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High prevalence of laminopathies among patients with metabolic syndrome

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Constitutional laminopathies, such as the Dunnigan familial partial lipodystrophy, are severe diseases caused by mutations in A-type lamins and share several features with metabolic syndrome (MS). In this study, we hypothesized that MS may be, in some cases, a mild form of laminopathies and use the abnormal cell nucleus phenotype observed in these diseases as a primary screening test in patients suffering from common MS.

Nuclear shape and lamin A nucleoplasmic distribution abnormalities were systematically searched in lymphoblastoid cells of 87 consecutive patients with MS. In parallel, five genes encoding either the A-type lamins or the enzymes of the lamin A maturation pathway were systematically sequenced (*LMNA*, *ZMPSTE24, ICMT, FNTA* and *FNTB).* We identified 10 MS patients presenting abnormal nuclear shape and disturbed lamin A/C nuclear distribution. These patients were not clinically different from those without nuclear abnormalities except that they were younger, and had higher triglyceridemia and SGPT levels. Three of them carry a heterozygous mutation in *LMNA* or in *ZMPSTE24,* a gene encoding one of the lamin A processing enzymes. All three mutations are novel missense mutations predicted to be damaging. Both lymphoblastoid cells and skin fibroblasts from the patient carrying the mutation in *ZMPSTE24,* showed accumulation of lamin A precursor, indicating an alteration of the lamin A processing, confirmed by functional study.

Together, these results show for the first time, that a significant proportion of MS patients exhibits laminopathies and suggest that systematic investigation of lamin A and its partners should be performed at the diagnosis of this syndrome.

INTRODUCTION

The metabolic syndrome (MS) is a cluster of metabolic abnormalities affecting 30% of North Americans and 23% of Europeans (1,2). These abnormalities include abdominal adiposity, impaired fasting glucose and hyper-insulinemia both reflecting insulin resistance, dyslipidemia, hepatic steatosis and high blood pressure (1). Interestingly, MS-associated features such as insulin resistance and dyslipidemia are also frequently observed in monogenic disorders such as the Dunnigan familial partial lipodystrophy (FPLD) (3,4), suggesting that such inherited disorders could provide important insights for understanding typical MS.

FPLD belongs to laminopathies, a large group of human diseases due to mutations in genes encoding the nuclear lamins and associated proteins (5). A-type lamins form a nucleoplasmic network of structural proteins, the nuclear matrix; they are also localized at the inner surface of the nuclear envelope

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where they form the nuclear *lamina* together with other proteins such as B-type lamins or nucleoplasmic domain of some nuclear envelop proteins. Lamins have multiple functions, including the maintenance of the nuclear structure and shape, heterochromatin organization and regulation of gene transcription (6).

A-type lamins, lamin A and C, are both encoded by *LMNA* but differ from each other because of an alternative splice site in *LMNA* exon 10. Lamin C is produced directly in its mature form, whereas lamin A is first produced as a precursor, the prelamin A, which then undergoes a series of four post-translational modifications (4). To generate mature lamin A protein, prelamin A is first farnesylated by the farnesyltransferase, a heterodimer enzyme encoded by the *FNTA* and *FNTB* genes, then cleaved either by the zinc metalloprotease ZMPSTE24 or by RCE1. The following steps include a carboxy-methylation by the isoprenyl-cysteine carboxyl methyltransferase (encoded by *ICMT*) and a second cleavage by the zinc metalloprotease ZMPSTE24. In humans, genetic variants in *ZMPSTE24* are known to cause two different laminopathies (i.e. Mandibuloacral dysplasia, MAD and Restrictive Dermopathy) (7,8), while *ZMPSTE24*-null mice exhibit decreased blood glucose and insulin concentrations as well as an altered lipid metabolism (9). There has been considerable interest in the association between common *LMNA* single nucleotide polymorphisms (SNPs) and components of the MS with conflicting results reported, some of them suggesting modest positive associations, whereas others, based on studies with larger sample size, did not demonstrate any convincing evidence for an effect of *LMNA* SNPs on risk for type 2 diabetes mellitus or MS (10–21). However, it is not known whether rare DNA sequence variants of *LMNA* or of the four other genes encoding enzymes involved in lamin A maturation collectively contribute to common MS.

