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MFN2-associated lipomatosis: clinical spectrum and impact on adipose tissue

Running title: *MFN2*-associated lipodystrophic syndrome

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ABSTRACT

Background. Multiple symmetric lipomatosis (MSL) is characterized by upper-body lipomatous masses frequently associated with metabolic and neurological signs. *MFN2* pathogenic variants were recently implicated in a very rare autosomal recessive form of MSL. *MFN2* encodes mitofusin-2, a mitochondrial fusion protein previously involved in Charcot-Marie-Tooth neuropathy (CMT).

Objective. To investigate the clinical, metabolic, tissular, and molecular characteristics of *MFN2*-associated MSL.

Methods. We sequenced *MFN2* in 66 patients referred for altered fat distribution with one or several lipomas or lipoma-like regions and performed clinical and metabolic investigations in patients with positive genetic testing. Lipomatous tissues were studied in three patients.

Results. Six patients from five families carried a homozygous p.Arg707Trp pathogenic variant, representing the largest reported series of *MFN2*-associated MSL. Patients presented both lipomatous masses and a lipodystrophic syndrome (lipoatrophy, low leptinemia and adiponectinemia, hypertriglyceridemia, insulin resistance and/or diabetes). CMT was of highly variable clinical severity. Lipomatous tissue mainly contained hyperplastic unilocular adipocytes, with few multilocular cells. It displayed numerous mitochondrial alterations (increased number and size, structural defects). As compared to control subcutaneous fat, mRNA and protein expression of leptin and adiponectin was strikingly decreased while the CITED1 and FGF21 thermogenic markers were strongly overexpressed. Consistently, serum FGF21 was markedly increased, and ^{18}F-FDG-PET-scan revealed increased fat metabolic activity.

Conclusion. *MFN2*-related MSL is a novel mitochondrial lipodystrophic syndrome involving both lipomatous masses and lipoatrophy. Its complex neurological and metabolic phenotype

justifies careful clinical evaluation and multidisciplinary care. Low leptinemia and adiponectinemia, high serum FGF21 and increased ^{18}F-FDG body fat uptake may be disease markers.

Keywords

Mitofusin 2; genetics; lipomatosis; neuropathy; lipodystrophy; dyslipidemia; insulin resistance; mitochondria; leptin; thermogenic markers

INTRODUCTION

Multiple symmetric lipomatosis (MSL, MIM 151800) or Launois-Bensaude lipomatosis is a rare disorder characterized by the development of unencapsulated masses of adipose tissue, especially in the neck and upper trunk [1, 2]. In most patients, it is associated with high alcohol intake [2, 3], though several familial forms have been reported. The first genetic form to be identified was the MERRF syndrome (Myoclonus Epilepsy and Ragged Red Fibers), usually due to pathogenic variants in the mitochondrial *MT-TK* gene [4]. More recently, rare patients with biallelic *MFN2* pathogenic variants have been shown to display an autosomal recessive form of MSL, associated or not with axonal neuropathy and metabolic complications [5, 6]. Among them, the metabolic phenotype of only four patients, all adolescents, has been studied, with characterization of surgical biopsies of adipose tissue masses in three cases [5].

The *MFN2* gene, located in the nuclear genome, is ubiquitously expressed. It encodes mitofusin-2, a mitochondrial outer membrane protein of the dynamin-related GTPase family, involved in mitochondrial fusion and in endoplasmic reticulum-mitochondria interactions [7, 8]. This protein could also regulate a number of functions including whole-body energy metabolism and aging [9-13]. A high number of *MFN2* variants, located all along the gene, have been shown to be responsible for the autosomal-dominant and axonal form of Charcot-Marie-Tooth (CMT2A) [14, 15]. Notably, all patients with MSL due to *MFN2* carry the same pathogenic variant, p.Arg707Trp, in the homozygous state or in the compound heterozygous state. The rare patients carrying biallelic *MFN2* pathogenic variants other than p.Arg707Trp have never been reported to display lipomatosis [14, 16-21], but present with early-onset and

usually severe CMT2A. Individuals carrying only one *MFN2* p.Arg707Trp mutated allele were not reported to develop MSL but can present mild signs of CMT [18].

Adipose tissue is critical for whole-body metabolic homeostasis and fat distribution is closely linked to cardiometabolic risk [22, 23]. Anatomically distinct adipose tissues vary in embryological origins, gene expression profiles, and adipokine secretion patterns. In the case of MSL, the mechanisms by which the p.Arg707Trp variant, in the homozygous or compound heterozygous state, leads to localized adipose tissue overgrowth, remains largely unknown. Previous reports prior to the identification of *MFN2* as a disease-causing gene suggested that adipose tissue masses observed in MSL could originate in brown fat [24, 25]. This idea was strengthened by the primary localization of lipomatous regions in the supraclavicular and interscapular regions, known to contain thermogenic adipose tissue [26]. The recent report by Rocha *et al* describing *MFN2*-associated MSL revealed that affected adipose tissue displays UCP1-negative unilocular adipocytes with enlarged and disorganized mitochondria, reduced levels of mtDNA, increased expression of genes involved in mitochondrial oxidative stress, and a strong decrease in leptin and adiponectin expression [5].

Here, we take advantage of our national recruitment of patients as an expert center of lipodystrophic diseases to study the clinical, metabolic, tissular, and molecular characteristics of six patients, representing the largest series of *MFN2*-associated MSL reported to date.

MATERIAL AND METHODS

Patients

This study includes six patients from five independent families. Two patients were referred to our French reference center for rare disorders of insulin secretion and insulin sensitivity and clinical files of all patients were reviewed in this center. Informed written consent was obtained from each individual. This study was approved by the Comité de Protection des Personnes Ile-de-France 5 (Paris, France).

