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LIPE-related lipodystrophic syndrome: clinical features and disease modelling using adipose stem cells

Short title: Lipodystrophy due to HSL defects

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ABSTRACT

Objective

The term Multiple Symmetric Lipomatosis (MSL) describes a heterogeneous group of rare monogenic disorders and multifactorial conditions, characterized by upper-body adipose masses. Biallelic variants in *LIPE* encoding hormone sensitive lipase (HSL), a key lipolytic enzyme, were implicated in three families worldwide. We aimed to further delineate *LIPE*-related clinical features and pathophysiological determinants.

Methods

A gene panel was used to identify pathogenic variants. The disease features were reviewed at the French lipodystrophy reference center. The immunohistological, ultrastructural, and protein expression characteristics of lipomatous tissue were determined in surgical samples from one patient. The functional impact of variants was investigated by developing a model of adipose stem cells (ASCs) isolated from lipomatous tissue.

Results

We identified new biallelic *LIPE* null variants in three unrelated patients referred for MSL and/or partial lipodystrophy. The hallmarks of the disease, appearing in adulthood, included lower-limb lipoatrophy, upper-body and abdominal pseudolipomatous masses, diabetes and/or insulin resistance, hypertriglyceridemia, liver steatosis, high blood pressure, and neuromuscular manifestations. Ophthalmological investigations revealed numerous auto-fluorescent drusen-like retinal deposits in all patients. Lipomatous tissue and patient ASCs showed loss of HSL and decreased expression of adipogenic and mature adipocyte markers. *LIPE*-mutated ASCs displayed impaired adipocyte differentiation, decreased insulin response, defective lipolysis, and mitochondrial dysfunction.

Conclusions

Biallelic *LIPE* null variants result in a multisystemic disease requiring multidisciplinary care. Loss of HSL expression impairs adipocyte differentiation, consistent with the lipodystrophy/MSL phenotype and associated metabolic complications. Detailed ophthalmological examination could reveal retinal damage, further pointing to the nervous tissue as an important disease target.

78 **SIGNIFICANCE STATEMENT**

79

80 Multiple Symmetric Lipomatosis (MSL), characterized by upper-body pseudo-
81 lipomatous masses, can be associated with alcoholism or due to rare monogenic disorders. Its
82 pathophysiology remains elusive. We identified in three unrelated patients new biallelic
83 variants in *LIPE,* encoding the key lipolytic enzyme hormone sensitive lipase (HSL), and
84 defined the disease clinical features. MSL co-existed with peripheral lipoatrophy, diabetes
85 and/or insulin resistance, hypertriglyceridemia, liver steatosis, hypertension, neuromuscular
86 signs and previously undescribed drusen-like retinal deposits. We isolated adipose stem cells
87 from pseudo-lipomas. HSL expression was abolished, leading to major defects in adipocyte
88 differentiation, insulin response, lipolysis, and mitochondrial function.

89 Loss of HSL leads to a multisystemic disease with lipodystrophic syndrome and
90 neurological alterations. Cellular modeling reveals the major role of HSL for adipocyte
91 differentiation.

92

93 **INTRODUCTION**

94

95 Multiple Symmetric Lipomatosis (MSL), also known as Launois-Bensaude Lipomatosis or
96 Madelung disease is a generic term used to describe several rare disorders. MSL is characterized
97 by the progressive development of voluminous masses of adipose tissue, mostly localized in
98 the neck and upper body, and frequently associated with metabolic complications and
99 neurological manifestations. MSL was first described and most frequently encountered in
100 alcoholic patients (1). A few genetic forms have also been characterized. MSL could affect
101 ~20% of patients with the myoclonic epilepsy and ragged red fibers (MERRF) syndrome due
102 to variants in the mitochondrial *MTTK* gene (2, 3, 4). Two autosomal recessive form of MSL
103 have been described. Among them, MSL due to *MFN2* variants has been reported in a dozen
104 unrelated families (5, 6, 7). *MFN2* encodes mitofusin 2, a protein involved in mitochondrial
105 fusion and cellular energy metabolism (8). In addition, biallelic null variants in the *LIPE* gene
106 have been reported in 2 unrelated families of Israeli-Arab and Italian origin, and in 3 patients
107 from an Old Order Amish community with MSL (9, 10, 11, 12). *LIPE* encodes the hormone-
108 sensitive lipase (HSL), a key enzyme for triglyceride hydrolysis expressed during adipocyte
109 differentiation (13, 14, 15). At the clinical level, the different MSL subtypes cannot be easily
110 distinguished. The pathophysiological mechanisms underlying the development of lipomatous
111 masses remain also very poorly understood. A better understanding of these mechanisms is
112 important for the management of patients and could also expand our knowledge on more
113 common related polygenic conditions. As an example, the *LIPE* null variants responsible for
114 the autosomal recessive form of MSL have been reported as susceptibility factors to
115 hypertriglyceridemia and type 2 diabetes in the heterozygous state (9).
116 We and others have previously shown that *MFN2*-related MSL is a multisystem disease
117 characterized by pseudo-lipomatous masses but also by lipoatrophy, insulin resistance-