In this study, we hypothesized that MS may represent in some cases, a mild form of laminopathy and used the cell nucleus phenotype observed in several of these diseases (22) as a screening test in patients suffering from common MS. For this purpose, 100 consecutive patients with MS were systematically enrolled for clinical data collection, and 87 were investigated for nuclear shape abnormalities and lamin A nucleoplasmic distribution. Concomitantly, *LMNA* and four other genes encoding proteins involved in the lamin A maturation pathway were investigated by direct sequencing.

RESULTS

Cell studies

Among the 100 patients enrolled in the study, 87 were available for cell investigations.

Out of these 87 patients, 10 (11.5%) harbored the two selection criteria for abnormal nuclear phenotype (Tables 1 and 2). Only one patient harbored just one criterion (24% of dysmorphic nuclei) but as there was no lamin A/C staining abnormalities, he was classified as having a normal phenotype. Interestingly, two of the 10 patients with abnormal phenotype were siblings. In order to confirm these abnormalities on another cell type, we analyzed the cellular phenotype in cultured dermal fibroblasts which were available for seven of the 10 patients with abnormalities. We confirmed the abnormal phenotype identified in lymphoblastoid cells for all seven with a high rate of dysmorphic nuclei (mean 30%) and an abnormal lamin A/C staining (reduced signal, heterogeneous staining with aggregates). Blebbing nuclei were frequent and associated with the reduction or the absence of lamin B1 staining in blebs (Fig. 1). For five of the 10 patients, the nuclear pattern of NuMA (Nuclear Mitotic Apparatus) protein was abnormal with a heterogeneous staining that could reflect a profound nuclear matrix defect (Supplementary Material, Fig. S1). Four of the 10 patients with abnormalities exhibited significant reduction (15–56%) of lamin A expression on western blot analyses. Prelamin A was undetectable for all patients, except for patient 23. This patient showed 47% of his cells positive for prelamin A by immunofluorescence (IF) with quite a low and heterogeneous intensity staining which could explain the absence of detection by western blot technique.

Clinical and biological features of the MS from patients with nuclear abnormalities

The clinical and biological characteristics of the 10 patients with nuclear abnormalities are summarized in Tables 1 and 2. In summary, four patients (patients 12, 23, 45, 67) had a very severe metabolic phenotype either because of insulin resistance or cardiac disease. Noticeably, patient 9 was enrolled after a loss of 40 kg following bariatric surgery and presented a normalization of glycemia at the time of cell phenotyping.

Patients with nuclear abnormalities ($n = 10$) tended to be younger than patients without nuclear abnormalities ($n = 77$) (Table 1). No anthropometrical difference was evidenced between the two groups [particularly no difference in BMI, waist circumference (WC), WC/BMI] except for thigh circumference (TC), which was slightly higher in patients with nuclear abnormalities. The frequency of diabetes was no different in both groups but the age at diagnosis tended to be lower in the group with nuclear abnormalities. No difference was evidenced in the frequency of treatment with statins or in the number of MS criteria (4.4 ± 0.27 versus 4.09 ± 0.09, $P = 0.25$). Interestingly, the group with nuclear abnormalities tended to have higher levels of triglycerides (3.39 ± 0.89 versus 2.14 ± 0.16 mmol/l, $P = 0.03$) and of alanine aminotransferase (ALT) (**(**66 ± 20 versus 43.1 ± 2.8 UI/l, $P = 0.03$) than the group without.

Molecular studies

Molecular studies were performed for all 100 patients. In the group of patients with nuclear abnormalities, direct sequencing of the five genes revealed two new heterozygous missense mutations in *LMNA* : the first one (patient 15), in position c.1232 G > A, resulted in a Glycine to Aspartate exchange in amino acid 411 which is located in the chromatin binding domain of both lamin A and lamin C, upstream and near to nuclear localization signal (NLS) localized at amino acids 417–422 (Fig. 2). The second single-nucleotide substitution (patient 45), c.1893G > A, changed the amino acid Glycine into Aspartate at position 631, located in the C-terminal domain specific of lamin A (Fig. 2).

