Study of *LPIN1*, *LPIN2* and *LPIN3* in rhabdomyolysis and exercise-induced myalgia

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Summary

Background: Recessive *LPIN1* mutations were identified as a cause of severe rhabdomyolysis in pediatric patients. The human lipin family includes two other closely related members, lipin-2 and 3, which share strong homology and similar activity. The study aimed to determine the involvement of the *LPIN* family genes in a cohort of pediatric and adult patients (n=171) presenting with muscular symptoms, ranging from severe (CK>10 000 UI/L) or moderate (CK<10 000 UI/L) rhabdomyolysis (n=141) to exercise-induced myalgia (n=30), and to report the clinical findings in patients harbouring mutations.

Methods: Coding regions of *LPIN1, LPIN2* and *LPIN3* genes were sequenced using genomic or complementary DNAs.

Results: Eighteen patients harboured two *LPIN1* mutations, including a frequent intragenic deletion. All presented with severe episodes of rhabdomyolysis, starting before age 6 years except two (8 and 42 years). Few patients also suffered from permanent muscle symptoms, including the eldest ones (≥40 years). Around 3/4 of muscle biopsies showed accumulation of lipid droplets. At least 40% of heterozygous relatives presented muscular myalgia. Nine heterozygous SNPs in *LPIN* family genes were identified in milder phenotypes (mild rhabdomyolysis or myalgia). These variants were non-functional in yeast complementation assay based on respiratory activity, except the *LPIN3*-P24L variant.

Conclusion: *LPIN1*-related myolysis constitutes a major cause of early-onset rhabdomyolysis and occasionally in adults. Heterozygous *LPIN1* mutations may cause mild muscular symptoms. No major defects of *LPIN2* or *LPIN3* genes were associated with muscular manifestations.

A concise sentence take-home message of the article: *LPIN1* gene mutations but neither *LPIN2* nor *LPIN3* constitute a major cause of early-onset rhabdomyolysis with lipid droplets and occasionally in adulthood.

Introduction

Metabolic myopathies cover a wide clinical spectrum, extending from recurrent rhabdomyolysis to exercise-induced muscle pain, or permanent muscle pain with weakness (Ohkuma et al 2009). Multiple origins have been identified for this heterogeneous group, but it is mostly attributed to a defect of ATP production or utilization, such as elicited by fatty acid oxidation (FAO) deficiencies which constitute the majority of known causes (van Adel and Tarnopolsky 2009, Laforet and Vianey-Saban 2010, DiMauro et al 2010).

Autosomal recessive lipin-1 gene (*LPIN1*) mutations were identified as a cause of severe recurrent rhabdomyolysis (Zeharia et al 2008) and later recognized as one of the most frequent causes in paediatric patients (Michot et al 2010). A single heterozygous mutation has also been described in two adult patients who developed statin-induced myopathy (Zeharia et al 2008), and an association between the metabolic syndrome and its associated phenotypes and common genetic variants of the *LPIN1* gene was described (Loos et al 2007, Wiedmann et al 2008, Fawcett et al 2008). Accordingly, lipin-1 appears to play a critical role in severe early-onset myoglobinuria and may also be involved in milder muscular diseases in adults.

Lipin-1 is most abundantly expressed in adipocytes and skeletal muscle (Reue and Zhang 2008, Donkor et al 2007). It plays a dual role: in the cytoplasm it acts as a phosphatidate phosphatase 1 (PAP1) converting phosphatidic acid to diacylglycerol, the precursor of both triglycerides and phospholipids (Donkor et al 2007, Han et al 2006); whilst in the nucleus lipin-1 functions as a transcriptional co-activator through interaction with transcription factors which regulate the expression of genes involved in energy pathways (Donkor et al 2008, Finck et al 2006, Sugden et al 2010). Three lipin-1 protein isoforms are generated by alternative splicing, specifically involved in either PAP1 activity, transcriptional function or lipid droplets biogenesis (Han and Carman 2010, Han et al 2007, Peterfy et al 2005, Reue and Brindley 2008, Wang et al 2011).