118 associated metabolic abnormalities and neurological involvement (5, 6, 7). We report here the
119 consequences of new biallelic pathogenic *LIPE* variants, identified in three unrelated patients,
120 at the clinical, tissular and cellular levels. Cellular disease modelling was obtained by isolating
121 adipose stem cells (ASCs) from lipomatous tissue and submitting them to adipocyte
122 differentiation *in vitro*.

123 **METHODS**

124

125 **Patients**

126 This study included three unrelated women with biallelic variants in the *LIPE* gene. Clinical
127 data collection was coordinated at the French reference center for rare diseases of insulin
128 secretion and insulin sensitivity. We obtained written informed consent for all genetic studies
129 as well as for the use of photographs shown in Figure 1. The study was approved by the
130 institutional research ethics board (CPP Ile de France 5).

131

132 **Genetic studies**

133 Genomic DNA was extracted from peripheral blood leukocytes using standard procedures. A
134 panel of genes used for routine diagnosis of lipodystrophic syndromes, including *LIPE* and
135 other genes, was analyzed. Exons and flanking intronic sequences of 23 genes were captured
136 from fragmented DNA with the SeqCapEZ enrichment protocol (Roche NimbleGen). Paired-
137 end massively parallel sequencing was achieved on a MiSeq platform (Illumina). Bioinformatic
138 analysis was performed using the Sophia Genetics DDM pipeline®. *LIPE* variants were
139 confirmed by Sanger sequencing with the Big Dye Terminator v3.1 sequencing kit (Thermo
140 Fisher) after PCR amplification and analyzed on a 3730XL device with the SeqScape v2.7
141 software (Applied Biosystems).

142

143 **Histological and immunohistological studies**

144 Samples of axillar lipomatous masses were obtained from patient A after surgical ablation for
145 functional discomfort. Control adipose tissue was obtained from subcutaneous abdominal
146 plastic surgery in 3 non-obese, non-diabetic women aged 40.5 ± 14.2 years (16). Light
147 microscopy was performed on 10% zinc-formol–fixed paraffin-embedded tissue sections,

148 stained with hematoxylin-eosin, and with Sirius Red to detect collagen fibers. For
149 immunohistochemical studies, tissue sections were probed with antibodies directed against
150 CD68 (PA0273, Leica), and CD163 (NCL-CD163, Leica). Electron microscopy procedures are
151 described in Supplemental Methods.
152
153 **Adipose stem cell isolation and culture**
154 Adipose stem cells were isolated from surgical samples of lipomatous masses in patient A and
155 of subcutaneous abdominal adipose tissue in controls. Adipose tissue samples were
156 enzymatically digested with collagenase B (0.2%). After centrifugation, stromal vascular
157 fraction was filtered, rinsed, plated and cultured in α-MEM with 10% Fetal Calf Serum (FCS),
158 2 mmol/L glutamin, 1% Penicillin/streptomycin (10,000 UI/mL), 1% Hepes and Fibroblast
159 Growth Factor-2 (145 nmol/L). After 24 h, only adipose stem cells adhered to plastic surfaces,
160 while other cells were removed after culture medium replacement (17).
161
162 **Adipocyte differentiation of ASCs**
163 The differentiation of ASCs was induced in DMEM with 10% Fetal Bovine Serum, 1 µmol/L
164 insulin, 500 µmol/L 3-isobutyl-1-methylxanthine (IBMX), 1 µmol/L dexamethasone and 1
165 µmol/L rosiglitazone. Cells were cultured in this medium for 7 days, then in adipogenic
166 maintenance medium (DMEM with 1 µmol/L insulin) for an additional 7 days. Mature
167 adipocytes were obtained within 14 days (17).
168
169 **Western blot analysis**
170 Frozen fat tissue or ASCs were solubilized in Laemmli buffer containing dithiothreitol. Lysates
171 were subjected to SDS-PAGE, blotted onto nitrocellulose membranes, and probed with
172 antibodies directed against HSL (#4107), FAS (#3180), IRβ (#3025), ERK1/2 (#9102),

