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A recurrent homozygous *LMNA* missense variant p.Thr528Met causes atypical progeroid syndrome characterized by mandibuloacral dysostosis, severe muscular dystrophy, and skeletal deformities

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Abstract

Atypical progeroid syndromes (APS) are premature aging syndromes caused by pathogenic *LMNA* missense variants, associated with unaltered expression levels of lamins A and C, without accumulation of wild-type or deleted prelamin A isoforms, as observed in Hutchinson-Gilford progeria syndrome (HGPS) or HGPS-like syndromes. A specific *LMNA* missense variant, (p.Thr528Met), was previously identified in a compound heterozygous state in patients affected by APS and severe familial partial lipodystrophy, whereas heterozygosity was recently identified in patients affected by Type 2 familial partial lipodystrophy. Here, we report four unrelated boys harboring homozygosity for the p.Thr528Met, variant who presented with strikingly homogeneous APS clinical features, including osteolysis of mandibles, distal clavicles and phalanges, congenital muscular

dystrophy with elevated creatine kinase levels, and major skeletal deformities. Immunofluorescence analyses of patient-derived primary fibroblasts showed a high percentage of dysmorphic nuclei with nuclear blebs and typical honeycomb patterns devoid of lamin B1. Interestingly, in some protrusions emerin or LAP2 α formed aberrant aggregates, suggesting pathophysiology-associated clues. These four cases further confirm that a specific *LMNA* variant can lead to the development of strikingly homogeneous clinical phenotypes, in these particular cases a premature aging phenotype with major musculoskeletal involvement linked to the homozygous p.Thr528Met variant.

KEYWORDS

cell nucleus, lamins, muscular dystrophy, premature aging, progeria

1 | INTRODUCTION

Atypical progeria syndromes (APS) are premature aging syndromes caused by dominant or recessive missense *LMNA* variants, whose clinical spectrum is continuously growing (Barthélémy et al., 2015; Doubaj et al., 2012; Garg et al., 2009; Guo et al., 2016; Hisama et al., 2011; Soria-Valles et al., 2016). From a pathophysiological point of view, they differ from typical Hutchinson-Gilford progeria syndrome (HGPS) (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003), HGPS-like syndromes (Barthélémy et al., 2015), restrictive dermopathy (Navarro et al., 2004; Navarro et al., 2005), or mandibuloacral dysplasia type B (MAD-B) (Ben Yaou et al., 2011; Shackleton et al., 2005), in that APS patients do not accumulate farnesylated and toxic prelamin A isoforms, either wild-type or truncated (as prelamin A Δ 50, also called progerin). APS patients express in most cases unaltered levels of lamins A and C, which carry the missense amino acid variants, and are thus classified as lamin A processing “proficient” premature aging syndromes, opposite to HGPS and HGPS-like syndromes, which are lamin A processing “deficient” syndromes (Barthélémy et al., 2015; Doubaj et al., 2012; Kane et al., 2013). The phenotypes of APS patients are heterogeneous and often largely overlap with the clinical features of patients affected with mandibuloacral dysplasia (MAD, OMIM #248370), classical HGPS, or atypical Werner syndrome (Chen et al., 2003); they have also been referred to as “non-classical” progeria (Hennekam, 2006), progeria-like syndromes, neonatal (congenital) progeria, or cardiocutaneous progeria (Garg et al., 2009; Madej-Pilarczyk et al., 2008; Madej-Pilarczyk et al., 2009; Plasilova et al., 2004; Renard et al., 2009). These syndromes vary in severity, depending on the nature, zygosity, and location of the underlying pathogenic *LMNA* variant, as well as on the organ systems affected by the particular variant (see Doubaj et al., 2012; Guo et al., 2014, for a review on APS). The natural history of different forms of APS is being defined in the current years, with the continuous identification of novel cases, mostly due to private *LMNA* variants, which are often identified at a rather young age (Garg et al., 2009; Schultz et al., 2019; Toni et al., 2019; Yukina et al., 2021). Their pathophysiology is linked to the presence of missense-altered lamins A/C and is presumably due to abnormal interactions of the mutated proteins with their numerous molecular partners (Bercht Pflieger et al., 2015; Simon et al., 2010).