In the same group, a third new heterozygous missense mutation was characterized in *ZMPSTE24* for patient 23 in

Table 1. Main characteristics of the 100 patients with metabolic syndrome included in the cohort

	Total population, $n = 100$[a]	Patients without nuclear abnormality, $n = 77$	Patients with nuclear abnormalities, $n = 10$	*P*-value
Age (years)	56 ± 0.9	56.6 ± 1.0	50.2 ± 3.3	**0.03**
BMI (kg/m^2)	37.1 ± 0.7	36.6 ± 0.8	39.1 ± 1.2	0.28
WC (cm)	119.8 ± 1.4	118.8 ± 1.5	125 ± - 2	0.16
TC (cm)	55.5 ± 0.6	54.8 ± 0.7	59 ± 1.3	**0.04**
Fat mass (%)	39.4 ± 0.7	38.8 ± 0.7	40 ± 3	0.3
WC/TC	2.16 ± 0.02	2.17 ± 0.02	2.13 ± 0.06	0.3
TC/BMI	1.52 ± 0.02	1.53 ± 0.02	1.51 ± 0.02	0.7
Frequency of diabetes (*n*, %)	82 (82)	62, 81	9, 90	0.49
Frequency of hypertension (*n*, %)	80 (80)	59, 77	9, 90	0.45
Frequency of statin TRT (*n*, %)	60 (60)	48, 62	6, 60	0.9
Number of criteria of MS	4.12 ± 0.009	4.09 ± 0.09	4.4 ± 0.27	0.25
HDL cholesterol (mmol/l)	1.09 ± 0.04	1.12 ± 0.04	0.97 ± 0.13	0.3
Triglycerides (mmol/l)	2.23 ± 0.17	2.14 ± 0.16	3.39 ± 0.89	**0.03**
Alanine aminotransferase (UI/l)	46.86 ± 3.9	43.1 ± 2.8	66 ± 20	**0.03**
Gamma glutamyl transpeptidase (UI/l)	70.34 ± 9.71	63.9 ± 8.5	100 ± 43	0.2
Slight lipodystrophy (of lower limb) (*n*, %)	4 (4)	(2) (2.6)	2 (20)	**0.006**
Muscular complaint (*n*, %)	4 (4)	(2) (2.6)	2 (20)	**0.006**
Cardiac rhythm abnormalities (*n*, %)[b]	3 (3)	(2) (2.6)	1 (10)	0.31

Bold values indicate *P*-value <0.05.
MS, metabolic syndrome as assessed by NCEP-ATPIII; WC, waist circumference; TC, thigh circumference.
Quantitative variables are expressed as mean ± SD and qualitative variables as number with percentage.
P for comparison between patients with and without nuclear abnormalities.
[a]Among the 100 patients enrolled in the study, 87 were available for cellular investigations.
[b]Cardiac rhythm abnormalities due to coronaropathy were excluded from this analysis.

position c.1312 C > T and leads to a Leucine to Phenylalanine substitution in position 438 at the protein level (Fig. 2). Interestingly, this patient was the only one to harbor prelamin A accumulation in fibroblast cells. ZMPSTE24 protein expression was not reduced in this patient compared with wild-type control (Fig. 1C). Moreover, study of the prelamin A processing by an ELISA approach revealed a significant reduction (more than 50%) of mature lamin A production for this patient compared with control (Fig. 3).

None of the three identified mutations were present in a population of 120 unrelated control individuals. Transcripts sequencing showed the presence of both mutated and non-mutated alleles indicating that the mutant alleles are expressed. Family study was not possible for any of the mutated patients because their parents were not available.

No missense or splice sites variation or copy-number variations (CNV) in the five genes was identified in the seven remaining patients with nuclear defects.

In the group of the 77 patients without nuclear defects and in the group of 13 patients not tested for these nuclear abnormalities, no missense or splice sites variation was identified either in *LMNA* or in *ZMPSTE24*; regarding the three other tested genes, two missense mutations in heterozygous condition were detected in *FNTA*: p.P27L and p.T375A. However, as these variations were not associated with nuclear defects, they have not been considered as lamin altering mutations for the time being.

Expression studies on lymphoblastoid cells were conducted in two subgroups of seven patients with and eight without the nuclear defects, both groups being matched for age and sex. No significant differences were noted between the two groups either in the levels of lamin A/C transcripts expression or in the protein levels (Supplementary Material, Fig. S2).