173 phosphor-Tyr204-ERK1/2 (#9101) from Cell Signalling Technology; SREBP1c (SC-366),
174 PPARγ (SC-7273), phosphotyrosine residues (αPY, SC-7020), Akt (SC-8312), phospho-
175 Ser473-Akt (SC-7985-R), ATGL (SC-67355) from Santa Cruz Biotechnology; and C/EBPα
176 (#18311-1-AP, Proteintech), ADIPOQ (#MA1-054, Invitrogen), PLIN1 (ab3526, Abcam) and
177 IRS1 (#07-247, Upsate). Tubulin (T5168, Sigma-Aldrich) was used as a loading control. The
178 immune complexes were detected by chemiluminescence and quantified using the ImageJ
179 (NIH) software.
180
181 **Oil Red-O staining, gene expression analyses, lipolysis, insulin response, and**
182 **mitochondrial function studies**
183 These experiments, performed on ASCs submitted to adipocyte differentiation for 14 days, are
184 described in the Supplemental Methods section.
185
186 **Statistical analyses**
187 Data were expressed as means ± standard error of the mean (SEM) using the GraphPad Prism
188 6 software. Statistical significance was evaluated using non-parametric Mann-Whitney tests
189 with a threshold at $p < 0.05$.
190
191
192 **RESULTS**
193
194 **Identification of rare pathogenic variants in *LIPE***
195 Three women referred for MSL (patient A) and/or partial lipodystrophy (patients B and C) were
196 found to carry new germline biallelic null variants in *LIPE* (Fig. 1A). Patient A was
197 homozygous for the frameshift variant c.2828del; p.(Glu943Glyfs*22). Patient B was

198 compound heterozygous for two frameshift variants: c.1890_1891del; p.(Leu631Glyfs*57),
199 and c.2077del; p.(Arg693Valfs*76). Patient C carried a nonsense variant in the homozygous
200 state: c.1261C>T; p.(Gln421*). The variants identified in patient A and B were absent from
201 databases reporting variants from the general population (gnomAD, ExAC, Exome Variant
202 server). The variant identified in patient C was present in gnomAD at very low frequency (6.10^{-}
203 ^{5}), but was never observed in the homozygous state.
204
205 **Clinical picture of patients with *LIPE* pathogenic variants**
206 **Patient A** was a 54-year old woman born from asymptomatic first-cousin parents. She had
207 progressively developed lipomatous masses in the nape, shoulders, back, axillae, arms,
208 abdomen and pubis since the age of 40, leading to iterative surgical ablation for functional
209 discomfort. On clinical examination, she also presented with lipoatrophy of lower limbs and
210 forearms (Table 1, Fig. 1B). She was diagnosed with insulin resistance (fasting insulinemia
211 13.8 mU/L, increased to 133 mU/L after 2h-oral glucose tolerance test), hypertriglyceridemia
212 and low HDL-cholesterol (1.05 mmol/L) at age 35, hypertension at age 49, and fasting
213 hyperglycemia at age 53. Hypercortisolism was ruled out. Liver steatosis with increased liver
214 enzymes was present since the age of 45, in the absence of alcohol consumption. She had *pes*
215 *cavus* and complained of paresthesia. Cardiac examination and resting electrocardiogram were
216 normal. While visual acuity was normal, ophthalmological fundus monitoring revealed
217 numerous disseminated drusen-like retinal flecks at the age of 45. They appeared as bilateral
218 sub-retinal auto-fluorescent deposits of the posterior pole, without neovascularization, and were
219 confirmed by optical coherence tomography.
220
221

222 **Patient B**, born from asymptomatic non-consanguineous parents, was referred at age 76 for fat
223 accumulation in face and neck, lipomatous masses in axillar, abdominal and pubic areas, and
224 lower limb lipoatrophy (Table 1). She was investigated for cushingoid morphotype, infertility,
225 spaniomenorrhea and hirsutism at the age of 30, and hypercortisolism was ruled out.
226 Lipomatous masses had progressively appeared since the age of 40. Diabetes with insulin
227 resistance (fasting insulinemia 16.4 mU/L) was diagnosed at the age of 55. She also had
228 hypertriglyceridemia, low HDL-cholesterol, and high blood pressure. She displayed
229 paroxysmal atrial fibrillation at the age of 76 without sign of cardiomyopathy. Liver enzymes
230 were increased and echography showed hepatosplenomegaly. She complained of walking
231 difficulties due to asymmetric painful hypertrophy of the calves and *pes cavus*. Creatine
232 phosphokinase was increased and electromyogram revealed axonal sensorimotor
233 polyneuropathy. Magnetic resonance imaging (MRI) showed increased fat depots and
234 dystrophic features of the right gastrocnemius muscle (Table 1, Fig. 2B). She underwent
235 cataract surgery at the age of 67. Fundus examination at the age of 68 showed bilateral small
236 sub-retinal deposits disseminated throughout the posterior pole, in the absence of decreased
237 visual acuity.
238
239 **Patient C**, born from asymptomatic first-cousin parents, had progressively developed upper-
240 body adipose overgrowth since the age of 35. She underwent a surgical resection of cervical
241 and axillar pseudo-lipomatous masses at the age of 40 and 51. Clinical examination at the age
242 of 57 showed accumulation of fat in anterior and posterior neck, shoulders, arms and axillar
243 folds, contrasting with lipoatrophy of lower limbs (Fig. 1C, Fig. 2C). Patient C was diagnosed
244 with dyslipidemia (hypertriglyceridemia and low HDL-cholesterol) at the age of 40, then with
245 diabetes at the age of 47. She had severe hypertension since the age of 28, with macronodular
246 adrenocortical hyperplasia, in the absence of specific hormonal alterations. Abdominal MRI

