscle pain and cramp	Prodrome	Seizure	Coma	Respiratory failure	Cardiac symptom	Liver disease	CK	Familial history	Consanguinity	Lipid droplets distribution by fiber type
Mutations																
1	8m	F	vomiting, diarrhea	NA	NA	NA	+	−	+	−	−	+	243–1006	−	−	type 1>
2	4y	M	gait disturbance	+	−	−	−	−	−	−	+	−	150	+	−	type 1>
3	5y	M	gait disturbance	+	−	−	−	−	−	−	+	−	69	−	−	type 1>
4	5m	F		+	−	NA	NA	−	−	−	−	+	55	−	−	type 1>
5	6m	M		−	NA	NA	+	−	−	−	−	+	2000–4000	−	−	type 1>
6	11m	M	diarrhea	−	+	NA	−	+	+	−	+	+	128–618	−	−	type 1>
7	13y4m	F	muscle weakness	−	+	−	−	−	−	−	−	+	127	−	−	type 1>
8	27y	M	muscle weakness	+	−	−	−	−	−	−	+	−	757–1697	−	−	type 1>
9	35y	F	gait disturbance	−	+	−	−	−	−	−	+	−	654	+	−	type 1>
No Mutations																
10	15y	M	muscle cramp	−	−	+	−	−	−	−	+	−	587	−	−	< type2
11	67y	M	gait disturbance	−	−	−	−	−	−	−	−	−	4904	−	−	< type2
12	37d	F	metabolic acidosis	+	+	NA	−	−	−	−	+	−	44–200	−	−	type1 = type2
13	4m	F		NA	NA	NA	NA	NA	NA	NA	+	NA	NA	NA	NA	type1 = type2
14	4m	M	dyspnea	−	−	NA	−	−	−	−	+	−	112	+	−	type 1>
15	7m	M	developmental delay	+	+	NA	+	−	−	−	−	−	67	−	−	type 1>
16	1y	M	status epilepticus	−	−	NA	−	+	+	−	−	−	1593	−	−	type 1>
17	1y6m	F	metabolic acidosis	−	−	−	−	−	−	−	−	−	330	+	−	type 1>
18	1y1m	M		+	−	NA	−	−	−	+	+	−	559	−	−	type 1>
19	1y2m	F	developmental delay	+	+	NA	+	+	+	+	−	−	NA	−	−	type 1>
20	1y6m	M	albinism	+	+	NA	−	−	−	−	−	−	200–300	−	−	type 1>
21	2y2m	M	diarrhea	NA	+	−	+	−	+	−	−	−	163900	−	−	type 1>
22	2y7m	M	developmental delay	−	+	−	−	−	−	−	−	−	603	−	−	type 1>
23	3y	M	status epilepticus	NA	NA	NA	−	+	+	−	−	−	2034	−	−	type 1>
24	3y	F	developmental delay	+	−	−	−	−	−	−	−	−	63	−	−	type 1>
25	3y	M	developmental delay	NA	+	NA	−	−	NA	+	−	−	NA	−	−	type 1>
26	4y	F	periodic paralysis	+	−	−	−	+	−	−	−	−	162	−	−	type 1>
27	5y7m	M	developmental delay	−	+	−	−	−	−	−	−	−	47	−	−	type 1>
28	6y	M	dyspnea, abdominal pain	−	−	−	+	−	−	+	−	−	15	+	+	type 1>
29	13y8m	F	muscle weakness	+	NA	−	−	−	−	−	−	−	normal	−	−	type 1>
30	30y	F	lumbago	−	−	+	−	−	−	−	−	−	NA	−	−	type1 = type2
31	40y	F	diplopia, muscle cramp	−	−	−	−	−	−	−	−	−	NA	−	−	type 1>
32	49y	F	hypokalemic myopathy	+	−	−	−	−	−	−	−	−	3480	−	−	type 1>
33	54y	F	weakness	+	−	−	−	−	−	−	−	−	623	−	−	type 1>
34	59y	F	dyspnea, weakness	+	−	−	−	−	−	+	−	−	878	−	−	type 1>
35	66y	F	gait disturbance	−	−	+	−	−	−	−	−	−	49	−	−	type 1>
36	69y	M	gait disturbance	−	−	+	−	−	−	−	−	−	400	−	−	type 1>
37	75y	F	gait disturbance	−	−	+	−	−	−	−	−	−	5418	−	−	type 1>