The mammalian lipin family includes two other closely related members, lipin-2 and lipin-3, encoded by distinct genes on separate chromosomes. All three mammalian lipins share 44-48% overall identity and strong homology in their C-terminal moiety (C-LIP domain). Importantly, the latter contains conserved motifs for PAP1 activity and interaction with nuclear transcriptional coactivators (Finck et al 2006, Donkor et al 2009). Accordingly, all three lipins exhibit PAP1 activity (Donkor et al 2007, Harris et al 2007) suggesting some degree of functional complementation, as observed in *fld* mice where hepatic PAP1 activity results from increased *LPIN3* gene expression (Donkor et al 2007). Finally, homozygous mutations in *LPIN2* are responsible for the syndrome of chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anaemia (Majeed syndrome) (Ferguson et al 2005), while lipin-3 is not currently known to be involved in human disease.

The purpose of the study was to i) determine the involvement of the *LPIN* gene family in patients presenting with various muscular symptoms of unknown origin, ranging from severe rhabdomyolysis to exercise-induced myalgia at any age, ii) improve the clinical description of the patients with *LPIN1* mutations and their heterozygous relatives, and iii) describe the histological characteristics of muscle biopsies of affected patients.

Methods

Patients

We retrospectively studied a cohort of 171 paediatric and adult patients presenting with a wide clinical spectrum of muscular symptoms of unknown origin. Because of this clinical heterogeneity, we assigned patients to one of two subgroups (Figure 1): group 1 with one or recurrent episodes of rhabdomyolysis and group 2, with permanent and/or exercise-induced myalgia and no history of myoglobinuria. Relatives of patients (parents and healthy siblings) were considered separately to this cohort.

Group 1 included 141 patients of diverse ethnic origins, coming from an international recruitment. They were subdivided according to the following characteristics reflecting disease severity: plasma creatine kinase (CK) < or > 10 000 UI/L (normal <150) during episodes of rhabdomyolysis, and age of onset $\leq$ 6 years, 7-16 years, > 16 years (Figure 1). The number of episodes per patient was variable in all subgroups of patients (1 - >20). The rhabdomyolysis episodes were mostly triggered by fever and/or exercise, more rarely by fasting, anaesthesia or statins. Between episodes, most patients (85%) had normal daily life physical activities, clinical examination and normal or occasionally slightly elevated CK (up to 700 UI/L).

Group 2 consisted of 30 paediatric and adult patients followed in different French hospitals, suffering from exercise-induced myalgia and/or permanent myalgia worsened by exercise, but who never had myoglobinuria. Myalgia coincided with statin administration in 7 adult patients. Muscular testing was normal or subnormal and plasma CK levels ranged from normal to slightly increased (peak levels < 500 UI/L). EMG was normal in all patients tested.

In all patients of both groups, extensive metabolic work-up excluded the common identified causes of rhabdomyolysis (van Adel and Tarnopolsky 2009, Laforet and Vianey-Saban 2010, DiMauro et al 2010). Plasma amino acids, acylcarnitine profile, lactate and glucose were normal, as were urinary organic acids. Muscle histology performed in most patients, was normal or showed lipidosis, predominance of type I muscle fibers with type II atrophy and only rarely subsarcolemmal aggregates of mitochondria with ragged-red fibers.

Patient consent and clinical description

All patients gave informed consent to the studies, after approval by the Necker ethics board committee. Clinical data were given by each referring physician who participated to the study, including ethnicity which was assessed regarding to the possibility of a founding effect.

Histology

Skeletal muscle, heart and liver specimens were immediately frozen in liquid nitrogen-cooled isopentane and serial cross-sections were stained with hematoxylin-eosin, modified Gomori trichrome, Periodic Acid of Schiff, Oil red and Red Sirius (heart). Muscle histochemical staining for cytochrome *c* oxidase (COX) was performed using a modified method (Possekel et al 1995). For electron microscopy, the muscle specimens were fixed with glutaraldehyde (2.5 %, pH 7.4), post fixed with osmium tetroxide (2 %), dehydrated and embedded in resin (EMBed-812, Electron Microscopy Sciences, USA). Ultrathin sections were stained with uranyl acetate and lead citrate.