MFN2 screening

Genomic DNA (gDNA) was extracted from peripheral blood leukocytes using standard procedures. *MFN2* was screened by Sanger sequencing. To this purpose, all *MFN2* coding exons and their flanking intronic sequences were amplified by PCR. Purified PCR products were sequenced with the Big Dye Terminator sequencing kit (Applied Biosystems), and run on an automated sequencer (ABI 3730xl). Sequences were analyzed with SeqScape v2.7 (Applied Biosystems). Primer sequences are available upon request.

Leptin, adiponectin, and FGF21 measurements

Leptin and FGF21 (Quantikine; R&D Systems – Bio-Techne, Lille, France), as well as total adiponectin (Bühlman, Basel, Switzerland), were measured by ELISA according to manufacturers' recommendations. All measurements were performed in duplicates. The intraseries coefficients of variation (CV) were <3.5%, <3.5%, and <5% for leptin, FGF21, and adiponectin dosages, respectively; the interseries CV were <5.5%, <5.2%, and <7% for the same measurements.

Histology

Conventional light microscopy was performed on 10% zinc-formol-fixed paraffin-embedded 5-µm tissue sections, stained with hematoxylin-eosine. Adipocyte size was determined with a semi-automatic image analysis system (ImageJ, custom macro) in three randomly chosen regions. For immunohistochemical studies, tissue sections were probed with antibodies directed against alpha smooth muscle actin (1/300 dilution; M085, Dako, Trappes, France), CD34 (pre-diluted; PA0212, Leica, Nanterre, France), CD68 (pre-diluted; PA0273, Leica), CD163 (1/50; NCL-CD163, Leica), UCP1 (1/300; Abcam ab23841, Cambridge, UK), CITED1 (1/100; Novus H0000443S-M03, Bio-Techne, Lille, France), and FGF21 (1/300; Abcam ab171941, Cambridge, UK).

Electron microscopy

Ultrastructural analysis was performed on fat samples fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.4 at 4°C. Fragments were then post-fixed in 1% osmium tetroxide, dehydrated using graded alcohol series and embedded in epoxy resin. Semi-fine sections (0.5 µm) were stained with toluidine blue. Ultrastructure sections (60 nm) were contrasted with uranyl acetate and lead citrate and examined using an electron microscope (JEOL 1010, Tokyo, Japan) with an OSIS mega View III camera.

mRNA assays

Total RNA was extracted from fat samples stored in liquid nitrogen using the RNeasy lipid tissue mini-kit according to the manufacturer's instructions (Qiagen, Courtaboeuf, France). cDNA was synthetized from 1µg mRNA (High capacity cDNA reverse transcription kit, Thermo Fisher Scientific, Courtaboeuf, France). Real-time quantitative PCR (RT-qPCR) was performed with a LightCycler 480 using the SYBR Green I Master mix (Roche Diagnostics,

Meylan, France). Data were normalized to the expression of the *PPIA* (peptidylprolyl isomerase A) housekeeping gene. The list of primers used is available on request.

Western Blot analysis

Frozen fat tissue (300 mg) was solubilized in 500 µl 2.5X Laemmli buffer containing 150 mM dithiotreitol. Lysates were subjected to SDS-PAGE, blotted onto nitrocellulose membranes, and probed with antibodies directed against MFN2 (1/1000; Abcam, ab50838, Cambridge, UK), SREBP-1 (1/500; SC-366, Santa Cruz Biotechnology, TX, USA), PPARγ (1/500; SC-7196), adiponectin (1/1000; MAI-054, Affinity BioReagents, Golden United States), CITED1 (1/1000; Novus H0000443S-M03, Bio-Techne, Lille, France), FGF21 (1/1000; Abcam ab171941), PGC-1α (1/500; SC-5816), and tubulin (T5168, Sigma-Aldrich, St. Quentin Fallavier, France). Tubulin was used as a loading control.

^{18}F-FDG-PET scan

^{18}F-fluoro-2-deoxy-D-glucose (FDG) positron emission tomography (PET) with computed tomography (CT) was performed according to the EANM guidelines [27]. Patients fasted for at least 4.5 h. A Gemini Dual PET/CT camera (Philips) with time of flight (TOF) technology was used for imaging. Low-dose CT (120 kVp, 30–50 mAs) was acquired first, followed by PET acquisition 60–70 min after a weight-adjusted dose of 2.5 MBq/kilogram ^{18}F-FDG injection, covering a complete whole-body field of view, from the skull to the feet. Standardized uptakes values (SUV) were calculated for the regions of interest. FDG uptake in the liver was taken as a reference.

RESULTS

General characteristics of the studied patients

66 patients were referred to our unit for altered fat distribution and presented with one or several lipomas or lipomatous-like regions. *LMNA*-related familial partial lipodystrophy, which is occasionally associated with lipomas [28], was ruled out by genetic testing. In all patients, we sequenced the exons and intron-exon boundaries of the *MFN2* gene. Six patients from five independent families harbored biallelic pathogenic variants. The group of patients with a positive genetic diagnosis included five women and one man, ranging in age from 31 to 76 years old (mean: 54.5 ± 6.5 years). Patients P1 to P4 corresponded to sporadic cases of MSL, when P5 and P6 are siblings. P2, P4, P5 and P6 originated from Portugal, P1 and P3 from France (Table 1).