Table 1. Continued

Pt	Age	Sex	Clinical feature	Weakness	Muscle pain and cramp	Hypotonia	Prodrome	Seizure	Coma	Respiratory failure	Cardiac symptom	Liver disease	CK	Familial history	Consanguinity	Lipid droplets distribution by fiber type
No Available DNA																
38	4m	F		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	type1 = type2
39	7m	M	dyspnea	NA	NA	NA	NA	NA	NA	+	NA	NA	125	NA	NA	NC*
40	9m	F	developmental delay	NA	NA	+	–	+	–	–	–	–	NA	–	–	type 1>
41	9m	M	ketoacidosis	–	–	–	+	+	–	–	–	+	214	–	–	type 1>
42	1y	M	vomiting, diarrhea	NA	NA	NA	+	+	+	–	–	–	394	–	–	type 1>
43	1y	F	dyspnea, weakness	+	–	–	–	+	NA	+	–	–	12310	–	–	type1 = type2
44	5y	M	fever, arthralgia	+	+	NA	–	–	–	–	–	–	39	+	–	type 1>
45	21y	F	vomiting, diarrhea	–	–	–	+	–	–	–	–	–	NA	–	–	type1 = type2
46	23y	F	muscle pain	–	+	–	–	–	–	–	–	–	62180	–	–	type 1>
47	24y	F	lumbago	–	+	–	–	–	–	–	–	–	47–4797	–	–	type 1>

NC = not counted
NA = not available

containing vacuoles in skeletal muscle were predominantly observed in type 1 fibers (Fig. 4B). Patient 1 had type 2 fiber atrophy, whereas patients 2 and 3 showed type 2A fiber atrophy and type 2B fiber deficiency. On electron microscopy, there was an increase in number of lipid droplets and mitochondria. Incidentally, lipid droplets were often next to mitochondria (Fig. 5A, B).

MADD Patients. The clinical features of the 4 patients genetically confirmed to have MADD are summarized in Table 3. The diagnosis of MADD in patients 6 and 7 was initially made based on the results of urinary organic acid analysis by gas chromatography/mass spectroscopy. All 4 patients had the infantile form. They all had generalized muscle weakness and hypotonia. Serum CK levels varied from normal to 4000 IU/L. Hepatomegaly was documented in patients 4 and 5. Patient 5, who received L-carnitine and riboflavin treatment, had normal growth and development, except for some mild metabolic episodes, and is now 20 years old. Patient 6 had hypertrophic cardiomyopathy. He was treated with L-carnitine, but he died of pulmonary alveolar bleeding at the age of 1 year and 11 months. Patient 7 was always a slow runner and poor athlete with easy fatigability since her preschool years. She developed nausea and vomiting at age 13 years and started experiencing difficulty climbing stairs. She had proximal dominant muscle weakness and atrophy on examination at age 13 years and 4 months. After treatment with L-carnitine and riboflavin, muscle weakness was ameliorated.

In skeletal muscle, lipids were observed predominantly in type 1 fibers. Mitochondria were not as prominent as in PCD (Fig. 4C, D). Type 2 fiber atrophy was seen in patient 5. Electron-microscopic findings were similar to those seen in PCD patients: intracytoplasmic lipid droplets were markedly increased both in number and size, and lipid droplets were often present next to mitochondria (Fig. 5C, D).

NLSDM Patients. Patients 8 and 9 had mutations in *PNPLA2*. Patient 8 developed progressive weakness in the lower legs and fingers at age 20 years (article in submission); at age 27 years, echocardiogram revealed dilated cardiomyopathy with left ventricular enlargement. Serum CK was elevated from 757 to 1697 IU/L.[11] Patient 9 was a slow runner since childhood.[1] At age 33 years, she noticed weakness of all extremities and developed marked generalized muscle weakness at 35 years. Electrocardiogram (ECG) showed left ventricular hypertrophy, but echocardio-