MAD is a nosologically defined entity with autosomal recessive inheritance and major clinical overlap with most APS syndromes. It is characterized by postnatal growth retardation, progeroid facies, mandibular and clavicular hypoplasia, acroosteolysis, joint contractures, skin atrophy, and lipodystrophy (Simha et al., 2003; Young et al., 1971). This laminopathy is either primary, due to homozygous or compound heterozygous missense variants in *LMNA* (MAD-A), or secondary, due to biallelic loss of function variants in *ZMPSTE24* (MAD-B) (Agarwal et al., 2003; Novelli et al., 2002). Compared to HGPS patients, MAD patients usually present with different, though unique facial dysmorphism, more severe osteolysis, increased risk of fractures at a young age, but show milder growth retardation, longer persistence of scalp hair, milder lipodystrophy, and increased survival into adulthood (Hennekam, 2006).

On the other hand, typical HGPS is a sporadic, extremely rare, autosomal dominant disorder mostly due to a single recurrent, de novo synonymous variant in *LMNA* exon 11 (c.1824C>T, p.Gly608Gly) (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). Affected children display a very homogeneous clinical phenotype, including major growth retardation, a thin and beaked nose, alopecia, generalized lipodystrophy/lipoatrophy as well as amyotrophy, generalized osteopenia/osteoporosis with lyses of mandible, distal clavicles and phalanges, cutaneous alterations as hyper/hypopigmentation, or early sclerodermatous changes (Hennekam, 2006; Merideth et al., 2008). Average lifespan is estimated at 14.5 years, mostly due to myocardial infarction following disseminated and severe atherosclerosis (Hennekam, 2006).

Pathogenic missense variants in *LMNA* also cause tissue-specific disorders, collectively defined as “laminopathies” (OMIM *150330), which can selectively involve striated muscles, adipose tissue, peripheral nerve, bone, skin, and the cardiovascular system (Broers et al., 2004; Jacob & Garg, 2006; Schreiber & Kennedy, 2013; Worman & Bonne, 2007). Many patients present with overlapping phenotypes among tissue specific and systemic disorders (Benedetti et al., 2005; Garg et al., 2002; van der Kooij et al., 2002; Vantghem et al., 2004). In particular, to the best of our knowledge, the association of myopathy and progeroid features associated with *LMNA* or *ZMPSTE24* variants was described to date in 10 patients (Ben Yaou et al., 2011; Garg et al., 2009; Kirschner et al., 2005; Lombardi et al., 2007; Luo et al., 2014; Madej-Pilarczyk et al., 2008; Madej-Pilarczyk et al., 2009; Zirn et al., 2008).

The *LMNA* gene encodes lamins A and C, major isoforms of A-type lamins, produced by alternative splicing, constituting the intermediate filament family group V. A-type lamins form together with B-type lamins, lamins B1–B3, the nuclear *lamina*, a filamentous meshwork underlying the inner nuclear membrane, which is involved in nuclear mechanical stability, nuclear pore positioning and in the regulation of gene expression patterns. Besides their positioning at the nuclear periphery, lamins A/C are also major and conserved components of the nucleoskeleton, where they serve as a scaffold for fundamental nuclear processes such as chromatin organization, epigenetic regulation, DNA replication, transcription, and repair (Prokocimer et al., 2009).

Under physiological conditions, lamin A is produced as a precursor, prelamin A, that undergoes four post-translational processing steps in order to yield mature lamin A including: Cystein farnesylation of the CaaX motif (C, Cystein, a, aliphatic, X, any amino acid) at its C-terminal end, cleavage of the last 3 amino acids (aaX), carboxymethylation of the farnesylated C-terminal cystein and a final cleavage of the last 15 amino acids including the methylated and farnesylated Cystein residue (Sinensky et al., 1994). Consequently, mature lamin A is physiologically non-farnesylated. On the other hand, lamin C is smaller and non-farnesylated, and is directly translated into its mature form. As all intermediate filaments, lamins A and C contain a N-terminal head domain, an α -helical rod domain, and a globular C-terminal tail domain.

Here, we report and compare the clinical and cellular phenotypes of four young patients with a very homogeneous phenotype that can be nosologically classified as an APS featuring muscular dystrophy and major skeletal abnormalities, linked to the *LMNA* homozygous p.-Thr528Met variant (NM_170707.4:c.1583C>T, exon 9). The unaffected parents of the children, each carrying the same heterozygous variant, were in part clinically explored and are reported. The clinical phenotypes as well as the molecular and cellular findings are discussed and placed in the context of other laminopathies.