Molecular basis of the disease

All patients carried the recurrent *MFN2* c.2119C>T pathogenic variant (p.Arg707Trp; reference mRNA: NM_014874.3) in the homozygous state. None of them was found to carry any additional pathogenic or likely pathogenic variant in the complete coding sequence of *MFN2* or in its flanking intronic sequences. The frequency of the p.Arg707Trp variant in the general population, estimated at 2.10^{-4} (gnomAD browser), is low. In this regard, patient P4 was born from consanguineous parents. Consanguinity was not established in other families, although it was very likely in the family of P1 (Table 1). The p.Arg707Trp variant is located in the protein carboxy-terminal coiled coil heptad-repeat domain HR2, which is believed to be critical for homotypic interactions of mitofusin-2 and outer mitochondrial membrane fusion [29].

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Sex	F	M	F	F	F	F
Age at last investigation	76 years	31 years	67 years	53 years	55 years	45 years
Origin	France	Portugal	France	Portugal	Portugal	Portugal
Consanguinity	not established but endogamy	not known	not known	yes parents are first cousins	not known	not known
Weight (kg)	48	69	66	73	53.6	77
Height (m)	1.60	1.74	1.59	1.63	1.46	1.56
Body mass index ($kg.m^{-2}$)	18.7	22.8	26.1	27.5	25.1	31.6
Genotype at the *MFN2* locus	p.Arg707Trp homozygous	p.Arg707Trp homozygous	p.Arg707Trp homozygous	p.Arg707Trp homozygous	p.Arg707Trp homozygous	p.Arg707Trp homozygous

Table 1. General characteristics of patients

Clinical and biological presentation of patients

Natural history of the disease

There is a great heterogeneity in the chronology of the onset of disease manifestations in the different patients of the series and in the severity of these manifestations, leading some clinical features to be overlooked in certain individuals (Table 2). In patients P1 and P4, neurological signs occurred first during childhood, followed by the development of lipomatous masses at the age of 25 and 30 years, respectively. Metabolic complications were diagnosed several dozen years later, after the diagnosis of *MFN2*-associated MSL. P2 was first diagnosed with *acanthosis nigricans*, hyperglycemia and severe insulin resistance at the age of 10 years, then with adipose overgrowth at the age of 11 years, whereas mild neurological signs appeared in adulthood. In patient P3, all clinical signs started during adulthood, neurological signs appearing last. In patients P5 and P6, lipomatous masses appeared first in childhood, followed by metabolic alterations and paucisymptomatic neuropathy during adulthood (Table 2).

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age at first lipoma-like fat masses	30 years	11 years	35 years	25 years	6 years	2 years
Age at diagnosis of hyperglycemia, insulin resistance, or liver steatosis	76 years (not investigated prior to *MFN2* testing)	10 years	adulthood	53 years (not investigated prior to *MFN2* testing)	55 years	adulthood
Age at first neurological signs	childhood	26 years	65 years	childhood	54 years	adulthood
Age at genetic diagnosis	76 years	31 years	67 years	53 years	55 years	45 years

Table 2. Natural history of the disease

Lipomatosis and lipoatrophy

All patients presented with multiple lipoma-like adipose masses. Although adipose overgrowth affected the posterior part of neck and/or thorax in all patients, other lipomatous masses developed in various localizations depending on the cases: scalp (P4), chin (P4, P5, P6), tongue (P3), anterior chest (P3, P4, P5), back (P2), shoulders and arms (P1, P3, P4, P5, P6), thighs (P2, P4, P5, P6) and/or abdomen (P4, P5, P6). Lipomatous masses also strikingly varied in size: P1, aged 76, developed two fat masses in left arm and nape, with a larger diameter of 4 and 6 cm, respectively. P2, P5 and P6 had giant dorsocervical lipomatous masses that required surgery removal procedures. None of the patients had adipose overgrowth in forearms nor lower legs (Table 3, Figure 1). The lipomatous masses were unencapsulated and frequently difficult to delineate as observed in magnetic resonance imaging (MRI) showing a similar signal intensity in the regions of adipose tissue accumulation and in the adjacent subcutaneous adipose tissue (Figure 2 and data not shown). Apart from lipomatous areas, patients presented with a marked subcutaneous lipoatrophy in the four limbs in P1 and P2, and in lower limbs and forearms in others (Table 3, Figure 1). Visceral adipose tissue was also hardly detectable on abdominal CT or MRI (performed in patients P1, P2, P4, P5, data not shown). Consistently, and despite the presence of localized

adipose overgrowth, the percentage of total body fat mass was either decreased (in P1, P2, P4, as assessed by dual-energy X-ray absorptiometry - DEXA) or found to be normal (in P5, as measured by impedancemetry). In addition, the serum leptin level, which is a relevant biomarker of the white adipose tissue mass, was extremely low with respect to the patients' body mass index (mean concentration: 0.98 ± 0.28 $\mu g.L^{-1}$, patients P1 to P5) (Table 3).

Metabolic presentation

Most patients had low high density lipoprotein (HDL)-cholesterol (mean: 0.90 ± 0.34 $mmol.L^{-1}$) and high serum triglycerides (mean: 2.66 ± 0.97 $mmol.L^{-1}$). Insulin resistance is a common feature of the disease, as shown by the presence of *acanthosis nigricans*, increased fasting or 120 min-oral glucose tolerance test (OGTT) insulinemia, and/or M-value measured during hyperinsulinemic euglycemic clamps. In accordance, adiponectin levels were low (1.03 ± 0.43 $mg.L^{-1}$), and liver steatosis was diagnosed in all investigated patients. Diabetes diagnosed in patient P4 was successfully treated by diet. Metformin treatment was initiated at the age of 19 years in patient P2 for hyperglycemia with severe insulin resistance (Table 3).