247 showed liver steatosis. Liver function tests were normal except for increased gamma glutamyl
248 transferase levels, in the absence of alcohol consumption. Neuromuscular examination revealed
249 *pes cavus*. Cardiac investigations showed left ventricular hypertrophy linked to high blood
250 pressure. Routine ophthalmological examination at the age of 50, in the absence of visual
251 complaints, revealed a very high number of symmetrical sub-retinal yellowish deposits
252 disseminated in the macula, mid-peripheral and far-peripheral retina. These deposits were
253 intensely auto-fluorescent, and induced abnormalities in the structure of retinal pigmentary
254 epithelium, as assessed by optical coherence tomography (Table 1, Fig. 2D). Corrected visual
255 acuity and anterior segments were normal, as well as electro-retinogram and electro-oculogram.
256 Sequencing of a panel of genes involved in macular or retinal dystrophies with drusens/flecks,
257 including *ABCA4*, *BEST1*, *EFEMP1*, *IMPG1*, *IMPG2*, *PLA2G5*, *PRPH2*, *RDH5*, *RLBP1*, and
258 *TIMP3*, did not reveal any pathogenic variant.
259
260 **Characteristics of patient lipomatous tissue**
261 We studied surgical axillar lipomatous samples from patient A. Although it was not enclosed
262 in fibrous capsules, pseudo-lipomatous tissue displayed a particular architectural organization
263 with numerous lobules visible after Sirius red staining (marker of collagen), evocative of areas
264 of cell regeneration (Fig. 3A, a). Pseudo-lipomatous tissue was composed of unilocular white
265 adipocytes (Fig. 3A, b). It displayed inflammatory features with infiltrates of monocytes and
266 macrophages, as assessed by the positivity of CD68 and CD163 staining (Fig. 3A, c,d).
267 Western blot studies revealed a complete loss of HSL expression in lipomatous tissue (Fig. 3B).
268 The protein expression of the adipocyte differentiation marker peroxisome proliferator-
269 activated receptor gamma (PPARγ) was decreased in adipose tissue from patient A as compared
270 to controls. The protein expression of two markers of mature adipocyte, adiponectin (ADIPOQ)
271 and fatty acid synthase (FAS), was severely reduced (Fig. 3C). Electron microscopy of

272 lipomatous masses revealed no abnormalities in size and morphology of mitochondria (Fig.
273 3D).
274
275 **Defect in adipocyte differentiation of patient adipose stem cells (ASCs)**
276 The decrease in the expression of adipogenic and mature adipocyte markers in pseudo-
277 lipomatous tissue suggested a defect in adipocyte differentiation. To address this issue, we
278 isolated ASCs from lipomatous tissue of patient A and compared their *in vitro* adipocyte
279 differentiation with that of control ASCs.
280 Protein expression of HSL was detectable from day 7 onwards in control cells and further
281 increased during differentiation. In contrast, as expected from lipomatous tissue data, HSL
282 expression was completely absent in patient's ASCs submitted to adipocyte differentiation (Fig.
283 4A). Adipocyte differentiation was confirmed by accumulation of cytoplasmic lipid droplets
284 stained by Oil Red-O after 14 days of differentiation in control ASCs. In contrast, lipid
285 accumulation was markedly impaired in ASCs from patient A (Fig. 4B). Consistently, and in
286 accordance with the data obtained in the patient's lipomatous tissue, the protein expression of
287 mature adipocyte markers (ADIPOQ, FAS,PLIN1 and ATGL) was strongly decreased in ASCs
288 from patient A as compared to control ASCs after 14 days of *in vitro* adipocyte differentiation.
289 Mutated ASCs showed a low to normal expression of adipogenic proteins (PPARγ, C/EBPα,
290 SREBP1c) (Fig. 4C). In addition, the mRNA expression level of *LIPE* and of adipogenic and
291 mature adipocyte genes was decreased in patient ASCs as compared to controls (Fig. 4D).
292 Taken together, our results strongly suggest that *LIPE* null variants impair the gene activation
293 cascade required for proper adipocyte differentiation.
294
295 **Altered function of adipocytes harboring *LIPE* pathogenic variants**
296 We then studied the functional characteristics of patient ASCs after 14 days of adipocyte
297 differentiation. We first evaluated the efficiency of lipolysis, known to be strongly regulated by