2 | MATERIALS AND METHODS

2.1 | Informed consent and cell lines culture

Informed consent was obtained from all parents and patients, complying with the ethical guidelines of the institutions involved. The parents gave written informed consent for publication of the patient's pictures. Blood samples were used to establish Epstein–Barr virus (EBV) immortalized lymphoblastoid cell lines while primary fibroblasts were obtained from a skin biopsy for Patients 1 and 2 and one HGPS patient carrying the typical c.1824C>T, p.Gly608Gly heterozygous variant. The two control cell lines, AG08498 and GM08398, were obtained from the Coriell cell repository (<https://catalog.coriell.org/>). All the cell lines were cultured in DMEM medium containing 15% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), 2 mm/mL L-glutamine, and 100 U/mL penicillin–streptomycin (Invitrogen-Life Technologies) in a humidified atmosphere of 5% CO₂ at 37°C. All cell lines prepared, stored, and delivered for research according to the French regulation by the Biological Resource Center (CRB AP-HM Biobank; NF S96-900 & ISO 9001 v2015

Certifications), la Timone Hospital, Marseille, whose cell lines were declared to the French Ministry of Health (declaration no. DC-2008-429) and whose use for research purposes was authorized by the French Ministry of Education, Research and Innovation (authorization nos. AC-2011-1312; AC-2017-2986).

2.2 | Genomic analysis

The coding sequences and intronic boundaries of *LMNA* and *ZMPSTE24* were PCR-amplified on peripheral blood-extracted DNA, following standard procedures. The amplicons were then sequenced using an ABI-PRISM 3130XL 16 capillaries automatic sequence analyzer (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's recommendations. Sanger sequencing was performed in both orientations in order to exclude sequencing artifacts. The electropherograms were visualized and interpreted by comparison to genome database reference sequences using the Sequencher 4.8 software (Gene Codes Corp). Nomenclature used to describe sequence variations was the one proposed by the Human Genome Variation Society available at <http://www.hgvs.org/mutnomen/>. *LMNA* and *ZMPSTE24* reference cDNA sequences used for genomic exploration and variant nomenclature are respectively GenBank NM_170707.4 and NM_005857.3.

2.3 | Transcriptional analyses

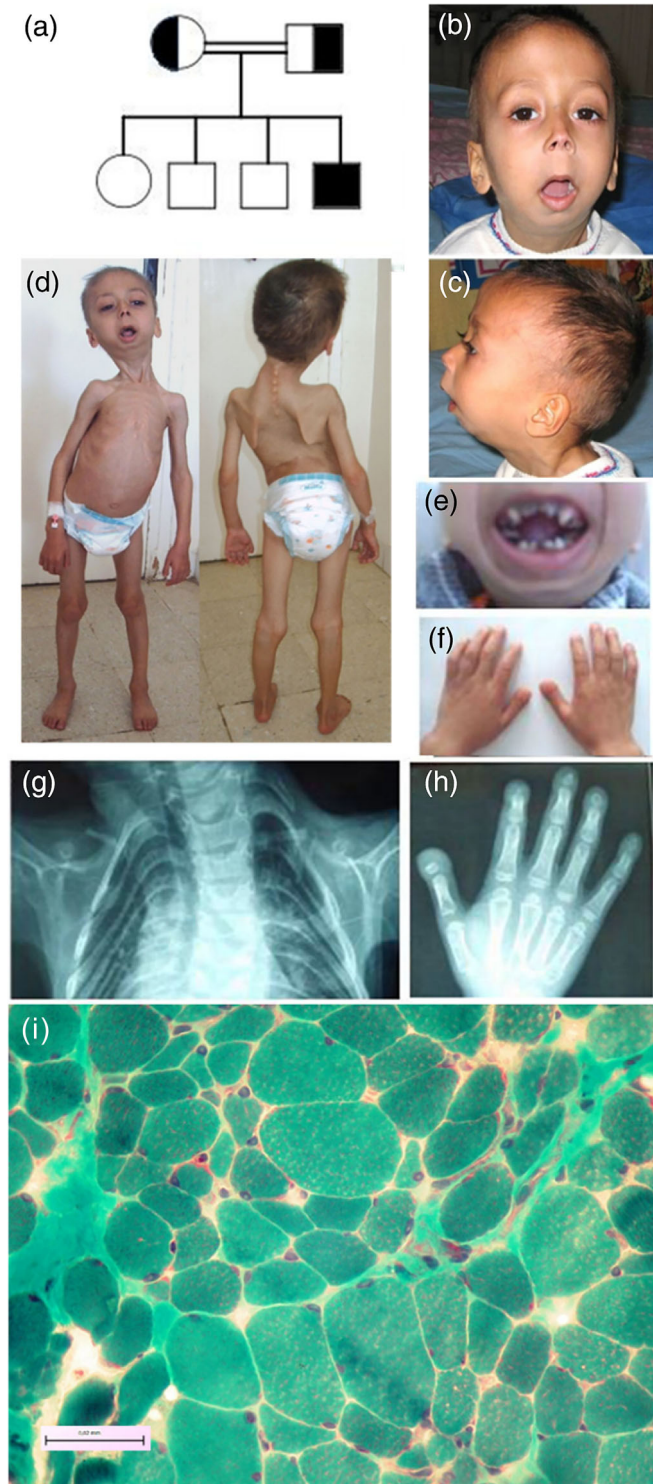
Total RNA was extracted with Trizol (Roche Diagnostics) from lymphoblastoid cell lines, following the manufacturer's instructions. Transcriptional analyses encompassing the variant were performed using a forward primer located in exon 7 and a reverse in exon 12. Primer sequences are available upon request. RT-PCR products were analyzed on agarose gel and directly sequenced, as described for genomic sequences.