Neurological manifestations

Neurological manifestations were observed in all patients. Patients experienced different features: *pes cavus* or flat feet with calcanean valgus, lower limb pain, distal weakness of the upper and/or lower limbs, wasting of distal muscles especially in the lower limbs, absent Achilles reflexes associated in some patients with brisk patellar reflexes, distal paresthesia. Achilles tendon contractures together with arthropathic feet were observed in patient P1. In all investigated patients, electromyography confirmed the diagnosis by showing an axonal sensorimotor polyneuropathy (Table 3).

Bone abnormalities

We and others previously reported that patients with congenital generalized lipodystrophy (CGL) present specific bone alterations, including focal sclerotic and osteolytic lesions, and fat bone marrow alterations evocative of serous transformation [30]. Therefore, we performed skeletal radiographs and whole-body MRI in three patients of the series (P1, P4, and P5). Bone imaging revealed centromedullary cystic lesions of tibial, peroneal and femoral epiphysis, metaphysis and diaphysis regions. The bone marrow MRI signal was similar to fluid as revealed by short tau inversion recovery (STIR) sequence analysis (Figure 2 and data not shown).

Other manifestations

Several patients presented with additional clinical features observed in other mitochondrial disorders: deafness (P1, P5), precocious puberty, recurrent constipation with occasional episodes of diarrhea (P2) and/or hypothyroidism (P2, P3) (Table 3). Considering that mitochondrial diseases are often associated with cardiac manifestations [31], patients P1, P2, P4, P5, and P6 underwent cardiac investigations by means of electrocardiogram, heart ultrasound imaging, and Holter monitoring. Apart from a mild aortic valvular regurgitation in patient P6, none of them was found to present any cardiac sign. No patient reported any current or past history of alcohol abuse.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Metabolic characteristics						
Areas of fat accumulation	nape and upper back, proximal upper limbs	back, thighs	thorax, proximal upper limbs, tongue	head, chin, nape, proximal upper limbs, thorax, abdomen	chin, neck, proximal upper and lower limbs, thorax, abdomen	chin, neck, proximal upper and lower limbs, thorax, abdomen
Lipoatrophy	generalized	generalized	lower limbs, forearms, hands	lower limbs, forearms	lower limbs, forearms	lower limbs, forearms
Body mass index ($kg.m^{-2}$)	18.7	22.8	26.1	27.5	25.1	31.6
% ot total body fat mass	6.8	8.2	nd	18.9	34.0*	nd
Leptin ($\mu g.L^{-1}$)	0.80	1.40	0.62	1.10	1.60	nd
Adiponectin ($mg.L^{-1}$)	0.62	nd	1.00	1.31	1.00	nd
Triglycerides ($mmol.L^{-1}$)	3.60	2.95	2.10	2.99	0.97	3.35
HDL-cholesterol ($mmol.L^{-1}$)	0.70	0.67	1.39	1.24	0.90	0.52
LDL-cholesterol ($mmol.L^{-1}$)	1.02	2.32	1.64	4.07	5.09	1.53
Total cholesterol ($mmol.L^{-1}$)	3.35	4.33	3.98	6.70	6.43	3.56
Fasting glucose ($mmol.L^{-1}$)	4.8	4.4	5.3	5.7	5.0	6.5
2h-OGTT glucose ($mmol.L^{-1}$)	9.0	6.4	7.0	12.1	nd	nd
Fasting insulin ($pmol.L^{-1}$)	98	313	nd	56	72	nd
2h-OGTT insulin ($pmol.L^{-1}$)	1320	1312	> 1500	636	nd	nd
M-value	9.2	nd	nd	6.2	16.2	nd
Insulin resistance and/ or diabetes	yes	yes	yes	yes	yes	fasting hyperglycemia
Glycated haemoglobin (%, $mmol.mol^{-1}$)	5.9 41.0	5.5 37.0	5.2 33.0	5.9 41.0	4.7 27.9	nd
Treatment	no	metformin	no	no	no	fibrate
Hepatic steatosis	yes	yes	yes	yes	yes	nd
Neurological presentation						
Clinical manifestation	pes cavus, tendon retraction, lower limb pain, amyotrophy, feet and hand paresthesia, Achilles areflexia	lower limb pain, flat feet with calcanean valgus	pes cavus, lower limb paresthesia and amyotrophy, calf muscle fasciculations, Achilles areflexia	pes cavus, hand paresthesia, quadricipital weakness, Achilles areflexia	lower limb weakness and pain	lower limb weakness and pain
Electromyogram abnormalities	axonal sensorimotor (mainly motor) polyneuropathy	axonal mainly motor polyneuropathy	four limbs-axonal sensorimotor polyneuropathy	four limbs-axonal sensorimotor polyneuropathy	sensorimotor neuropathy	nd
Treatment	orthopaedic shoes, surgical treatment	thermoformed soles	no	orthopaedic shoes	no	no
Other signs						
	deafness, breast cancer	precocious puberty, severe acne, constipation/diarrhea, hypothyroidism	hypothyroidism	thyroid and breast cancer	deafness	-

Table 3. Clinical and biological features of patients
Body fat mass was evaluated by dual-energy X-ray absorptiometry (DEXA) except for patient 5 (, by impedancemetry). Insulin-stimulated glucose disposal rate (M-value), measured during an euglycemic hyperinsulinemic clamp after 100 min-infusion of 80 mU.m^{-2}.min^{-1} insulin as previously described [32], is expressed in mg.kg^{-1} of fat free mass.min^{-1}. Insulin resistance was defined by acanthosis nigricans, and/or fasting insulinemia > 70 pmol.L^{-1}, and/or 2h-OGTT insulinemia > 800 pmol.L^{-1}, and/or M-value < 10. Hepatic steatosis was assessed by echography and/or computed tomography.*
OGTT: 75g oral glucose tolerance test; nd: not determined.