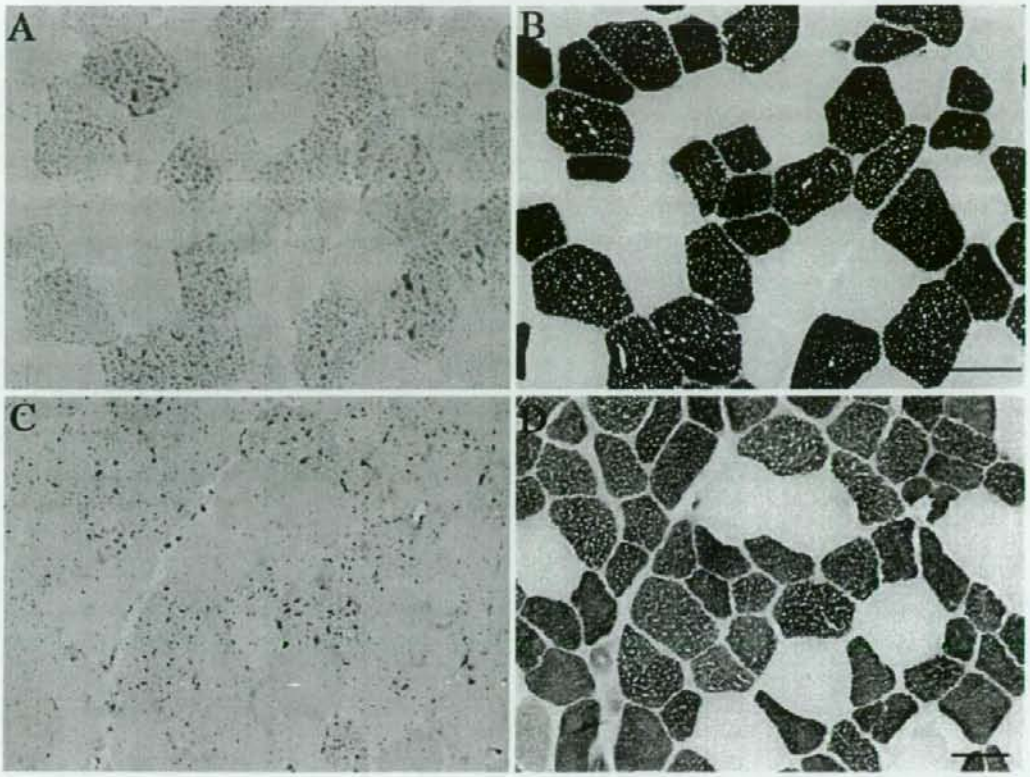

FIGURE 2. Lipid accumulation in type 2 fibers of patients with no mutations in known genes associated with LSM: patient 10 **(A, B)** and patient 11 **(C, D)**. Lipid droplets stained with oil-red-O **(A, C)** are only seen in type 2 fibers (routine adenosine triphosphatase stain) **(B, D)**. Bar = 50 μm.

gram was normal. Serum CK was elevated to 654 IU/L. In both patients, peripheral blood smear revealed lipid-containing vacuoles in leukocytes, namely Jordan's anomaly. Both patients had numerous lipid droplets mainly in type 1 fibers in addition to variation in fiber size. Surprisingly, there were scattered rimmed vacuoles within the myofibers (Fig. 1E, F), which were demonstrated to be autophagic vacuoles on electron microscopy (Fig. 5F). Interestingly, increased lipid droplets were seen between myofibrils where mitochondria appeared pyknotic (Fig. 5E).

DISCUSSION

Among all LSM cases, we identified mutations in known causative genes in only 24% of the cases. This brings to our attention two possibilities: the existence of yet-unknown causative genes, and secondary increase of lipid in muscle under a variety of metabolic alterations without inheritance.

Analysis of muscle lipids demonstrated an increase in the amount of TG, but not FFA. The accumulated lipid droplets in the cytoplasm of skeletal myofibers are therefore likely to be mainly composed of TG. Although, theoretically, triglyceride accumulation should occur in NLSDM and NLSDI, in which genes encoding TG hydrolase or its activator are mutated, it is accumulated in virtually all patients analyzed regardless of the causative gene. Reduction of mitochondrial fatty acid metabolism may negatively regulate the hydrolysis of TG in cytosol.