2.4 | Western blotting

Cells were lysed in 300 μ L of NP-40 lysis buffer (Invitrogen), protease, and phosphatase inhibitors cocktail (18611284, Thermo Scientific) were added to a final concentration 1 \times . Cell extracts were sonicated three times for 30 s, incubated 30 min at 4°C, and centrifuged 10 min at 10,000 rpm at 4°C. Forty micrograms of total proteins were loaded in each lane, separated in 7% Tris-Acetate precast Criterion gels (Biorad) and transferred on a PVDF membrane (Millipore). Membranes were blocked for 1 h in 1:2 diluted blocking buffer for near-infrared fluorescent western blotting (Rockland). Blocked membranes were incubated for 1 h at room temperature (RT) with corresponding primary antibodies in addition to an anti-GAPDH (MAB374, Millipore) as a loading control, all in a blocking solution for 1 h at RT. After several washes in PBST, membranes were incubated with IR-Dye 800CW or 680 conjugated secondary antibodies (1/5000, LI-COR[®] Biosciences) for 1 h at RT. Bound antibodies were detected and analyzed on an Odyssey[®] V3.0 imaging system (LI-COR Biosciences), according to the manufacturer's instructions.

2.5 | Immunofluorescence microscopy

The immunofluorescence analyses followed previously described protocols (Navarro et al., 2004). Briefly, cells were fixed in 4% paraformaldehyde solution for 15 min, permeabilized in 0.5% Triton X-100 in PBS for 5 min, and washed in PBS. Primary antibody incubations were performed for 45 min at RT diluted in PBS supplemented with 2% BSA and followed by several washing in PBS.



Slides were incubated with secondary antibodies for 45 min at room temperature. Secondary antibodies were obtained from Molecular Probes Company (Life technologies): Alexa 488-conjugated goat anti-mouse (1:400), Alexa 488-conjugated goat anti-rabbit (1:400), and Alexa 594-conjugated donkey anti-goat (1:400). The cells were then washed twice for 10 min in PBS, incubated with DAPI (Sigma-Aldrich) at 100 ng/mL for 10 min, and finally washed three times for 5 min in Tween 20 at 0.1% in PBS. The slides were then mounted in Vectashield mounting medium (Vector), coverslipped, and sealed.

2.6 | Primary antibodies list

Anti-lamin A/C (sc-20681), anti-prelamin A (sc-6214), and anti-progerin (sc-81611) were from Santa Cruz. Anti-lamin A (ab8980), anti-lamin B1 (ab16048), anti-emerin (ab40688), anti-Lap2 Alpha (ab 5162), and anti-H3K9-me3 (ab8898) were obtained from Abcam. Anti-NuMA (Ab-1) was purchased from Calbiochem.

3 | CLINICAL REPORTS

3.1 | Case 1

The proband was the fourth child of consanguineous unaffected parents who were first cousins (Figure 1a; Tables 1 and 2). He was born at term with birth weight of 4.2 kg (1.5 SD). At the age of 10 months, he presented marked axial weakness with loss of head control. His mother noticed thin skin, progressive loss of hair, and growth retardation at 14 months of age. Two months later, he developed progressive rigid spine and scoliosis. When evaluated at the age of 3 years (Figure 1b,c), he had a height of 89 cm (−1.5 SD), a weight of 8 kg (−3 SD), and was microcephalic, head