Characteristics of lipomatous tissue

Histological features of adipose masses

We performed histological studies of lipomatous tissue biopsies obtained from two female patients undergoing surgery of lipoma-like masses of left arm (patient P1) or of periumbilical abdominal region (patient P4). They were compared to subcutaneous abdominal fat samples previously obtained from plastic surgery in three healthy, non-obese (BMI: 20.4, 21.2 and 25.6), non-diabetic women [33]. In the two patients, lipomatous masses were not circumscribed nor enclosed in fibrous capsules. The histological appearance of adipose tissue was rather that of white fat with prevailing unilocular normal sized adipocytes (Figure 3A and data not shown). Nevertheless, we also observed multilocular adipocytes in about 1/50 cells for P1 and 1/20 cells for P4 (Figure 3A). Such multilocular adipocytes were absent in controls. In patients, adipose tissue was highly vascularized, with a high number of small blood vessels. This was confirmed by staining with antibodies directed against smooth muscular actin (data not shown) and against CD34, a marker of early hematopoietic and vascular-endothelium tissue (Figure 3B). Multilocular adipocytes were preferentially localized in the vicinity of vessels in patients. In contrast to controls, inflammatory infiltrates positive for the CD68 and CD163 markers of monocyte and macrophage lineages were also observed (Figure 3C and data not shown), with a small number of lipogranuloma in P1 and P4. We did not observe any significant fibrotic regions in patient or control samples. The

UCP1 (uncoupling protein 1) brown fat marker seemed to be weakly expressed both in patients and in controls (Figure 3D). In contrast, the staining of the CITED1 (Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 1) and FGF21 (fibroblast growth factor 21) thermogenic markers was markedly increased in lipomatous tissues (Figure 3E-F), with a very high expression of FGF21.

Ultrastructural morphology of adipocytes

Electron microscopy was performed in lipomatous samples obtained in patients P1 and P4, and in the same controls as for histological studies. Consistent with previous results, we observed that patients' lipomatous tissues were mainly composed of unilocular adipocytes, although some of them displayed numerous additional peripheral lipid droplets (Figure 4A). Adipocytes from patients were characterized by an enlargement of the cytoplasmic rim, which was packed with a very high number of mitochondria (Figure 4B). The mean length of the minor axis of mitochondria measured in patients was threefold higher than that of mitochondria in normal white adipocytes (Figure 4C). A number of ultrastructural abnormalities were also observed, such as disorganized cristae, or double membranes structures engulfed in mitochondria (Figure 4B).

Transcript and protein profiles in lipoma-like regions from patients

Gene expression was evaluated in subcutaneous lipomatous tissue from patients P1 (adipose overgrowth region of left arm), P4 and P5 (periumbilical lipoma-like regions) and in the same subcutaneous abdominal fat control samples as for histological studies (Figure 5A-B). Protein expression studies were performed in lipomatous samples from P1 and P4 patients (Figure 5C). *MFN2* expression was found to be similar in patients and controls. Several adipogenic markers showed a slight decrease in mRNA and/or protein expression in patients' fat masses

as compared to adipose tissue from controls, including sterol regulatory element binding transcription factor 1 (*SREBF1*), CCAAT/enhancer binding protein alpha (*CEBPA*) and peroxisome proliferator activated receptor gamma (*PPARG*). Most importantly, leptin and adiponectin were markedly underexpressed in lipomatous samples from patients, consistent with the low serum concentrations measured in the patients' sera (Figure 5A, Table 3). Notably, the relative expression of thermogenic markers was strongly lower than that of white adipocyte markers, both in controls and in patients (data not shown). Among them, *UCP1* expression was below the detection threshold. Several other thermogenic markers displayed low expression levels in subcutaneous adipose tissues from controls and in lipoma-like masses from patients: PR/SET domain 16 (*PRDM16*), transmembrane protein 26 (*TMEM26*), iodothyronine deiodinase 2 (*DIO2*), PPARG coactivator 1 alpha (*PPARGC1A*) (Figure 5B). However, we observed a marked overexpression of *CITED1* and *FGF21*, which exhibited a more than 10-fold expression increase as compared to controls, both at the transcript and at the protein levels (Figure 5B-C). These latter data are consistent with our previous results of immunohistological studies and suggest that certain thermogenic markers could play a role in the pathogenic process.

Measurement of FGF21 in the serum of patients

The major increase in *FGF21* expression at the transcript and protein levels led us to measure this adipokine in the serum of patients with *MFN2*-associated MSL (P1 to P5). We observed very high levels of serum FGF21 in these patients as compared to healthy normal-weighted controls (n=10). We also found elevated levels of serum FGF21 in patients with other forms of acquired or genetically-determined generalized lipodystrophic syndromes (n=13), as previously described [34], but FGF21 levels in patients with *MFN2*-associated MSL were significantly higher (Figure 6). Therefore, in combination to the decreased serum levels of

leptin and adiponectin, high FGF21 serum levels may be a valuable biomarker of *MFN2*-associated MSL.

Metabolic activity of adipose tissue in patients

The identification of positive thermogenic markers in lipomatous masses led us to perform ^{18}F-fluoro-2-deoxy-D-glucose positron-emission tomography and computed tomography (^{18}F-FDG PET-CT) to evaluate the presence of adipose tissue with increased metabolic activity [35] in patients P1, P2, P4 and P5. Consistent with our histological and MRI observations (Figures 2 and 3), the patients' lipomatous masses were not clearly delineated from adjacent adipose tissue on ^{18}F-FDG PET scans. Most importantly, we observed an increased ^{18}F-FDG uptake in adipose tissue, mostly located in lipomatous depots but also in subcutaneous lipoatrophic regions (Table 4). This latter point was clearly evidenced in patient P4, who displayed some remaining subcutaneous lower limb fat (Figure 7 and data not shown).