Molecular investigations

Coding regions and flanking splice sites of the *LPIN1* (NM_145693.1), *LPIN2* (NM_014646.2) and *LPIN3* (NM_022896.1) genes were sequenced using genomic or complementary DNAs (cDNAs) prepared from fibroblasts, lymphocytes or skeletal muscle. Screening for the common *LPIN1* intragenic deletion (c.2295-863_2410-27del) was systematically performed by long range PCR as previously described (Michot et al 2010).

Parents' genotype was established to determine the allelic transmission of the *LPIN1, LPIN2* or *LPIN3* variants.

LPIN1 single nucleotide polymorphisms (SNPs) (intron 1, rs10192566; intron 1 rs11693809; intron 9, rs33997857; intron 11, rs6744682; intron 13, rs2577262; intron 13, rs6708316; intron 18, rs2716609; 3'UTR rs2577256), associated with the metabolic syndrome and its related phenotypes (Loos et al 2007, Wiedmann et al 2008, Fawcett et al 2008) were examined by nucleotide sequencing.

Plasmid construction

The coding sequences of *LPIN2* and *LPIN3* cDNAs were cloned in pGemT-easy plasmid (Promega) according to standard procedures (Michot et al 2010). After site-directed mutagenesis with the Quick Change II kit (Stratagene, San Diego, CA), normal and mutated

cDNAs were subcloned into the yeast shuttle vector YEp51 (Zeharia et al 2008). The *LPIN* coding sequences of resultant clones were entirely verified by nucleotide sequencing before yeast transformation.

Functional characterization of Lipin-2 and lipin-3 mutants in yeast

Wild-type (WT-W303) and Δ*pah1* strains harboring an empty plasmid (YEp51) or plasmids encoding the normal human *LPIN 2* or *3* (YEp-*LPIN* 2 and 3) and the mutant cDNAs (YEp-P149L, YEp-P623S for *LPIN2* and YEp-P24L, YEp-S226L for *LPIN3*) were grown on glucose and glycerol medium for 3 and 7 days at 30°C as previously described (Zeharia et al 2008).

Results

LPINs sequencing

*LPIN*s gene sequencing revealed 18 patients with two *LPIN1* mutations. Nine other patients harboured only one heterozygous variant (one in *LPIN1*, four in *LPIN2* and four others in *LPIN3*). No patients have been found with two mutations in the *LPIN2* or *LPIN3* genes.

LPIN1

Recessively inherited mutations

18 patients (aged 22 months – 46 years) were homozygous or compound heterozygous for *LPIN1* mutations. All presented with one or recurrent bouts of rhabdomyolysis (group 1). These episodes were severe in all cases (CK > 10,000 UI/L) and occurred before age 6 years (mean age of onset 30 months) except in two patients: one developed his first bout of rhabdomyolysis at age 8 years and the other one was an active man until he developed rhabdomyolysis at age 42 years. Remarkably, recessive *LPIN1* mutations were identified neither in patients presenting milder rhabdomyolysis (CK < 10,000 UI/L), nor in patients

from group 2, with myalgia. The incidence of recessively-inherited *LPIN1* mutations reaches 37% of the patients when strict inclusion criteria are considered (CK > 10,000 UI/L, age of onset ≤ 6 years), ie 19% of all the patients with severe myolyses.