DISCUSSION

The past decade has seen the emergence of links between the nuclear envelope and several diseases (23). Undoubtedly, is the identification of more than 10 different diseases resulting from mutations within *LMNA* along with a further seven diseases or anomalies due to defects in other lamin-associated nuclear envelope proteins that has revitalized interest in these proteins. Our study is the first to systematically assess typical features of laminopathies such as nuclear shape abnormalities in a cohort of individuals with MS. Following this approach, 10 patients (about 11%) displayed abnormalities of the nuclear shape and lamin A/C nuclear distribution. Moreover, these patients also presented a reduction or the absence of lamin B1 staining in blebs which is another typical feature seen in laminopathies (9,24). To avoid a possible effect due to cell transformation in lymphoblastoid cells (i.e. overestimation of nuclear abnormalities), we analysed skin fibroblasts for seven out of the 10 patients and confirmed the results in all cases.

Three of the 10 individuals with anomalies of nuclear envelope had functionally significant sequence variations in *LMNA* or *ZMPSTE24*. It has been already observed that *LMNA* mutations may be characterized in individuals referred for lipodystrophy and/or android adiposity, insulin resistance or altered glucose tolerance (25) but to our knowledge, we provide here the first study describing the prevalence of laminopathy in MS. In our cohort of patients with

Table 2. Clinical, molecular and cell characteristics of the 10 patients with nuclear abnormalities

Patient 9	Patient 10	Patient 12[a]	Patient 15	Patient 17	Patient 23	Patient 35[a]	Patient 45	Patient 62	Patient 67
Main clinical features									
Women, 37 years, BMI = 40.1 Diabetes at 35	Women, 68 years, BMI = 46.6, No diabetes	Women, 50 years, BMI = 41.5 Diabetes at 37 Insulin resistance (2.23UI/kg/day) Fatty liver HyperTG Slight lipoatrophy	Men, 48 years, BMI = 36.5 Diabetes at 48 Fatty liver HyperTG Referred for hyperthyroidism	Men, 49 years, BMI = 32.7 Diabetes at 30 Fatty liver Severe hyperTG Neuromuscular complaint	Men, 43 years, BMI = 41 Diabetes at 39 Dilated cardiomyopathy (LVEF = 25%) Fatty liver HyperTG	Men, 51 years, BMI = 37.4 Diabetes at 47 HyperTG Post-intensive care muscular weakness Kidney neoplasm (death 6 months later)	Women, 44 years, BMI = 37.1 Diabetes at 26, Insulin resistance (2UI/kg/day) CAD Severe hyperTG Fatty liver Slight lipoatrophy	Women, 70 years, BMI = 39.3 Diabetes at 40, hyperTG	Men, 47 years, BMI = 38 Diabetes at 37 Sudden death on ventricular arrhythmia Myalgias
Molecular defects									
NI	NI	NI	*LMNA* exon 7 Heterozygous; p.G411D, C-terminal domain	NI	*ZMPSTE24 exon 10* Heterozygous; p.L438F, C-terminal domain	NI	*LMNA* exon 11 Heterozygous; p.G631D, C-terminal domain	NI	NI
Immununofluorescence									
Dysmorphic nuclei (types of abnormalities), %									
LC : 40 F : 47 (L, B)	LC : 52 F : 23 (B)	LC : 30 F : NA (B)	LC : 32 F : NA (L, B)	LC : 50 F : 24 (B)	LC : 37 F : 28 (L, B)	LC : 26 F : NA (B)	LC : 29 F : 38 (L, B)	LC : 31 F : 17 (L, B, M)	LC : 32 F : 31 (L, B, M)
Lamin A/C staining									
Heterogeneous with polar clustering of staining (40% of nuclei)	Aggregates (8% of nuclei)	Aggregates (10% of nuclei)[b]	Heterogeneous; reduced with aggregates (12% of nuclei)[b]	Reduced with aggregates (9% of nuclei)	Aggregates (4% of nuclei)[b]	Reduced with aggregates (7% of nuclei)[b]	Reduced with aggregates (9% of nuclei); rare cytoplasmic staining	Reduced with aggregates (5% of nuclei)	Reduced with aggregates (3% of nuclei)
Prelamin A staining									
Absent	Absent	Absent[b]	Absent[b]	Absent	Positive in 47% of nuclei	Absent[b]	Absent	Absent	Absent
Lamin B1 staining									
Absent in blebs	Reduced or absent in blebs	Absent in blebs[b]	Reduced in blebs[b]	Reduced or absent in blebs	Reduced or absent in blebs	Reduced in blebs[b]	Absent in blebs; rare cytoplasmic staining	Reduced or absent in blebs	Reduced in blebs
NuMA staining									
Heterogeneous	Heterogeneous	Homogeneous[b]	Homogeneous[b]	Heterogeneous	Homogeneous	Homogeneous[b]	Homogeneous	Heterogeneous	Heterogeneous
Western blot (expression versus control)									
Lamin A/C									
Reduction of 38% for Lamin A	Normal	Reduction of 15% for Lamin A	Reduction of 55% for Lamin A	Reduction of 56% for Lamin A	Normal	Normal[b]	Normal	Normal	Normal
Prelamin A									
Absent	Absent	Absent[b]	Absent[b]	Absent	Absent	Absent[b]	Absent	Absent	Absent