We identified 3 PCD patients with mutations in *SLC22A5*. Their clinical characteristics were consistent with the typical PCD symptoms with severe hypoglycemia, dilated cardiomyopathy, and progres-

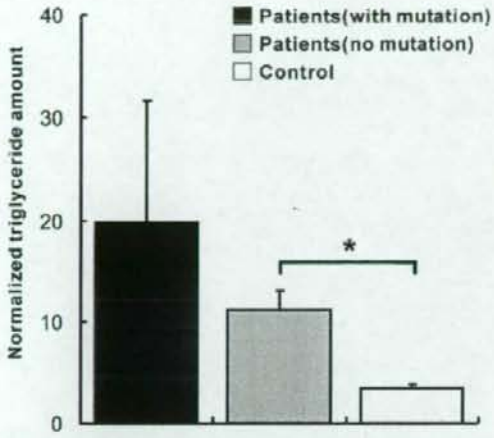

FIGURE 3. TLC analysis of lipid composition of skeletal muscle with LSM. The bars represent the mean triglyceride (TG) amount which is normalized with cholesterol (Cho) content. Values are shown for patients with mutation (black bar; $N = 2$), patients with no mutation (gray bar; $N = 12$), and in controls (white bar; $N = 4$). Error bars represent standard error of means. Note the remarkable increase of TG in patients with mutations. $^*P < 0.05$ (Student's *t*-test).

sive muscle weakness, as reported elsewhere.[11,12] A positive response to L-carnitine treatment was seen in all 3 patients, a feature that has been shown to be characteristic of PCD.[11]

Among the patients with MADD, 2 had a good response to riboflavin. Olsen et al. noted that riboflavin-responsive MADD may result from defects in ETFDH combined with general mitochondrial dysfunction.[15] In support of this notion, both of our patients who responded to riboflavin had mutations in *ETFDH*. With regard to CoQ_{10} levels, however, our case contradicts the recent report.[7] Although we measured CoQ_{10} levels in only 2 patients due to sample size limitation, the finding of a normal CoQ_{10} level in a patient with the *ETDFH* mutation is still relevant for clinicians, because it indicates that *ETFDH* mutations may not always be associated with CoQ_{10} deficiency. Further studies are necessary to determine whether there is a detailed relationship between the *ETFDH* mutation and CoQ_{10} deficiency.

The first step of the mitochondrial β-oxidation cycle is catalyzed by four fatty acyl-CoA dehydrogenases (very long, long, medium, and short chain), all of which are affected in MADD. We previously reported that very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency does not show increased lipid droplets in muscle.[13] In contrast, MADD is characterized pathologically by lipid storage, raising the possibility that lipid droplets may not accumulate when one of the four acyl-CoA dehydrogenases, such as VLCAD, is defective.

Our patients with NLSDM presented with distal myopathy and cardiac symptoms, accompanied by lipid accumulation in muscle and peripheral leukocytes, suggesting multisystemic lipid accumulation. Notably, in the patient with NLSDM, mitochondria on electron microscopy were pyknotic, in stark contrast to those in PCD and MADD. This morphological difference is contrary to that expected from function of each causative gene, because PCD and MADD have defects in the mitochondrial β-oxidation cycle, whereas NLSDM is due to a defect in cytoplasmic TG hydrolysis. In addition, rimmed vacuoles were observed in the 2 NLSDM patients and not in the other LSM patients. Together with the fact that both patients had progressive, rather than episodic, muscle disease, these clinicopathological peculiarities should reflect a distinct pathomechanism that is yet to be elucidated. Clearly, further studies

Table 2. Identified mutations.

Patient	Age	Gender	Gene name	Nucleotide change	Amino acid change
1	8 mo	F	*SLC22A5*	c.396G>A* c.844C>T	p.W128X p.A282X
2[11,12]	4 y	M	*SLC22A5*	-91_22del†	
3[11,12]	5 y	M	*SLC22A5*	-91_22del†	
4	5 mo	F	*ETFDH*	c.1519T>G*	p.Y507D
5	6 mo	M	*ETFDH*	c.1208C>T†	p.A403V
6	11 mo	M	*ETFA*	c.284T>G†	p.L95W
7	13 y	F	*ETFDH*	c.524G>A*	p.R175H
				c.1774T>G	p.C592R
8	27 y	M	*PNPLA2*	c.477_478insCCTC†	Frameshift 176X
9	35 y	F	*PNPLA2*	c.477_478insCCTC†	Frameshift 176X

*Compound heterozygous.
†Homozygous.

Table 3. Clinical summary of MADD patients.