Episodes of rhabdomyolysis were mostly precipitated by febrile illnesses, and occasionally by intense exercise, anesthesia and/or fasting. Of note, the bout frequency decreased with age. Six patients and five siblings (not studied) died during episodes of rhabdomyolysis. Where documented, cause of death was cardiac arrhythmia, possibly due to hyperkalaemia. Between rhabdomyolysis episodes, patients were healthy except six patients who presented exercise intolerance and a moderate limb girdle muscular deficit with Gowers sign. Only one patient required wheel-chair for long walks. Two of these patients had scapular amyotrophy with rigid spine after 10 years. An apparent mild chronic myolysis was noted in 4 patients. Interestingly, these patients include the two eldest patients, respectively 45 and 46 years). Conversely, two other young adults are top-level athletes and one woman had an uneventful vaginal delivery. No signs of neuropathy were noted and the electromyograms in a few patients tested were normal. In all patients, heart and liver ultrasounds were normal between episodes of rhabdomyolysis when studied, however cardiomegaly and/or dilated cardiomyopathy were noted at autopsy of two siblings who died in the context of rhabdomyolysis or sepsis. Interestingly, autopsies of patients revealed either normal heart (3/6) and liver (5/6), or cardiomyopathy (one patient: infiltration of adipocytes and mild fibrosis (Figure 2a, b), two other patients: dilated cardiomyopathy) and liver steatosis (Figure 2c, d). Average weight percentiles and normal fat distribution were found in all patients, confirmed by dual-absorptiometry in two patients tested (data not shown). All patients had normal plasma levels of cholesterol triglycerides, adiponectin and leptin. Oral glucose tolerance test and HbA1c level were normal in three patients tested (data not shown).

A total of 13 different mutations, including six new mutations, were identified (Figure 3). Mutations were scattered throughout the *LPIN1* coding region and most (n=11) generated

a premature stop codon, directly or as a consequence of frameshifts. The only missense mutation was located in the C-LIP domain. The majority of Caucasian patients displayed the intragenic deletion (c.2295-863_2410-27del; Supplementary data, eTable 1) encompassing exon 18, reinforcing our hypothesis of a founder effect (Michot et al 2010).

At least 40% of parents and siblings, heterozygous carriers of one of the proband's mutations (either stop mutations or the common deletion), reported frequent cramps and/or exercise-induced myalgia.

Heterozygous variants in LPIN1

A heterozygous nucleotide variant (c.1621A>G, p.Ile541Val) and a heterozygous deletion of one aminoacid (c.856_858delTCT, p.Ser286del) were identified in one and three patients respectively (Supplementary data, eTable 1). We were unable to detect additional mutations in *LPIN1* or in the two other *LPIN* genes for these patients. Although they have not been reported as polymorphisms in databases (dbSNP on NCBI; Ensembl) and not found in 250 control chromosomes, they are considered to be likely polymorphisms since they involve poorly conserved amino acids. Accordingly, the potential effect of the p.Ile541Val variant was predicted to be benign by the PolyPhen software (http://genetics.bwh.harvard.edu/pph/).

cDNA isoforms

Unexpectedly, cDNA sequencing detected low levels of various alternative splicing events in patients, their relatives and controls (data not shown). The most frequently found isoform lacked exon 18 (NM_145693.1), encoding part of the conserved C-LIP domain. This has not previously been reported in databases (Aceview on NCBI and Ensembl). This splicing produces a frameshift and results in a premature stop codon eight amino acids downstream. It was found in both lymphocytes and skin fibroblasts, but not in the skeletal muscle of two patients tested. This alternative splicing event was not explained by SNPs in the exon/intron boundaries of *LPIN1* exons.

LPIN2* and *LPIN3

No recessively-inherited mutations were identified in the *LPIN2* and *LPIN3* genes in patients in the present cohort.

Single heterozygous nucleotide variants and their characterization by yeast Δpah1 complementation assay

Eight single heterozygous nucleotide variants were identified, affecting *LPIN2* in four patients: three in group 1 (one patient with mild rhabdomyolysis at age 8 years and two patients with severe rhabdomyolysis in adulthood, including one who received statin medication; no myalgia between episodes of rhabdomyolysis) and one from group 2; and *LPIN3* in four patients: three in group 1 (myolysis at ages 2, 3 and 35 years, no myalgia between episodes of rhabdomyolysis) and one in group 2 (Supplementary data, eTable 1). No further alteration in the two other *LPIN* genes was detectable in all cases. All variants resulted in private missense variation, not described as polymorphisms and not found in 250 control chromosomes. They were inherited from heterozygous asymptomatic parents. The PolyPhen sofware predicted four changes to be "probably damaging" (p.Ser226Leu ; p.Pro24Leu; p.Arg522Trp in *LPIN3* gene) or "possibly damaging" (p.Pro149Leu in *LPIN2* gene). Others variants were predicted to be "benign" (Supplementary data, eTable 1).