CAD, coronary artery disease; NI, no mutation identified; NA, not available; ND, not done; hyperTG, hyperTriGlyceridemia; LC, lymphoblastoïd cells; F, fibroblasts; LVEF, left ventricular ejection fraction; L, lobulation; B, blebs; M, micronuclei.

[a]Related patients.

[b]Studies realized on lymphoblastoïd cells.

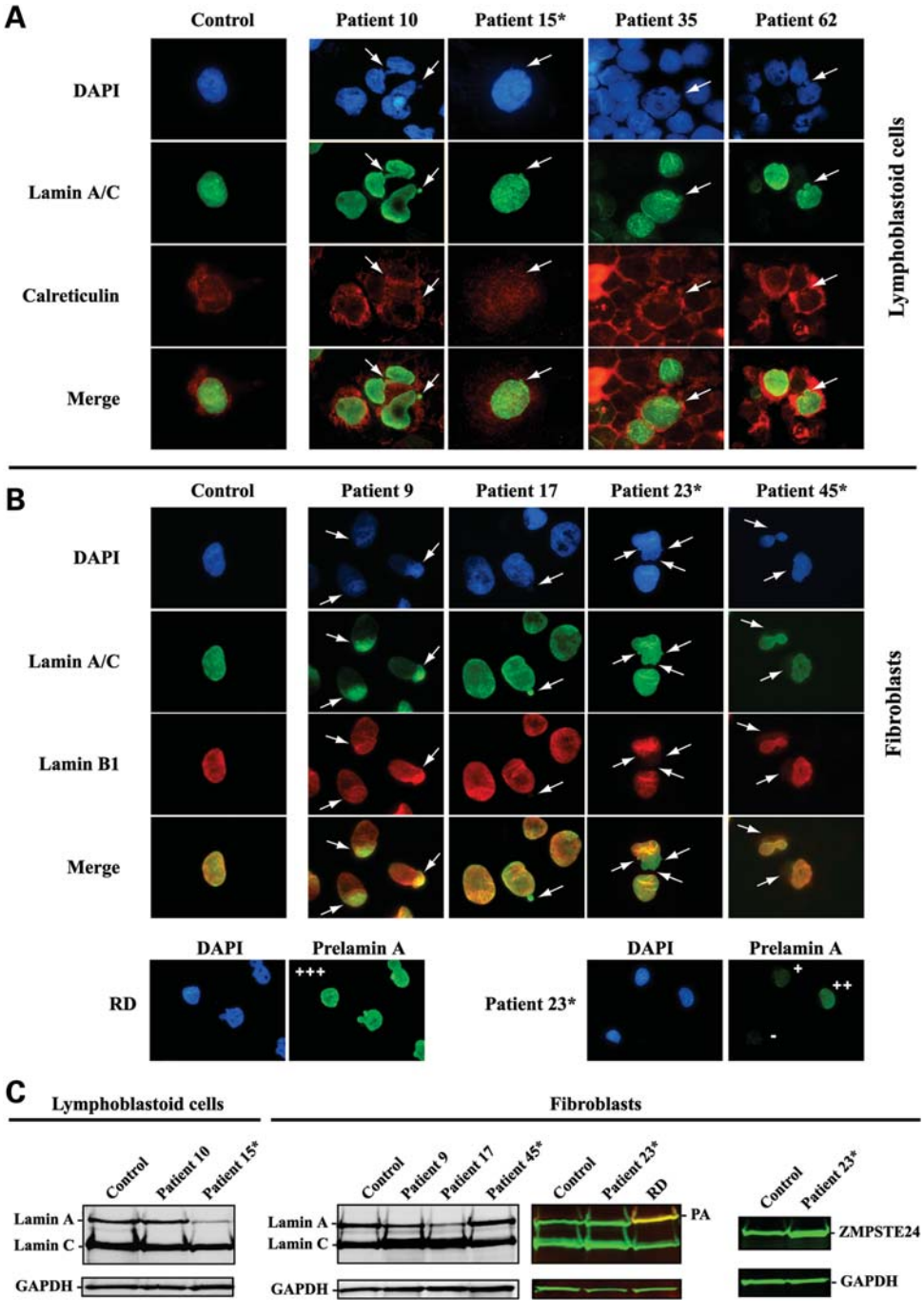

Figure 1. Immunofluorescence and western blot analysis from different patients and controls. (**A**) Lymphoblastoid cells from a non-obese, non-diabetic control (aged 36 years) and four different patients (10, 15, 35 and 62). Lamin A/C and calreticulin were detected with specific antibodies and nuclei were counterstained with DAPI. (**B**) Cultured skin fibroblasts from a non-obese, non-diabetic control (aged 42 years) and four different patients (9, 17, 23 and 45). Lamin A/C, lamin B1 and prelamin A (for patient 23) were detected with specific antibodies and nuclei were counterstained with DAPI. For prelamin A analysis, cells from restrictive dermopathy *ZMPSTE24*-deficient patient (RD) were used as positive control. Arrows indicate some of nuclear shape or protein staining abnormalities observed in patients but not in control cells. Asterisk indicates patients with *LMNA* or *ZMPSTE24* mutations. (Minus) indicates negative staining and (plus) low, (double plus) medium or (triple plus) high staining. (**C**) Western blot analysis of nuclear matrix proteins for lymphoblastoid cells (patients 10 and 15 versus a non-obese, non-diabetic control) and total proteins from fibroblasts (patients 9, 17 and 45 versus a non-obese, non-diabetic control). For fluorescent western blot, yellow signal indicates the presence of prelamin A protein, as the result of binding of anti-lamin A/C antibody (green signal) and anti-prelamin A antibody (red signal).

MS, genetic mutations affecting A-type lamins or ZMPSTE24 are far from being uncommon, with a prevalence of 3%. None of these three mutations was detected in a group of 120 healthy controls, nor was reported in the 1000 Genomes Project database (http://www.ncbi.nlm.nih.gov/projects).

Until now, mutations in *ZMPSTE24* have been shown to be responsible for Restrictive Dermopathy and MAD. This last

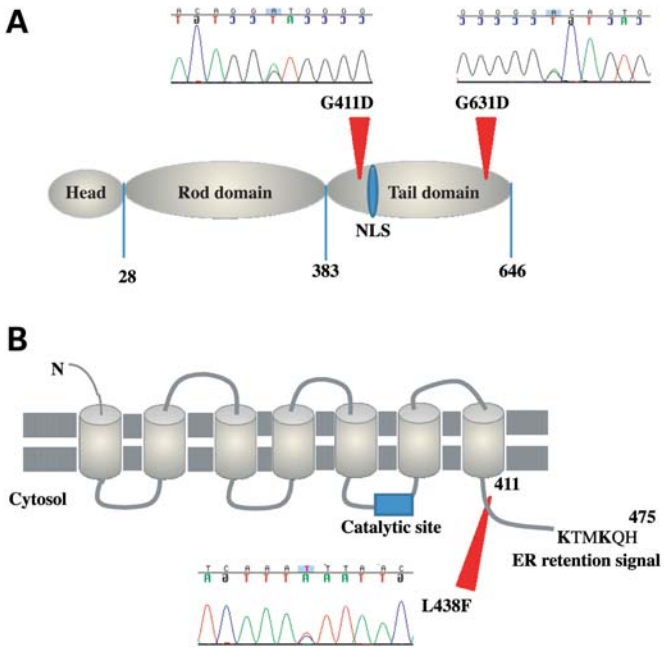

Figure 2. Models of protein structures for (**A**) Lamin A and (**B**) ZMPSTE24 and locations of the mutated residues (red arrows). Numbers represent AA residues. Experimental nucleotide sequences are shown for each variant.