Patient	Age	Gender	Clinical feature	Serum CK (IU/L)	CoQ_{10} level (μg/g muscle)	Treatment	Clinical course
4	5 mo	F	Muscle weakness, hepatomegaly	55	NA	NA	NA
5	6 mo	M	Muscle weakness, hepatomegaly	2000-4000	NA	L-carnitine riboflavin	Normal development after treatment
6	11 mo	M	Vomiting, hypertrophic cardiomyopathy	128-618	24.1	L-carnitine	Died at 2 years of age due to pulmonary alveolar bleeding
7	13 y 4 mo	F	Progressive muscle weakness	127	32.3	L-carnitine riboflavin	No muscle weakness at present

NA, not available. Normal range: CK, 57-197 IU/L; CoQ10, 32.1 ± 6.8 (mean ± standard deviation).

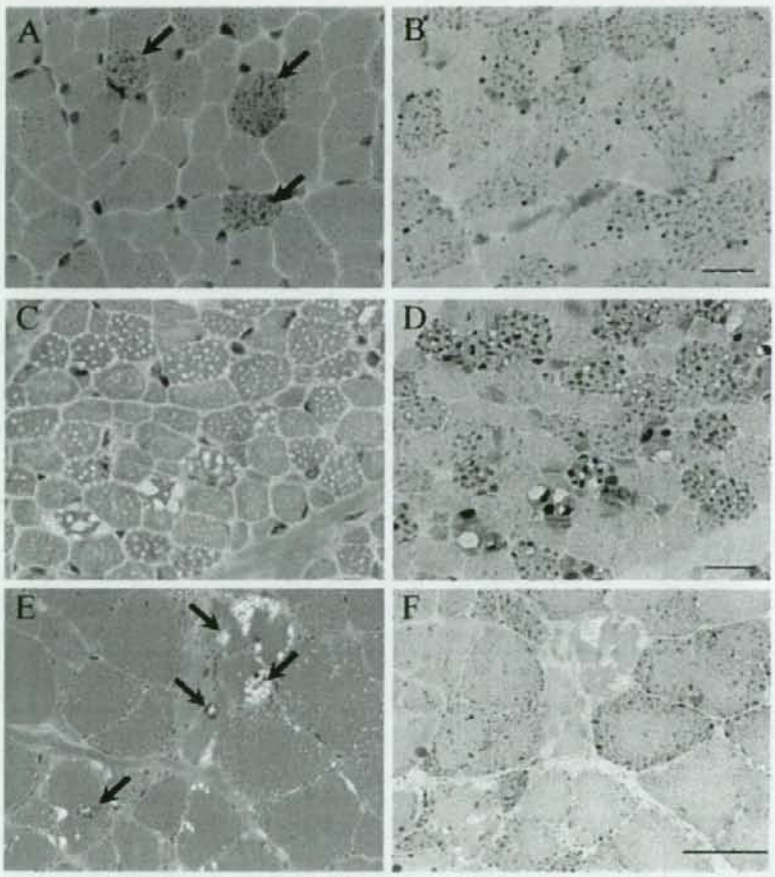

FIGURE 4. Routine histochemical staining of patients with LSM. Ragged-red–like fibers are seen on modified Gomori trichrome staining **(A)** and numerous lipid droplets are seen with oil-red-O **(B)** in a PCD patient, but not in an MADD patient **(C, D)**. Rimmed vacuoles are seen in myofibers of patients with NLSDM **(E)**, in addition to the characteristic numerous lipid droplets predominantly in type 1 fibers **(F)**. Bar = 20 μm.