FIGURE 1 Clinical, radiological, and histological features of Patient 1. (a) Pedigree of the family. The affected individual (homozygous variant carrier) is shown as a filled black symbol, the unaffected siblings (their variant carrier status was not analyzed) as unfilled symbols. Unaffected heterozygous variant carriers are shown with half-filled symbols. Squares denote males and circles denote females. Consanguinity is indicated with a double horizontal line. (b–h) Clinical features of Patient 1. (b, c) At the age of 3 years, showing progeroid facial features with prominent eyes, a thin and beaked nose, micrognathia with a small open mouth, sparse hair. (d) At the age of 6 years, the patient presents with pronounced scoliosis, rigid spine, generalized muscular atrophy. (e) Severe teeth overcrowding showing dental caries. (f) Rounded fingertips evocative of distal osteolysis. (g, h) Proband's radiographs at age 6. A marked clavicular hypoplasia (g) as well as distal phalanges acroosteolysis are evident (h). (i) Deltoid muscle biopsy of the proband (Trichrome Gomori staining) performed at the age of 6 years. Specific muscular dystrophy findings, including marked variations of fiber size and increased interstitial fibrosis can be observed. Scale bar: 0.2 mm.

TABLE 1 Comparative features of atypical progeria syndromes with and without myopathy, caused by different LMNA variants.

	p.T528M HOM	p.T528M HOM	p.T528M HOM	p.T528M HOM	p.R471C HOM	p.S143F HET	p.S143F HET	p.R527H/ p.V440M	p.T528M/ p.M540T	p.T528M/ p.S583L	P.T528M HET	p.T528M HET	p.T528M HET	
Bibliographic reference	This report	This report	This report	This report	Zirn et al. (2008)	Kirschner et al. (2005)	Madaj-Pilarczyk et al. (2008)	Lombardi et al. (2007)	Verstraeten et al. (2006)	Savage et al. (2004)	Savage et al. (2004)	Tu (2016)	Savage et al. (2004)	Araujo-Vilar et al. (2021)
Patient number in this report	Patient 1	Patient 2	Patient 3	Patient 4										
Geographic origin	Algerian 1	Turkish	Brazilian	Algerian 2										
Clinical features														
Sex/individuals	M	M	M	M	F	F	F	F	M	Several patients	Parents	2 F	5P	
Clinical description	Myopathy + progeria	Myopathy + progeria	Myopathy + progeria	Myopathy + progeria	Myopathy + progeria	Myopathy + progeria	Myopathy + progeria	Myopathy + MAD-A	Progeria	FPLD	Asymptomatic/mild metabolic syndrome	FPLD2	FPLD2	
Onset of myopathy	10 months	15 months	3–4 years	12 months	10 months	10 months	5 years	>22 years	–	–	–	–	–	–
Onset of progeroid features	14 months	3 years	1–3 years	2–3 years	3 years	>1 year	3 years	>22 years	>1 year	–	–	–	–	–
Mandibular hypoplasia	+	+	+	Discrete	+	+	+	+	–	–	–	–	–	–
Clavicular hypoplasia	+	+	+	+	+	+	+	–	+	–	–	–	–	–
Acroosteolysis	+	+	+	+	+	+	+	Milder	+	–	–	–	–	–
Alpecia/sparse hair	+	+	+	+	+	+	+	–	+	–	–	–	–	–
Thin/sclerotic skin	+	+	+	+	–	+	+	–	+	–	–	–	–	–
Lipodystrophy	Generalized	Generalized	Generalized	Preserved face	Partial	Generalized	Preserved face	Partial	Preserved face	Partial	–	–	–	–
Growth retardation	+	+	+	+	–	+	+	–	+	–	–	–	–	–
Microcephaly	+	–	+	+	+	–	–	–	+	–	–	–	–	–
Muscle weakness	+	+	–	+	+	+	+	+	–	–	–	–	–	–
Muscular Atrophy	+	+	+	+	+	+	–	–	–	–	–	–	–	–
Rigid spine	+	–	–	+	+	+	–	–	–	–	–	–	–	–
Scoliosis	+	+	+	+	–	–	–	–	–	–	–	–	–	–
Contractures	Achilles	Elbow, knees	Achilles, elbow	–	Achilles, knees, elbow	–	Achilles, knees, elbow wrists, fingers	–	–	–	–	–	–	–
Cardiac disease	–	Tachycardia attack	–	–	–	N	Atrial fibrillation	–	Nr	–	–	–	–	–
Osteopenia/coxa valga	–	+	+	–	+	+	+	–	Nr	–	–	–	–	–