298 HSL. Glycerol, a product of triglyceride lipolysis, was measured in cell supernatants in the
299 basal state and after stimulation by forskolin, an adenylate cyclase activator. Basal lipolysis was
300 not different in patient and controls (Fig. 4E). However, whereas we observed a 3-fold increase
301 in the release of glycerol after forskolin treatment in control cells, the level of glycerol remained
302 unchanged in the supernatants of ASCs from patient A, indicating an altered lipolytic response
303 to cAMP (Fig. 4E). We then investigated the cellular insulin response. Upon insulin stimulation,
304 the activation of proximal and distal signaling intermediates (IRβ, IRS1, Akt/PKB, and
305 ERK1/2) was decreased in ASCs from patient A as compared to control ASCs (Fig. 4F).
306
307 **Mitochondrial dysfunction in ASCs harboring pathogenic *LIPE* variants**
308 Since mitochondrial function contributes to adipocyte differentiation (18, 19), and since genes
309 encoding mitochondrial proteins have been previously involved in monogenic forms of MSL,
310 we hypothesized that *LIPE* pathogenic variants could alter mitochondrial functions. The
311 analysis of basal oxygen consumption rates (OCR) of ASCs from patient A and controls did
312 not reveal any significant difference. However, the patient's ASCs displayed a major decrease
313 in their maximal respiratory capacity, as indicated by a significantly lower increase in OCR
314 after addition of the uncoupling agent FCCP (Fig. 5A). Of note, the mitochondrial mass
315 evaluated by MitoTracker staining was not decreased (data not shown). The mitochondrial
316 membrane potential, evaluated by the capacity of the JC1 cationic dye to form aggregates, was
317 significantly decreased (Fig. 5B). In addition, oxidative stress, assessed by the production of
318 reactive oxygen species (ROS), was increased in cells from patient as compared to control cells
319 (Fig. 5C).
320
321

322 **DISCUSSION**

323

324 The *LIPE*-associated MSL syndrome is a very rare disease since only 6 families have been
325 reported worldwide, if we include patients from the current study and previous datasets (Table
326 1). The variants identified in each index case are different and patients are from various origins
327 (French, Israeli-Arab, Italian, American Amish), consistent with independent mutational
328 events. All the molecular defects in *LIPE* reported to date are null variants including four
329 frameshift variants and one nonsense mutation. Consistent with another report (9), our data
330 show that biallelic frameshift variants result in a complete loss of HSL expression.

331

332 Considering the very low number of patients reported with *LIPE* pathogenic variants, we
333 integrated our clinical data with those described previously to draw the picture of the disease
334 (Table 1). *LIPE*-related MSL is a late-onset disease, since the age at referral for diagnosis ranges
335 from 23 to 76 years. Among the clinical signs, the following fours were the most common:
336 lipoatrophy, lipomatosis, metabolic complications and neuromuscular manifestations.
337 Unexpectedly, lipoatrophy is the predominant hallmark of the disease, since it was reported in
338 most patients. It usually affects lower limbs and sometimes forearms. In contrast, pseudo-
339 lipomatosis or areas of fat accumulation, present in all three patients from our study, was
340 reported in only half of previously published cases. Adipose masses are most frequently
341 observed in the neck, armpits, abdomen, and pubis. The metabolic complications observed in
342 nearly all patients are similar to those observed in lipoatrophic syndromes and include insulin
343 resistance and/or diabetes, hypertriglyceridemia, and liver steatosis. High blood pressure is also
344 frequent. These characteristics suggest that the term “lipodystrophic syndrome” would be more
345 appropriate than “multiple symmetric lipomatosis (MSL)” to describe the disease in patients
346 with *LIPE* variants. Notably, the term “lipomatous” has also been used to describe areas of fat