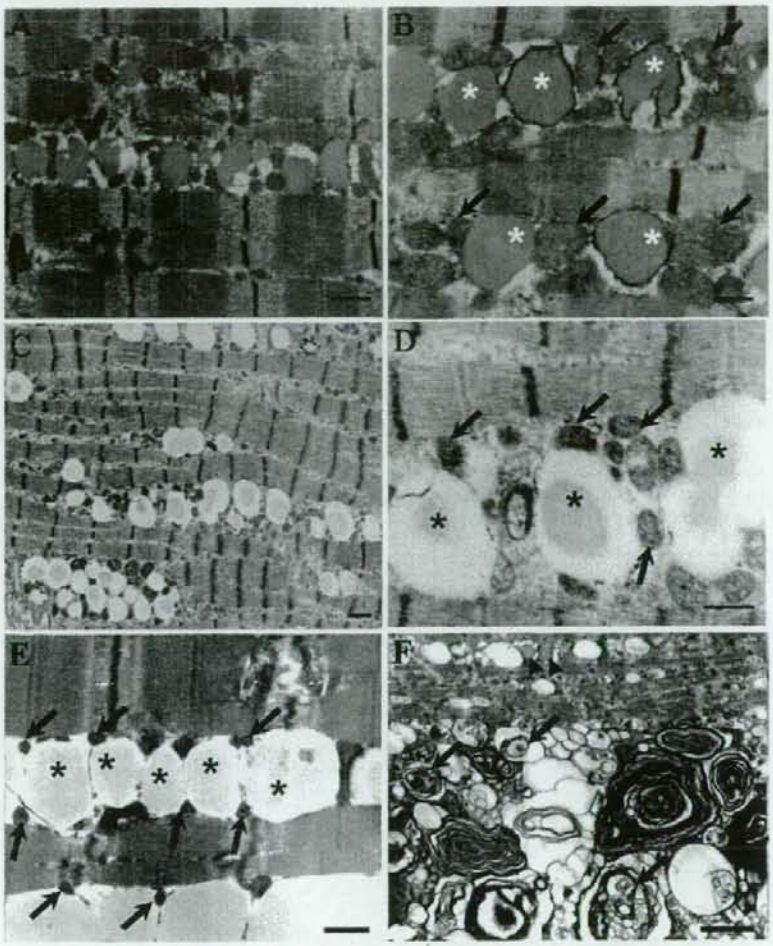

FIGURE 5. Electron microscopy findings. In a patient with PCD, markedly increased lipid droplets and increased numbers of mitochondria are seen **(A)**. On higher magnification, lipid droplets (asterisks) are seen next to mitochondria (arrows) **(B)**. In a patient with MADD, note the increase in the number of lipid droplets **(C)** and the proximity of these droplets (asterisks) with mitochondria (arrows) **(D)**, findings similar to those for PCD. In a patient with NLSDM, there is also a marked increase in the amount of lipid (asterisks) deposited within the myofibers; however, note that mitochondria appear pyknotic (arrows) **(E)**. In this patient, numerous autophagic vacuoles (arrows) in close proximity to lipid deposition (arrowheads) are also observed **(F)**. Bar = 1 μm in **(A)**, **(C)**, and **(F)**. Bar = 0.5 μm in **(B)**, **(D)**, and **(E)**.

are necessary to fully understand the mechanism of the disease.

CPT II deficiency has been reported to show increased lipid droplets in muscle.[3] However, in all patients whose samples were suitable for biochemical assays, normal enzymatic activity was seen. Furthermore, in our series, we had 7 patients with CPT II deficiency, but none showed lipid droplet accumulation on muscle pathology (data not shown). This suggests that lipid storage may not be a common pathological feature of CPT II deficiency, although analysis of a larger population of patients with CPT II deficiency would be needed to further support this contention.

In spite of an extensive genetic survey of known causative genes for LSM, we did not find mutations in 76% of the patients. One possible explanation for the absence of mutations in these patients is that

they may have secondary LSM, as intramuscular lipid content is known to be secondarily increased under a variety of conditions, including diabetes, renal disease, iatrogenic conditions, gastrointestinal disturbance, elevated plasma fatty acid levels, and high dietary fat intake.[18] The fact that 2 patients were taking antiepileptic drugs could be supportive of this notion. In the majority of patients without mutation, conditions associated with a secondary increase in muscle lipids were not seen. In addition, 2 patients had pathological features that differed from the rest of the patients in the form of lipid droplets almost exclusively in type 2 fibers, in contrast to the preference of lipid accumulation in type 1 fibers in all others, indicating the probability of a common pathomechanism, at least in these 2 cases.

Among the 28 patients without mutations in known causative genes, 5 had a positive family history and/or consanguinity (Table 1), suggesting that these individuals are likely to have primary genetic lipid disorders, rather than secondary LSM and the presence of additional yet-to-be-identified causative genes for LSM. Further analysis on biochemical analyses of accumulated metabolites and extended study of candidate genes involved in lipid metabolism will be helpful in the genetic diagnosis of LSM patients.

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