Δ*pah1* growth on glycerol medium was restored by the normal *LPIN2* and *LPIN3* cDNAs to levels comparable to that obtained with *LPIN1*. The two *LPIN2* mutants tested (P149L and P623S) and one *LPIN3* mutant (S226L) complemented similarly to their normal counterpart, whereas the *LPIN3*-P24L mutant cDNA was unable to fully complement Δ*pah1* phenotype (Figure 4) indicating functionally deleterious consequences of this substitution (the patient presented one moderate rhabdomyolysis at age 3 years).

Study of LPIN1 SNPs in patients with a single heterozygous nucleotide variant in LPIN1, LPIN2 or LPIN3.

The SNP genotype of the patients carrying only one heterozygous variant in *LPIN1*,

LPIN2 or *LPIN3* was established for 8 remarkable *LPIN1* polymorphisms, but no homogeneous genotype was found (data not shown).

cDNA isoforms in LPIN2 gene

cDNA sequencing also detected low levels of various alternative splicing events in patients, relatives and controls (data not shown). The most prominent isoforms lacked exons 11, and were not previously reported in databases (Aceview in NCBI and Ensembl). Alternative splicing was observed in both lymphocytes and skin fibroblasts, but not in the skeletal muscle of two patients tested. These alternative splicing events were not associated to SNPs in the exon/intron boundaries of *LPIN2* exons.

Histochemical analysis

Skeletal muscle biopsies of patients carrying two *LPIN1* mutations were re-evaluated. Most of them (ca. ¾) showed a similar histological pattern, compatible with a metabolic disorder. Histochemical analysis showed an increase of quantity and size of lipid droplets in the muscle fibers, with type I muscle fiber predominance and moderate atrophy of type II fibers (Figure 5a). Mitochondria aggregates were observed in two biopsies while COX histochemical staining was abnormal only in one biopsy and revealed a homogeneously weak COX activity in all muscle fibers (Figure 5e, with control COX staining in Figure 5d). Mitochondrial DNA mutations (MELAS A3243G, MERFF A8344G, NARP P8993G) and deletions have been excluded in muscle of these patients.

Further ultrastructural observations of three patients showed consistently scattered lipid droplets located between the normally structured myofibrils (Figure 5b), and occasionally demonstrated small clusters of mitochondria near the sarcolemmal membrane (Figure 5c).

Discussion

We recently showed that *LPIN1* mutations are involved in a large proportion of patients with severe rhabdomyolysis occurring in infancy after exclusion of FAO defects (Zeharia et al 2008, Michot et al 2010). The absence of an established molecular aetiology in at least one quarter of metabolic myopathies (Ohkuma et al 2009) prompted us to determine *LPIN1* gene status in patients with muscular symptoms of unknown origin. Importantly, our results confirm and extend previous observations. Recessive mutations in the *LPIN1* gene are a major cause of severe rhabdomyolysis occurring in childhood, as the incidence for patients suffering from severe rhabdomyolysis with onset before age 6 years and CK > 10 000 UI/L reaches 46% when we extend this series to the 17 previously described patients harboring two recessively inherited *LPIN1* mutations (Michot et al 2010), so far a total of 35 patients from 28 families (Supplementary data, eTable 1).,