347 accumulation in genetically-characterized lipodystrophy (20), further stressing the clinical
348 continuum between so-called "MSL" and lipodystrophic syndromes. Apart from lipodystrophy,
349 neuromuscular manifestations are present in nearly half of patients. Importantly, *pes cavus* was
350 described in the absence of diabetes, in two patients with biallelic *LIPE* variants reported by
351 Zolotov *et al*. (12) and in patient A from our study, arguing for a direct neuromuscular impact
352 of *LIPE* variants.
353
354 The three patients from our study present with countless sub-retinal yellowish deposits
355 disseminated in the macula and the peripheral retina that did not match with any description of
356 drusen or flecked-retina disorders (21). This is the first description of ophthalmological signs
357 in patients with *LIPE* pathogenic variants. Since they did not lead to visual complaints, their
358 diagnosis requires a systematic fundus examination. Their bilateral and symmetric disposition,
359 and their diagnosis at a young age in two patients (45-50 years old), strongly suggest that they
360 could be of genetic origin. Several clues argue for a role of *LIPE* pathogenic variants in the
361 development of these peculiar ophthalmological alterations: (i) HSL is expressed not only in
362 adipocytes, but also in other organs including neurological tissues such as retina (22) (ii) HSL
363 hydrolyzes triglycerides, but also retinyl and cholesterol esters, and alteration of neutral lipid
364 and cholesterol homeostasis is one of the mechanisms involved in drusen biogenesis (23, 24).
365
366 The study of the consequences of *LIPE* variants is of importance not only to improve diagnosis
367 and management of lipodystrophic syndromes, but also to better understand the role of HSL in
368 humans. HSL is one of the three enzymes of triglyceride lipolysis. Adipose triglyceride lipase
369 (ATGL) is able to catalyze the three successive enzymatic steps to release all non-esterified
370 fatty acids molecules from triacylglycerol, then HSL and monoglyceride lipase (MGL) are
371 thought to predominantly hydrolyze diglycerides and monoglycerides, respectively (13).

372 Several HSL-deficient mice models, which do not fully recapitulate the human disease, have
373 been generated (25, 26, 27, 28, 29). Although HSL-deficient mice show reduced abdominal fat
374 mass with normal body weight and decreased cAMP-stimulated lipolysis, they display
375 decreased levels of circulating triglycerides, and their glucose homeostasis, insulin sensitivity,
376 and liver lipid metabolism are very heterogeneous. This might be due, at least partly, to their
377 different genetic backgrounds. However, adipose-specific HSL knockout mice have been
378 described with age-dependent lipodystrophy, adipose tissue inflammation, systemic insulin
379 resistance and hepatic steatosis, suggesting that the functions of HSL in adipose tissue strongly
380 influence the whole-body metabolic homeostasis (29). How could decreased lipolysis due to
381 HSL deficiency be associated with insulin resistance and fatty liver in humans? Since HSL
382 plays a crucial role for proper adipocyte function and since its expression increases during
383 adipocyte differentiation (15), we made the hypothesis that *LIPE* null variants could alter
384 adipocyte differentiation. Human ASCs represent an appropriate model to address this issue.
385 During adipose differentiation of ASC *in vitro, LIPE* pathogenic variants lead to a severe
386 decrease in intracellular lipid droplets formation and in expression of adipocyte-specific
387 markers. Consistently, expression of adipocyte proteins were also downregulated in lipomatous
388 tissue obtained from a patient, consistent with data published by Albert *et al* (9). In accordance,
389 the insulin-mediated signaling pathways were strongly impaired in cells harboring pathogenic
390 *LIPE* variants. All these data were in agreement with the pathophysiology of other genetically-
391 determined lipodystrophic syndromes, which is mainly driven by primary defects in
392 differentiation, maintenance and/or functions of adipocytes (30). The mechanistic link between
393 HSL deficiency and adipocyte differentiation defects remains to be clarified. It was proposed
394 that HSL could act as a retinyl ester hydrolase, which provides ligands to activate the central
395 adipogenic factor PPARγ and/or the Retinoid X Receptor (RXR) (31, 32). Defective HSL-
396 mediated PPARγ activation could therefore be an important mechanism leading to defects in

397 adipocyte differentiation and maturation. Increased adipose tissue inflammation, described in
398 our study as well as by Albert *et al* (9), could also contribute to adipocyte dysfunction and
399 impaired differentiation. Finally, using the ASC model, we demonstrated the deleterious effect
400 of *LIPE* pathogenic variants on mitochondrial function, including decreased mitochondrial
401 respiration, decreased mitochondrial membrane potential and increased ROS production. These
402 data are consistent with those obtained previously in a model of hMADS adipocytes treated
403 with an HSL inhibitor, which severely decreased mitochondrial respiration (33). Mitochondrial
404 dysfunction is known to impair adipocyte differentiation and to contribute to metabolic diseases
405 such as obesity, diabetes and lipodystrophic syndromes (19, 34, 35). Other known genetic forms
406 of MSL are due to variants in *MTTK*, a mtDNA gene necessary for proper oxidative
407 phosphorylation and in *MFN2*, playing a major role in mitochondrial dynamics. Mitochondrial
408 dysfunction could therefore represent a pathophysiological mechanism shared by *MTTK*-,
409 *MFN2*-, and *LIPE*-related MSL.
410
411 The current study has some limitations. We only had the opportunity to study lipomatous tissue
412 and ASCs from one of the three patients with *LIPE* pathogenic variants. Control and lipomatous
413 tissue were both of subcutaneous origin, but originated from different depots, and we did not
414 obtain similar lipomatous masses from patients without *LIPE* variants for comparison.
415 However, these original clinical and cell modeling data, which increase the knowledge on the
416 consequences of *LIPE* pathogenic variants in humans, pave the way for future additional
417 studies.
418
419 In conclusion, biallelic null variants in *LIPE* lead to a multisystem disease associating features
420 of MSL and lipodystrophy syndrome with neuromuscular involvement which requires
421 multidisciplinary care. Attention should be drawn to possible specific retinal lesions that justify