	Age	**Gender**	**BMI**	**SUV max subcutaneous fat**	**SUV mean subcutaneous fat**	**SUV max liver**	**SUV mean liver**
Patients with *MFN2*-associated MSL							
P1	76	F	18.7	1.7	0.9	1.6	1.2
P2	31	M	22.8	4.8	2.8	4.0	2.3
P4	53	F	27.5	2.2	1.3	1.9	1.4
P5	55	F	25.1	1.49	na	1.65	na
Control values							
Control subject from this study (depicted in Figure 7)	58	F	26.7	0.4	0.2	2.4	1.9
Data from Chin BB et al, 2009 [36]	57.9 ± 13.6	F, n=23 / M, n=27	25.7	na	0.25 ± 0.1	na	2.5 ± 0.6
Data from Heusch et al, 2013 [37]	57 ± 13	F, n=10 / M, n=15	na	0.43 ± 0.24	0.28 ± 0.12	3.14 ± 0.56	2.55 ± 0.44
Data from Christen T et al, 2010 [38]:							
Lean subjects	66.0 ± 14.8	F, n=14 / M, n=12	21.7 ± 0.6	na	0.30 ± 0.09	na	na
Obese subjects	61.4 ± 10.4	F, n=15 / M, n=16	36.4 ± 3.5	na	0.33 ± 0.08	na	na
Data from Oliveira et al, 2015 [39]:							
Metabolically healthy lean individuals	49.6 ± 18.9	F, n=29 / M, n=31	23.0 ± 2.6	na	0.26 ± 0.1	na	na
Metabolically healthy obese subjects	50.1 ± 14.3	F, n=14 / M, n=6	30.7 ± 5.2	na	0.24 ± 0.06	na	na
Metabolically abnormal obese subjects	57.5 ± 15.5	F, n=29 / M, n=32	32.1 ± 4.5	na	0.24 ± 0.07	na	na

Data from Monteiro et al, 2017 [40]:							
Non-obese subjects	60.1 ± 14.6	F, n=52 / M, n=80	24.1 ± 3.1	0.6 (0.3)	0.28 (0.2)	na	2.1 (0.7)
Obese subjects	65.8 ± 10.2	F, n=13 / M, n=11	33.7 ± 2.7	0.7 (0.3)	0.26 (0.09)	na	2.4 (0.4)

Table 4. ^{18}F-FDG uptake of subcutaneous adipose tissue assessed by PET-scan in patients with MFN2-associated MSL as compared to controls

Mean and maximal Standardized Uptake Value (SUV) of adipose tissue were measured as described in Methods, with SUVmax of the liver as a reference. Individual data assessed during this study in patients P1, P2, P4 and P5 and in a control subject are presented, as well as previously published data obtained using similar protocols of ^{18}F-FDG-PET-scans, expressed as mean ± SD or median (IQR) [36-40]. na: not available.

DISCUSSION

As a French reference center for lipodystrophic diseases, we screened *MFN2* in all patients of our cohort referred for altered fat distribution and presenting with one or several lipomas or lipoma-like regions, of any size or location (n=66). In this regard, the six patients with positive genetic testing represent the largest series of *MFN2*-associated MSL reported to date, which completes the data obtained previously by Sawyer S. *et al* (3 patients) [6] and Rocha N. *et al* (4 patients) [5]. Multiple lipomata with facial lipodystrophy, without further description, were also mentioned in a patient with CMT2A and biallelic *MFN2* pathogenic variants [17]. In our series, 83% of patients (5/6) are women, an observation which might argue for a sex ratio imbalance. Nevertheless, in the eight patients reported to date in the literature, four are women and four are men, so that this hypothesis could not be confirmed. If we consider all of the 14 patients with *MFN2*-associated MSL available so far, including our dataset and previously published cases, 12 patients carry the c.2119C>T (p.Arg707Trp) pathogenic variant in the homozygous state and two patients carry it in the heterozygous state together with another molecular defect. Considering that these patients originate from different countries (Ireland, United-Kingdom, Portugal, Australia, France), the p.Arg707Trp disease-

causing variant is likely to lie in a mutation hotspot. Consistently, this variant affects a CpG dinucleotide and methylcytosines of CpG sequences are known to spontaneously deaminate to thymine leading to recurrent mutational events. Our data also underline the crucial role of this particular variant in the development of the adipose and metabolic disease since its presence is required for the development of *MFN2*-associated MSL in all reported cases. Indeed, the numerous other pathogenic variants reported in *MFN2* are associated with a phenotype restricted to neurological manifestations. Notably, a compound heterozygous genotype including the same variant (p.Arg707Trp/p.Gly108Arg) was identified in three siblings with CMT2A [16]. None of them was reported to present with lipomatous depots or lipodystrophy, but these features might not have been searched for, or reported in this cohort study of patients with CMT2A.

MFN2 is the first gene encoding a mitochondrial protein found to be responsible for a lipodystrophic syndrome. Indeed, one of the hallmarks of *MFN2*-associated MSL underlined by our study is the severe lipoatrophy, affecting subcutaneous and visceral adipose tissue, which contrasts with the presence of several lipomatous masses, potentially reaching a major volume. Leptin levels are severely decreased in patients with respect to their BMI, similarly to levels observed in generalized lipoatrophies [41]. Together with the decreased serum levels of adiponectin, this suggests that the whole adipose tissue, including lipomatous masses, displays severe endocrine defects. In accordance, and consistently with previous findings [5, 6], patients with *MFN2*-associated MSL present with hypertriglyceridemia, insulin resistance and liver steatosis, which are typical lipodystrophy-related metabolic complications. In addition, the bone osteolytic lesions, similar to those observed in generalized lipodystrophies, suggest a serous transformation of bone marrow associated with the total absence of medullar fat [30, 42].