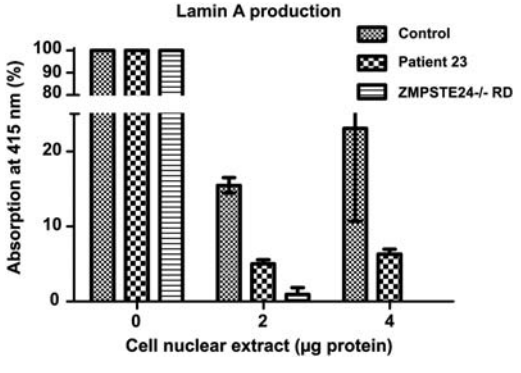

Figure 3. Study of the Prelamin A processing on fibroblasts from patient 23 harboring ZMPSTE24 p.L438F mutation compared with wild-type control and ZMPSTE24−/− RD subjects. A condition with 0 µg of cell nuclear extract was used as blanck (0). Two protein concentrations of cell nuclear extracts (2 and 4 µg) were tested. Lamin A production has been calculated by the difference between relative absorption observed with lamin A and prelamin A antibodies. Lamin A production is expressed as percentage normalized with sample containing no nuclear extract, referred as 100%. Each test was realized in triplicate and data were confirmed in two independent experiments. Data are mean ± SD.

syndrome associates insulin resistance, high risk for cardiovascular disease and lipodystrophy in patients with homozygous or compound heterozygous mutations. For the first time, we identified a heterozygous mutation in *ZMPSTE24* in an individual with MS. Bioinformatic prediction of the mutation consequences using the algorithm proposed on Polyphen site (http://genetics.bwh.harvard.edu/pph/) suggested a probably damaging effect. The substitution replaces an aliphatic AA by an aromatic and concerns a residue strongly conserved among species. This mutation does not cause a reduction in protein expression but probably reduces the enzymatic activity of ZMPSTE24 which is not totally compensated by the normal allele, as evidenced by the alteration of the prelamin A processing and nuclear prelamin A accumulation. The p.G411D lamin A mutation leads to the reduction of lamin A and C expression but seems to have no effect in the nuclear localization of the protein despite the vicinity of NLS. No functionally significant variations of the five sequenced genes were observed in the seven remaining individuals with nuclear abnormalities but the fact that two of these seven individuals are relatives is in favor of a constitutional cause for these abnormalities. In an attempt to go further in the characterization of molecular basis for nuclear abnormalities in this subgroup of seven patients, we screened also *RCE1*. This gene was not included initially in the design of the study given that the Ras Converting Enzyme, even if being responsible of the first cleavage of prelamin A, is not lamin A specific but is also involved in proteolytic processing of other farnesylated proteins, including Ras. Again, no variation of any kind was detected after direct sequencing. Because our selection criteria included nuclear lamin A distribution, we suggest that these seven patients presented a nuclear envelope or nucleoplasm-related disorders, involving a partner of lamins not investigated in this study such as Emerin (26) or Nesprin-1 and -2 (27) or a yet unknown partner. In these seven individuals, sequencing of other genes involved in nuclear envelope integrity is underway to find other functional variants that may be responsible for the nuclear abnormalities observed.

We evidenced no relationship between the clinical and biological characteristics of the patients and the presence of a laminopathy. Individuals with nuclear abnormalities were younger than those without, excluding an age effect. No differences in BMI, in adipose tissue repartition (particularly no differences in subcutaneous thigh development evaluated by TC/BMI) and in the frequency of diabetes or hypertension were observed. The severity of the metabolic phenotype in our 10 patients with laminopathy was heterogeneous; nevertheless triglycerides and ALT were significantly increased and median age and age at diabetes diagnosis were significantly lower, when compared with patients without laminopathy, indicating a more severe phenotype. It must be underlined that none of the 10 patients presented the characteristic morphotype of the Dunnigan syndrome.