TABLE 1 (Continued)

	p.T528M HOM	p.T528M HOM	p.T528M HOM	p.T528M HOM	p.T528M HOM	p.R471C HOM	p.S143F HET	p.S143F HET	p.R527H/ p.V440M	P.T528M/ p.M540T	P.T528M/ p.S583L	P.T528M HET	p.T528M HET	p.T528M HET
Other features	–	–	–	–	–	–	Freckles	Mottled pigmentation	–	Wormian bones	–	±Acanthosis nigricans	–	–
Creatine kinase	271 IU/L (15–130)	441 IU/L (15–130)	225 IU/L (0–171)	390 IU/L (15–130)	1808 IU/L (<70)	–	374 IU/L	4–5 x N	nl (56 IU/L)	Nr	–	–	–	–
Triglycerides	N	N	N	N	Nr	Nr	Nr	Nr	N	Nr	Elevated	±Elevated	–	–
Blood pressure	N	N	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Elevated	±Elevated	–	–
Insulin	N	Nr	Nr	Nr	N	N	Nr	Nr	N	Nr	Elevated	±Elevated	–	–
EMG	Myopathic	Nr	Nr	N	Nr	Nr	Myopathic	Myopathic	Myopathic	Nr	–	–	–	–
Indirect immunofluorescence analyses														
Available biological material	Fibroblast	Fibroblast	Nr	Nr	Nr	Nr	Muscle biopsy	Fibroblast	Fibroblast	Fibroblast	Nr	Nr	Nr	Nr
Dysmorphic nuclei	46%	35%					+	Nr	30%	36%				
Nuclear blebs	+	+					+	Nr	+	+				
Protrusion	+	+					+	Nr	+	+				
Honeycomb pattern	+	+					Nr	Nr	Nr	+				
Western blot analyses														
Lamin A	+	+					Decreased	N	N	N	Nr	Nr	+	+
Lamin C	+	+					N	N	N	N			+	+
Prelamin A	–	–					Nr	–	+	–			–	–
Progerin	–	–					Nr	–	Nr	–			–	–

Abbreviations: FPLD, familial partial lipodystrophy Dunnigan type; HET, heterozygous; HOM, homozygous; MAD-A, mandibuloacral dysplasia type A; N, normal; Nr, not reported; –, absent; +, present; ±, present or elevated in some cases.

TABLE 2 Clinical features of four patients harboring the same homozygous p.T528M LMNA variant.

	Patient 1	Patient 2	Patient 3	Patient 4
Geographic origin	Algerian 1	Turkish	Brazilian	Algerian 2
Consanguinity	+	+	+	+
Sex/individuals	M	M	M	M
Progeroid features				
Mandibular hypoplasia, clavicular hypoplasia, acroosteolysis, alopecia/sparse hair				
Thin/sclerotic skin growth retardation				
Onset	14 months	3 years	1–3 years	2–3 years
Lipodystrophy	Generalized	Generalized	Generalized	Preserved face
Microcephaly	+	–	+	+
Tachycardia attack	–	–	+	–
Myopathy				
Onset	10 months	15 months	3–4 years	12 months
Muscle weakness	+	+	–	+
Muscular atrophy	+	+	+	+
EMG	Myopathic	Nr	Nr	N
Creatine kinase	271 U/L (15–130)	441 U/L (15–130)	225 U/L (0–171)	390 U/L (15–130)
Skeletal deformities				
Scoliosis	+	+	+	+
Rigid spine	+	–	–	–
Contractures	Achilles	Elbow, knees	Achilles, elbow	–
Osteopenia/coxa valga	–	+	+	–
Indirect immunofluorescence analyses (Fibroblast)				
Dysmorphic nuclei	46%	35%	Nr	Nr
Nuclear blebs	+	+		
Protrusion				
Honeycomb pattern				
Western blot analyses				
Lamin A	+	+	Nr	Nr
Lamin C				
Prelamin A	–	–		
Progerin				
	p.T528M HOM	p.T528M HOM	p.T528M HOM	p.T528M HOM

Abbreviations: HOM, homozygous; N, normal; Nr, not reported; –, absent; +, present.

circumference (HC) of 47 cm (–2.5 SD). Physical examination revealed the following dysmorphic features: progeroid facies with moderate proptosis, beaked nose, pronounced micrognathia, small mouth, and dental overcrowding (Figure 1e) with severe caries as well as sparse discolored hair. He had rounded fingertips evocative of distal osteolysis, thin skin with visible veins, hypoplastic nails, generalized loss of subcutaneous adipose tissue, and a lumbar hyperpigmented skin patch. Furthermore, the patient presented with severe axial and proximal muscle weakness, hyporeflexia, lumbar hyperlordosis, rigid spine, and scoliosis. EMG on the deltoid muscle revealed spontaneous activity, maximal effort pattern—full interference, and low amplitude. Nerve conduction velocity of motor and sensory nerves was unremarkable. Radiography

confirmed phalanges and clavicles distal lyses (Figure 1g,h). Blood lipids, glucose, and CPK levels were normal.