In the present study, we also had the opportunity to report the natural history of the disease in six adult patients and thereby showed that all of them, by the age of 65 at the latest, eventually developed both lipodystrophic and neurological manifestations. However, chronological appearance of symptoms and clinical severity are very heterogeneous among patients, with independent disease course of lipodystrophy and neuropathy. Although lipomatous masses were easily diagnosed in all patients, neurological signs could go unnoticed for several years in several of them.

MFN2-associated MSL can be considered for differential diagnosis with several disorders. First, it shares a number of characteristics with the less rare form of MSL consecutive to alcoholism and further studies are needed to decipher the specific features that differentiate these clinical phenotypes. The diagnosis of *MFN2*-associated MSL might also be discussed in the patients with MERRF (Myoclonic Epilepsy with Ragged-Red Fibers) syndrome who develop multiple lipomatosis (~ 30% of cases) [43]. An axonal sensory polyneuropathy can be associated with MERRF [44]. However, the diversity of clinical signs is wider in MERRF, in which myopathy, cardiomyopathy, and ocular manifestations are frequently encountered. In our series, none of the patients was reported to present any of these comorbidities. Nevertheless, some of them present with other symptoms of mitochondriopathy (deafness, intestinal pseudo-obstruction). Larger phenotypic studies would be required to establish whether the *MFN2* p.Arg707Trp molecular defect is responsible for these features.

The p.Arg707Trp homozygous genotype is associated with a marked increase in the number of mitochondria in lipomatous regions from patients. Moreover, mitochondria exhibit major alterations such as oversize, and disorganized morphology. These data are consistent with

those obtained by Rocha *et al*., who showed highly dysfunctional mitochondria in *MFN2*-associated MSL [5]. Recently, another gene called *OPA3*, encoding a protein of the inner mitochondrial membrane, has been implicated in another metabolic syndrome associating lipodystrophy, neuropathy and ocular manifestations [45]. This paves the way to the identification of other genes involved in mitochondrial dynamics in the molecular etiology of adipose tissue and/or metabolic disorders.

The majority of adipocytes from lipomatous regions of patients with *MFN2*-associated MSL have the morphological characteristics of normal-sized white adipose cells, consistent with a previous report [5]. We also observed a few multilocular adipocytes, mainly localized near adipose vessels and containing a high number of small lipid droplets in their periphery. The presence of inflammatory features in lipoma-like tissues (macrophage infiltration, some lipogranulomas) could be reminiscent of what is classically observed in obese patients. However, reduced angiogenesis and fibrosis are also important drivers of adipose tissue dysfunction in obesity [46]. In contrast, we did not detect any fibrotic region, and we observed an increased vascularization in lipomatous tissues of patients with *MFN2*-associated MSL. The gene expression profile of patients' lipomatous depots also displays atypical features with characteristics of both white and brite/brown adipose tissues. Despite a normal expression of a number of mature adipocyte markers, leptin and adiponectin expression is markedly decreased. Simultaneously, two thermogenic factors were found to be overexpressed (FGF21, CITED1), when the expression of others, including UCP1, remains very low. Further studies are required to establish whether these characteristics are due to altered biological properties of white adipocytes, to the whitening of brite adipocytes, and/or to the cellular heterogeneity in the overgrown adipose tissue. The appearance of positive thermogenic markers is supported by the result of ^{18}F-FDG PET-CT scans, showing a

spontaneous increased uptake of glucose, mostly in lipomatous regions but also in lipoatrophic adipose tissue, at least in some patients with *MFN2*-associated MSL. Such an increase in FDG uptake has never been reported in normal-weight, overweight, or obese subjects [36-40] (Table 4, Figure 7). However, the level of this uptake is far lower than what is observed in brown activated adipose tissue [47, 48] or in hibernoma [49]. These data suggest that, in *MFN2*-associated MSL, adipose tissue could display some thermogenic properties, even if it does not present all the morphologic characteristics of brown adipose tissue. ^{18}F-FDG PET-CT imaging after activation of adipose tissue by cold exposure could provide further pathophysiological information.

Although FGF21 is an endocrine factor widely expressed in metabolically active organs [50], its increase in the serum of patients with *MFN2*-associated MSL is likely to originate, at least in part, from adipose tissue, as suggested by our transcriptional and protein expression studies. However, we cannot rule out the possibility that dysfunctional adipose tissue from patients with *MFN2*-associated MSL could also alter the production of FGF21 by the liver. In that setting, it has been recently shown in mice that exosomal miRNAs produced by brown adipose tissue regulate FGF21 production by the liver [51]. FGF21 promotes thermogenesis, glucose uptake, and metabolite oxidation [52, 53]. It has also been shown to protect against hyperglycemia and hyperlipidemia. In this regard, it is tempting to speculate that increased FGF21 originating from adipose tissue, could play a compensatory role to limit *MFN2*-associated metabolic complications. Indeed, in our series of patients, metabolic abnormalities are relatively moderate, contrasting both with the severe upper-body adipose tissue overgrowth and lower-body lipoatrophy. Altogether our data suggest that very low serum levels of adiponectin and leptin combined with a high level of FGF21 could constitute a useful set of disease biomarkers in *MFN2*-associated MSL.