Our results imply that a significant proportion of the common MS should be considered as a laminopathy and not only the severe forms as previously described (25). It thus extends the indications for screening for lamin A-related defect in diabetes or MS even in patients without major lipodystrophy. One of the 10 patients presented a dilated cardiomyopathy and another one ventricular arrythmia underlining the importance of this screening linked to the potential cardiac rhythm abnormalities and sudden death that might be associated with laminopathies (28–30).

In conclusion, laminopathies are frequent in patients with MS. Screening for genetic mutations in the A-type lamins and its partners can be recommended in these patients even without specific clinical signs of laminopathy.

MATERIALS AND METHODS

Patients' characteristics

A total of 100 consecutive patients attending the Department of Endocrinology of North Hospital, Marseille, for obesity (50%), diabetes (40%) or thyroid disorders (10%) and presenting the MS according to the 2005 revised ATPIII definition (31) were included between February 2006 and September 2009. The anthropometrical and clinical data of the population are shown in Table 1. Out of the 100 patients, two were siblings. Sex ratio was two out of three (M/F). Written informed consent was obtained from all participants and the study was approved by the local Ethics Committee.

Cell studies

IF microscopy. Blood samples were used to establish Epstein Barr Virus-immortalized lymphoblastoid cell lines. Fibroblasts were obtained from a skin biopsy. All analyses were performed at passage 2 for fibroblasts. Lymphoblastoid cells or fibroblasts from two non-obese, non-diabetic individuals were used as controls (aged 42 and 36, respectively).

We used an IF protocol described elsewhere (32). Primary antibodies list is available on request. The association of the two following criteria was considered to characterize an abnormal phenotype: (i) a percentage of dysmorphic nuclei up to 20% and (ii) an abnormal lamin A/C staining in the dysmorphic nuclei. These two criteria were chosen according to previously published data (25,33). A mean of 500 nuclei were analyzed for prelamin A staining and 200 nuclei for all other staining. IF was performed first on lymphoblastoid cells. Then, abnormal cellular phenotypes were confirmed, when available, on skin fibroblasts.

Western blotting. Protein extractions from cultured fibroblasts and nuclear matrix protein separation from EBV-immortalized lymphoblastoid cells were performed as previously described (24,34). Primary antibodies are available on request. We used WesternDot^{TM} 625 Western Blot kit (Molecular Probes, Eugene, USA), according to manufacturer's protocol, and BioSpectrum 500 imager (UVP, LLC Upland, CA) for detection. Protein expression studies were performed by densitometric analyses using LabImage 1D 2006 software. For patient 23, IR-Dye 700 and IR-Dye 800 conjugated anti-mouse and anti-goat antibodies were used and detected on an Odyssey Infrared Imaging System (LiCor Biosciences, Lincoln, NE).

Prelamin A processing study. Prelamin A processing was studied on fibroblasts at passage 5 using the 'Prelamin A Processing Cell-Free System Immunoassay' (Diatheva, Fano, Italy) following manufacturer's protocol. Two protein concentrations (2 and 4 μg/well) were used for each patients (patient 23 and ZMPSTE24-deficient RD patient) and control. Each point was tested in triplicate and data were confirmed in two independent experiments.

Molecular studies

DNA and total RNA was extracted from lymphoblastoïd cell lines obtained for each patient, on the MagnaPure (Roche) following the manufacturer's instructions. Real-time quantitative PCR assays for LMNA/C transcripts were performed on a Taq Man 7500 (Applied Biosystems) and direct sequencing for *LMNA, ZMPSTE24, ICMT, FNTA* and *FNTB* on the 3130XL sequencing (Applied Biosystems). Primers and probes are available on request. We compared expression levels of lamin A and lamin C transcripts with 2 probes: one located in a lamin A specific region (exon 11–12), the other in exon 2–3, targeting both lamin A and lamin C transcripts. qPCR was done in triplicates and two sets of experiments were performed.

Statistical analyses

The main clinical and metabolic characteristics of individuals with and without nuclear abnormalities were compared using the Fischer exact test for qualitative variables and the Mann–Whitney test for quantitative variables. It is unlikely that these methods, known to be valid for unrelated observations, could be influenced by the presence of one pair of relatives.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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