At the age of 6 (Figure 1d), the patient's muscle weakness and atrophy worsened. His progeroid features became more striking and his growth parameters further decreased (height: 96 cm [–4 SD]; weight: 9 kg [–4 SD]; HC: 47 cm [–2.5 SD]). In addition, he presented with Achilles tendons contractures. Deltoid muscle biopsy revealed marked variations of fiber size, and moderate increase in interstitial fibrosis (Figure 1i). Creatine kinase levels were now increased: 271 U/L (normal values: 15–130 U/L). Oral glucose tolerance test was normal. ECG and echocardiography exams were unremarkable, as well as cognitive functions. The patient died at the age of 10 following an acute chest infection.

3.2 | Case 2

The proband is a 4-year 5-month-old boy who is the second child of consanguineous unaffected parents who were first cousins (Figure 2a-d; Tables 1 and 2). Intrauterine growth retardation (IUGR)

was detected by fetal ultrasonography during prenatal follow-up. Motor and cognitive development were normal in the first year of life. He was delivered at term with low birth weight of 2 kg (-3 SD). The mother described that muscle weakness appeared by 15 months. Recurrent infections were seen after 2.5 years. At the age of 3, thin

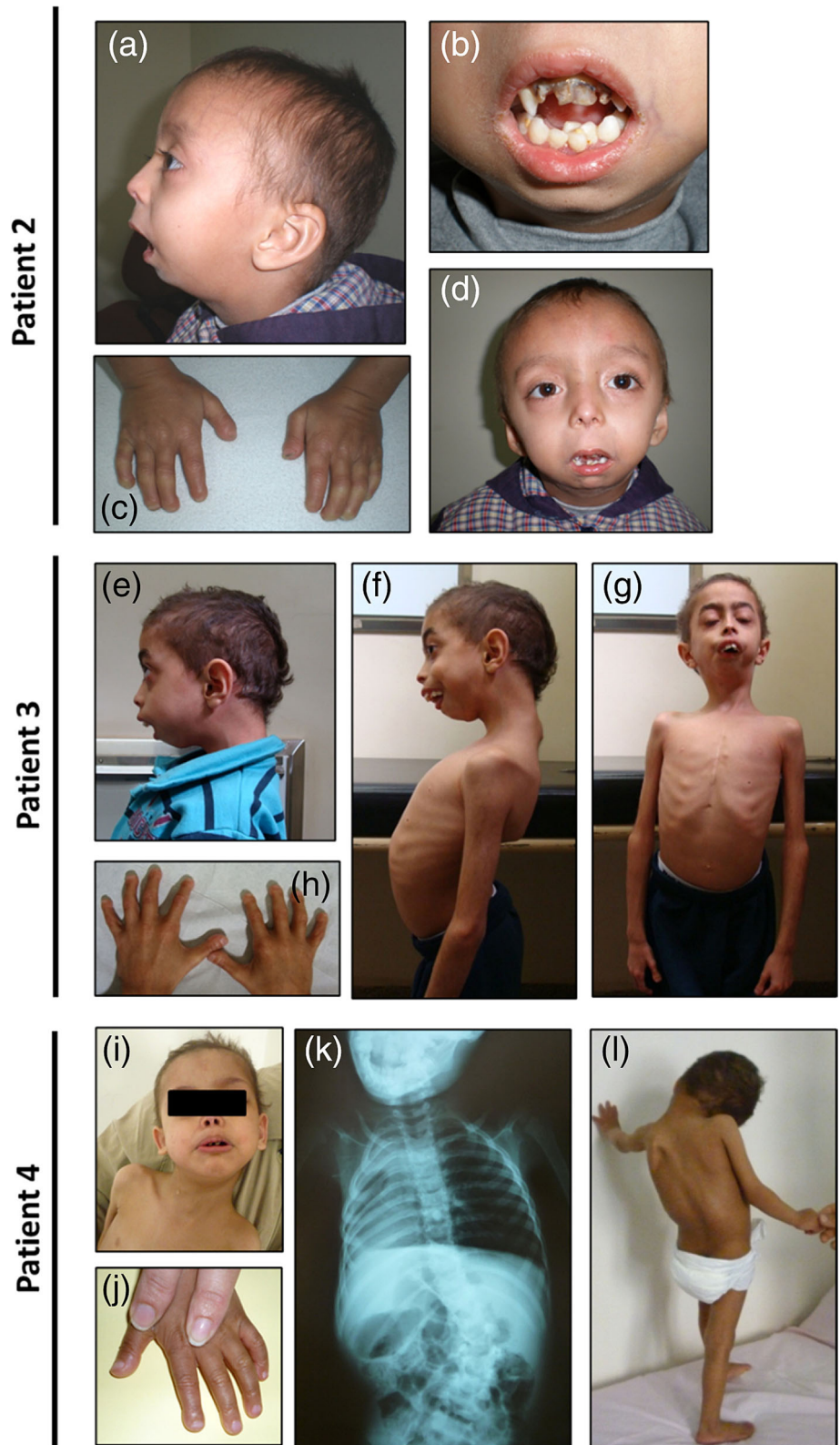


FIGURE 2 Clinical phenotypes of Patients 2, 3, and 4. (a–d) Clinical features of Patient 2: (a, b) Progeroid facial features with relatively large and low-set ears, micrognathia, open and small mouth. (c) Teeth overcrowding showing severe caries. (d) Rounded acro-osteolytic fingertips. (e–h) Clinical features of Patient 3: (e) Progeroid facial features with prominent eyes, sparse hair, micrognathia, open mouth. (f, g) The patient presents with pronounced scoliosis, rigid spine, generalized muscular atrophy, and teeth overcrowding. (h) Rounded acro-osteolytic fingertips. (i–l) Clinical features of Patient 4: (i) Patient at the age of 3 years, showing sparse hair, beaked nose, low-set ears. (j) At age of 6 years, typical rounded distal phalanges evocative of acroosteolysis are observed. (k) Scoliosis and shortened clavicles (osteolysis) can be noted on X-rays; (l) scoliosis, rigid spine, and major muscular atrophy are evidenced.

skin and sparse hair became apparent. At 4.5 years, joint contractures and scoliosis were evident at examination. He had dysmorphic findings such as a thin beaked nose, alar hypoplasia, and mandibular hypoplasia together with sparse hair, dystrophic nails, irregular teeth with severe caries (Figure 2a–d). He had rounded acro-osteolytic fingertips (Figure 2c), a thin-dry- and atrophic skin with visible (Figure 