MFN2-associated MSL affects multiple organs with potentially severe complications, and strongly impairs the patients' quality of life. It is mandatory that both neurologists and endocrinologists should be aware of the different disease characteristics. In addition, systematic skeletal radiographs should be performed in *MFN2*-associated MSL to detect and monitor bone lesions, inform patients, and avoid misdiagnosis of these lesions. This multidisciplinary care approach should ensure early diagnosis, allow the rapid initiation of physiotherapy, dietary and lifestyle preventive measures, as well as proper therapeutic management.

FIGURE LEGENDS

Figure 1. Clinical presentation of patients.

Lipomatous masses affecting dorso-cervical, proximal limbs and/or thoraco-abdominal regions (arrows), contrasting with lipoatrophy in other subcutaneous areas in patients P5 (A, B), P6 (C, D, E), P2 (F), P4 (G, I) and P1 (H, J). Surgical removal of giant lipomatous masses of the back (P2) and of the neck, shoulders and/or abdomen (P5 and P6) were previously performed.

Figure 2. MRI characteristics of lipomatous masses and bone alterations in patients with *MFN2*-associated MSL.

(A) Lipomatous masses. Patient 1: Axial T1-weighted image of the neck (a) shows an unencapsulated subcutaneous posterior lipomatous mass (white arrows). Patient 4: Coronal (b) and sagittal (c) T1-weighted images of the head and trunk reveal unencapsulated subcutaneous adipose overgrowth in the shoulders and the back (white arrows).

(B) Bone alterations. Patient 1: Coronal T1-weighted images of the thighs and proximal legs (a, b) show normal bone signal and subcutaneous lipoatrophy. Coronal T1-weighted and STIR (short tau inversion recovery) images of distal legs (c, d) reveal intramedullary cystic lesions with low T1 (c) and high STIR (d) signals, evocative of serous transformation of bone marrow fat (open arrows). Patient 4: The distal part of femurs (e, f) and tibias (g, h) presents abnormal bone marrow lesions characterized by hypointensity on T1-weighted images (e, g) and hyperintensity in STIR sequences (f, h) (open arrows). Note the loss of subcutaneous fat in the legs.

Figure 3. Histological and immunohistological features of lipomatous regions from patients with *MFN2*-associated MSL.

The different panels correspond to the staining obtained for subcutaneous white adipose tissue from one representative control and for lipomatous samples from two patients (P1 and P4). HE: haematoxylin-eosin. In panel A, black arrows indicate multilocular adipocytes.

Figure 4. Ultrastructural characteristics of lipomatous masses from patients with *MFN2*-associated MSL.

Images obtained by transmission electron microscopy (TEM) are presented for one representative control (subcutaneous white adipose tissue) and for one representative patient (P4) (lipomatous sample). **(A)** General view of adipocytes. L: lipid droplet. **(B)** Focus on the cytoplasmic rim and ultrastructural characteristics of mitochondria. Double arrows indicate the thickness of the cytoplasmic rim. m: mitochondria. Disorganized cristae and double membrane bound structures suggestive of mitophagy are pointed by black arrowheads. **(C)** Statistical comparison of the length of the minor axis of mitochondria in each patient versus controls using a non-parametric unpaired Mann-Whitney test (*: p values < 0.0001).

Figure 5. Gene and protein expression in lipomatous masses from patients with *MFN2*-associated MSL.

(A) Gene expression of adipocytes markers. RT-qPCR analysis was performed in lipomatous tissues from three patients and compared to gene expression in subcutaneous adipose tissues from two controls. Means of two independent experiments ± standard deviations (SD) are presented as fold changes relative to controls. **(B)** Gene expression of thermogenic markers. The same experiment was performed as in (A). **(C)** Protein expression of several adipocyte

and thermogenic markers assessed by Western blotting. The three lanes presented for each individual corresponds to three independent adipose tissue protein extractions.

Figure 6. FGF21 serum measurements.

FGF21 was measured in the sera of ten healthy individuals (mean ± SD: 40 ± 23 $ng.L^{-1}$), 13 patients with generalized lipodystrophy (mean ± SD: 193 ± 74 $ng.L^{-1}$), and five patients with *MFN2*-associated MSL (mean ± SD: 385 ± 181 $ng.L^{-1}$). Statistical comparisons between groups were made using non-parametric unpaired Mann-Whitney tests. *: $p < 0.001$ versus the control group; #: $p < 0.05$ versus the generalized lipodystrophy group.

Figure 7. ^{18}F-FDG-PET scan in patients with *MFN2*-associated MSL.

Maximum intensity projection images and axial slices from a ^{18}F-fluoro-2-deoxy-D-glucose positron-emission tomography (PET) with axial computed tomography (CT) slices at the indicated anatomical levels (A, B, C) in patients P1 (top) and P4 (middle). Mean and maximal Standardized Uptake Value (SUV) of adipose tissue were measured in regions of increased metabolic activity. The SUVmax of the liver was measured as a control reference. Images from patients were compared to those obtained in a female subject (age 58, BMI 26.7) investigated 30 years after complete surgical resection of pheochromocytoma (bottom). Note the increased uptake of ^{18}F-FDG in the lipomatous adipose tissue of patients P1 (section A) and P4 (A, B), but also in some subcutaneous lipoatrophic regions (B, C in P1; C in P4).

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTION STATEMENT

All the authors contributed to the data collection. C.Va., T.S., E.D., H.M., P.G., S.B., S.C-M, B.D., B.F., M.L., F.T. and C.Vi contributed to patient care. S.Z., A.B., P.F., O.L. C.Vi. and I.J. were involved in genetic analyses. A-S.C. and A.M. performed imaging studies. E.C., C.Va., P.C., M.A., M-C.V., J.R., C.Vi and I.J. participated in adipose tissue *in vitro* characterization. All the authors reviewed and approved the final article.

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