In addition, we identified two *LPIN1* mutations in two patients experiencing a first bout at 8 and 42 years of age, later than previously reported (Zeharia et al 2008, Michot et al 2010). The latter case suggests that *LPIN1* mutations should also be considered in adults with severe myolysis, but this remains an exception (only 1 adult with 2 *LPIN1* mutations out of the 29 patients with adult-onset rhabdomyolysis). By contrast, recessive *LPIN1* mutations were involved neither in mild rhabdomyolysis (CK < 10 000 UI/L) nor in myalgias without rhabdomyolysis. Indeed, recessively inherited *LPIN1*-related disease appears as a very severe form of rhabdomyolysis as 11 out of the 35 such patients and 10 siblings, not studied, died during a bout. Accumulation of lipid droplets was observed in a majority of muscle biopsies and in the liver biopsy of one patient, as observed in other metabolic disorders (Bruno and Dimauro 2008, Fischer et al 2007, Liang and Nishino 2010). No other organ seemed to be affected except possibly the heart. Interestingly, a few patients including the two eldest presented permanent muscle symptoms, as well as some relatives with heterozygous *LPIN1* mutations. In addition, heterozygous *LPIN1* gene nucleotide variants were separately

identified in three adult patients (Zeharia et al 2008) who received statin medications. Thus, heterozygous *LPIN1* mutations may produce mild muscular symptoms and lead to more severe consequences when combined with particular therapies targeting lipid metabolism (Nakajima et al 2011, Sarullo et al 2010, Shek and Ferrill 2001, Ucar et al 2000).

The 6 new recessively-inherited *LPIN1* mutations described here, and the total of 19 mutations reported so far when we include all the 35 patients with two *LPIN1* mutations (Michot et al 2010), are scattered in the coding sequence and induce a major structural modification, through premature stop codons or as a result of a large intragenic deletion. Only one missense mutation (p.Arg725His) was found in the C-LIP domain, combined with the common intragenic deletion. 86% of Caucasian families carry the common intragenic deletion, supporting a founder effect and underlying the usefulness of the long range PCR assay for rapid diagnosis (Michot et al 2010) . This deletion results in the absence of exons 18 and 19 at the mRNA level, removing 73 residues (766-838) within the conserved C-LIP domain, yet it preserves the motifs needed for PAP1 activity (DxDxT (678-682) and Ser 734) and for binding to nuclear receptors (LxxIL (689-693)). Significantly, it removes other motifs common to the haloacid dehydrogenase family (Finck et al 2006, Donkor et al 2009), suggesting that they play an important role in PAP1 activity. The deletion also removes some regions possibly required for lipin-1 homo- or hetero-dimerization which may further alter its activity (Liu et al 2010).

Lpin1 mutant mouse and rat models display a phenotype mainly characterized by lipodystrophy and neuropathy (Peterfy et al 2001, Mul et al 2011), two symptoms not observed in lipin-1 deficient patients. Since both *LPIN2* and *LPIN3* genes are highly expressed in human tissues, one hypothesis may be that lipin-2 and/or 3 compensate for decreased PAP1 activity (Donkor et al 2007). Considering that all three lipins have an overlapping tissue distribution, albeit at different levels (Donkor et al 2007, Zhou and Young 2005) (LPIN2 has a “net”: dictinct? expression in muscle, personal data not shown), share

similar functions of PAP1 activity and transcriptional co-activation (Donkor et al 2007, Finck et al 2006, Donkor et al 2009, Bou Khalil et al 2009), and associate in homo and hetero-oligomers for PAP1 activity (Liu et al 2010), we sequenced *LPIN2* and *LPIN3* genes in our cohort. Only a few heterozygous missense substitutions in *LPIN2* and *LPIN3* were detected in both groups of patients, predicted to be “possibly damaging” or “probably damaging” by the PolyPhen software. However study of the respiratory-deficient phenotype of the Δ*pah1* yeast did not confirm these predictions except for the P24L *LPIN3* variant. The hypothesis of an intragenic deletion on the other allele was excluded in all patients and no *LPIN1* SNP remarkable haplotype was noted, suggesting that most variants probably do not contribute significantly to muscular diseases.