422 further studies on the pathophysiological role of HSL in the retina. This work reveals the
423 multiple functional impacts of *LIPE* pathogenic variants on adipocyte differentiation and
424 functions and points to unexpected roles of hormone sensitive lipase in other organs.
425
426

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DISCLOSURE

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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554

555 **FIGURE LEGENDS**

556

557 **Figure 1. Genetic and clinical features in patients with biallelic *LIPE* pathogenic variants**

558 **A.** Human *LIPE* cDNA structure with positions of the *LIPE* variants identified in patients from

559 the current study (patients A, B and C) and from previous reports. The organization of hormone-

560 sensitive lipase (HSL) domains of are shown below.

561 **B.** Pictures of patient A showing lipomatous masses in the nape, shoulders, back, axillae, arms,

562 and abdomen (arrows) with lipoatrophy of lower limbs and forearms (arrowhead).

563 **C.** Pictures of patient C showing lipomatous masses in the anterior and posterior cervical areas,

564 as well as shoulders and armpits (black arrows).

565

566 **Figure 2. Imaging features in patients with biallelic *LIPE* pathogenic variants**

567 **A.** Axial computed tomography showing posterior cervical lipomatous regions in patient A

568 (arrow).

569 **B.** Coronal (a) and axial (b, c) T1-weighted magnetic resonance imaging of calves in patient B

570 showing an increased volume of the right medial gastrocnemius muscle, with increased fat

571 depots and dystrophic features, and muscular mild gadolinium contrast enhancement (c).

572 **C.** T1-weighted magnetic resonance imaging in patient C, showing accumulation of fat in the

573 sub-mandibular area (a, b: sagittal section; c: coronal section) with slight adipose infiltration of

574 the tongue muscle (a, arrow). Fat accumulation is also observed in the posterior cervical areas,

575 anterior and posterior thoracic regions (b), and in neck, shoulders, and axillary regions (d:

576 coronal section).

577 **D.** Ophthalmological multimodal fundus imaging of patient C. Fundus picture (a) showing

578 multiple yellow flecks with irregular edges disseminated in the macula and mid-peripheral

579 retina. Optic disc and retinal vasculature have a normal aspect. Short-wavelength fundus auto-

580 fluorescence (b). Most flecks are intensely auto-fluorescent. Larger flecks are surrounded by a
581 hypofluorescent halo. Spectral domain optical coherence tomography (c, d, e). Flecks
582 correspond to mound-shaped elevations of retinal pigmentary epithelium filled with iso- to
583 hyper-reflective content. Interruption of ellipsoid zone and internal limiting membrane are
584 observed on the apex of the fleck (d, arrow). Focal interruptions of outer retinal layers are
585 present (e, arrows).
586
587 **Figure 3. Characteristics of lipomatous tissue in patient A**
588 **A.** Histological and immunohistological features of lipomatous tissue from patient A (*LIPE*-
589 MSL), as compared to subcutaneous adipose tissue from one representative control. The
590 different panels (a-d) correspond to specific stainings, as indicated on the left. HE: hematoxylin-
591 eosin; SR: Sirius red. CD68 and CD163 are monocyte-macrophage markers.
592 **B.** Protein expression obtained by Western blotting of hormone-sensitive lipase in two samples
593 of lipomatous tissue from patient A, as compared to subcutaneous adipose tissue from two
594 representative controls. HSL: hormone-sensitive lipase.
595 **C.** Protein expression obtained by Western blotting of adipocyte markers in lipomatous tissue
596 from patient A, in comparison to subcutaneous adipose tissue from two representative controls.
597 PPARγ: peroxisome proliferator-activated receptor gamma; ADIPOQ: adiponectin ; FAS: fatty
598 acid synthase.
599 **D.** Ultrastructural characteristics of lipomatous tissue from patient A, as compared to
600 subcutaneous adipose tissue from one representative control. Double arrows indicate the
601 thickness of the cytoplasmic rim. m: mitochondria, L: lipid droplet.
602
603 **Figure 4. Defects in adipocyte differentiation and functions in ASCs harboring pathogenic**
604 ***LIPE* variants**