The pathogenic mechanism of *LPIN1*-related rhabdomyolysis remains to be elucidated. A defect in triglyceride and phospholipid synthesis has been suggested (Zeharia et al 2008) , since lipin-1 is an important modulator of circulating triacylglycerol levels in mice (Reue and Brindley 2008) and lipid droplets in yeast (Adeyo et al 2011). An energetic defect has also been proposed (Michot et al 2010, Finck et al 2006), since lipin-1 promotes fatty acid oxidation (FAO) under some conditions in adipose tissue and liver and its expression in human adipose tissue correlates with expression of genes involved in FAO and peroxisome proliferator-activated receptors (PPARs) (Donkor et al 2008, Yao-Borengasser et al 2006). Several findings in patient muscle samples such as lipid droplets, secondary carnitine palmitoyltransferase 2 defect in one patient (Michot et al 2010), secondary COX deficiency in another patient (de Lonlay-Debeney et al 1999), and in a few cases the presence of ragged-red fibers, suggest that *LPIN1* mutations lead to energetic defect, possibly due to PPARs and PPARγ coactivator 1-α dysfunction. Interestingly, various inflammatory inducers have recently been shown to decrease lipin-1 expression (Lu et al 2008, Tsuchiya et al 2009), while acute exercise (Higashida et al 2008) and fasting (Reue and Brindley 2008) increase lipin-1 expression. This is concordant with the majority of the present cases possessing a triggering

factor to induce a bout of rhabdomyolysis. The role of exercise may be variable as some patients regularly undertook physical exercise without adverse effects. In addition, heterozygous *LPIN1* gene nucleotide variants were separately identified in three adult patients with statin-induced rhabdomyolysis (Zeharia et al 2008), prompting to consider with caution statin medication in obligate heterozygotes (Nakajima et al 2011, Sarullo et al 2010, Shek and Ferrill 2001, Ucar et al 2000).

In conclusion, recessive *LPIN1* mutations constitute a major cause of severe and early rhabdomyolysis which can occasionally occur in adults. Most skeletal muscle biopsies show a histological pattern compatible with a metabolic disorder. Single heterozygous *LPIN1* mutations can also associate with moderate muscular symptoms. By contrast, no major defects of *LPIN2* or *LPIN3* genes have been associated with muscular diseases. Because of the high rate of death during bouts of rhabdomyolysis we advise proactive management when *LPIN1*-deficient patients complain of muscular pain and/or fever.

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Legends

Supplementary data, eTable 1: Molecular and clinical data of patients with *LPIN1*, *LPIN2* and *LPIN3* mutations or variants. The 17 previously reported patients with two *LPIN1* mutations (Michot et al 2010) are shown in shaded lines. NA : not available, CK : creatine kinase. Early : patient unable to precise the beginning of his symptoms.

Figure 1: **Decision tree for the distribution of the patients in the various subgroups of the cohort.**

Figure 2: **Histology of a patient at autopsy**. Right ventricle a) HES staining showing subepicardial and mediomural fatty replacement of myocardium. b) Sirius red staining showing dissociated tissue, infiltrated by adipocytes and interstitial fibrosis. Liver c) HES staining showing vacuoles. d) Oil Red O staining showing neutral fat in the vacuoles.

Figure 3: **Schematic representation of lipin-1 mutations at the protein level**. Previously reported mutations (Michot et al 2010) are shown below the protein bar and those described in the present report above. Mutations found in two or more unrelated patients are shown in bold; between brackets: number of patients carrying the mutation.

Figure 4: **Functional complementation assay in Δ*pah1* yeast strain**. The exogenous human proteins expressed in Δ*pah1* are indicated on the left. Empty: vector alone.

Figure 5: **Skeletal muscle morphology in patients carrying two LPIN1 mutations**: a) Oil-red O staining of a transverse section of muscle. b and c) Electron microphotographs of myofibres of two patients. Arrows: lipid droplets; arrowheads: mitochondrial aggregates. COX histochemical staining: d) control and e) patient.

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