605 **A.** Protein expression of hormone-sensitive lipase (HSL) obtained by Western blotting during
606 adipocyte differentiation (day 0 to day 14) in patient A (*LIPE*-MSL) and control ASCs.
607 **B.** Adipocyte differentiation assessed by Oil Red-O lipid staining. ASCs from patient A (*LIPE*-
608 MSL) and controls were studied after adipocyte differentiation for 14 days. Top: Representative
609 images of fluorescence microscopy after staining of intracellular lipids (Oil Red-O, red) and
610 nuclei (DAPI, blue). Bottom: Pictures of cell dishes stained by Oil Red-O and quantification of
611 Oil Red-O fluorescence normalized to DNA content (DAPI). Results are expressed as means ±
612 SEM of four independent experiments.
613 **C.** Protein expression of adipocyte markers obtained by Western blotting in patient A (*LIPE*-
614 MSL) and control ASCs after *in vitro* adipocyte differentiation for 14 days. Results are
615 representative of three independent experiments.
616 PPARγ: peroxisome proliferator-activated receptor gamma; C/EBPα: CCAAT/enhancer
617 binding protein alpha; SREBP1c: sterol regulatory element-binding protein 1c; ADIPOQ:
618 adiponectin; FAS: fatty acid synthase; PLIN1: perilipin-1 ; ATGL: adipose triglyceride lipase.
619 **D.** Gene expression of adipocyte markers obtained by RT-qPCR in patient A (*LIPE*-MSL) and
620 control ASCs submitted to adipocyte differentiation for 14 days. Results are expressed as means
621 ± SEM of three independent experiments. **: $p < 0.01$. *PNPLA2* encodes adipose triglyceride
622 lipase (ATGL).
623 **E.** Lipolytic function in patient (*LIPE*-MSL) and control ASCs submitted to adipocyte
624 differentiation for 14 days. Cells were incubated or not for 4 h with the adenylate cyclase
625 activator forskolin (FSK) as indicated. Lipolysis was evaluated by the production of glycerol
626 in supernatants relative to the protein content in each well. Results are expressed as means ±
627 SEM of three independent experiments. ns: non significant; *: $p < 0.05$; **: $p < 0.01$.
628 **F.** Activation of insulin signaling in patient (*LIPE*-MSL) and control ASCs submitted to
629 adipocyte differentiation for 14 days. Insulin-induced phosphorylation of insulin receptor β-

630 subunit (IRβ), insulin receptor substrate-1 (IRS1), protein kinase B (Akt/PKB), and
631 extracellular-regulated kinase (ERK)1/2 was evaluated by Western blotting. Results are
632 representative of three independent experiments.
633 PY: phosphotyrosine residues; Phospho-Akt/PKB: phospho-Ser473-Akt; Phospho-ERK1/2:
634 phospho-Tyr204-ERK1/2.
635
636 **Figure 5. Mitochondrial dysfunction in ASCs harboring pathogenic *LIPE* variants**
637 **A.** Cellular respiration assessed by the measurement of oxygen consumption rate (OCR) in
638 patient A (*LIPE*-MSL*, grey diamonds*) and control ASCs (*open squares*) previously submitted
639 to adipocyte differentiation for 14 days, using Seahorse at baseline and after sequential addition
640 of oligomycin (inhibitor of ATP synthase), carbonyl cyanide 4-
641 trifluoromethoxyphenylhydrazone (FCCP, uncoupling agent), and rotenone/antimycin A
642 (AA/R, inhibitors of the respiratory chain complexes I and III), as described in Supplemental
643 Methods. Results are means ± SEM of three independent experiments. *: $p < 0.05$.
644 **B.** Mitochondrial membrane potential assessed by JC-1 (tetrachloro-tetra-ethyl-
645 benzimidazolyl-carbocyanine iodide) cationic dye staining (aggregates to monomers ratio) in
646 patient (*LIPE*-MSL) and control ASCs previously submitted to adipocyte differentiation for 14
647 days. Results are expressed as means ± SEM of five independent experiments. **: $p < 0.01$.
648 **C.** Reactive oxygen species (ROS) production assessed by oxidation of 5-6-chloromethyl-2,7-
649 dichlorodihydro-fluorescein diacetate (CM-H2DCFDA) in patient and control ASCs previously
650 submitted to adipocyte differentiation for 14 days. Results normalized to DNA content
651 measured by DAPI, are expressed as means ± SEM of four independent experiments *: $p <$
652 0.05.
653
654