2a). Generalized joint stiffness (especially in elbows and knees) was also observed and X-ray evidenced acroosteolysis, scoliosis, and lumbar hyperlordosis, as well as osteopenia (data not shown). Cardiologic evaluation (ECG and echocardiography) was normal. At the age of 6, joint contractures and scoliosis became more striking. During that period, he had a tachycardia attack without any additional cardiac conduction disturbances. Normal laboratory findings were observed for lipid and glucose metabolism, whereas elevated creatinine kinase levels were detected of 441.0 U/L (normal values: 15–130 U/L).

3.3 | Case 3

The proband is a 10-year 3-month-old boy (Figure 2e–h; Tables 1 and 2), the third child born to consanguineous unaffected parents who were first cousins. He was born at term by vaginal delivery after an uneventful pregnancy. At birth, his weight was 3.8 kg (1.8 SD), his length 52 cm (–0.5 SD), Apgar 9/10. He had unremarkable newborn screening (phenylketonuria, congenital hypothyroidism, and sickle cell disease).

He showed unremarkable developmental milestones (walked without support at 12 months, said first words at 14 months), but failure to thrive was noticed within first 6 months of life. He also showed microcytic anemia. Subsequently, he successfully underwent surgery for congenital heart disease (interatrial communication—*ostium secundum* type). Despite the surgery, he continued to show poor growth and started to have upper respiratory airways infections, diarrhea, and pneumonia. He was referred for genetic evaluation when he was 3 years and 10 months because of growth failure and dysmorphic features. At that time, he weighed 11.9 kg (–2 SD) and was 94.5 cm tall (–1.5 SD), with OFC 49 cm (–1.5 SD). At 4 years, thin skin, nail dystrophy, and acroosteolysis of distal phalanges were noticed (Figure 2h). At 4.5 years, joint contractures and scoliosis were evident at examination (Figure 2f,g). At 9 years of age, he had a post-traumatic fracture of the skull and developed an extensive subgaleal hematoma but did not need surgical intervention.

His clinical evaluation showed dysmorphic features including thin beaked nose, alar hypoplasia and mandibular hypoplasia, sparse hair, and irregular teeth (Figure 2e–g). His skin showed an atrophic pattern with visible veins on the scalp. Neurological examination showed joint limitations (elbows and knees), generalized muscle hypotrophy with areflexia, but normal muscle strength. X-rays evidenced bilateral *coxa valga*, acroosteolysis, kyphosis with thoracic scoliosis, as well as osteopenia. Spine MRI showed hypoplasia of many cervical vertebrae and agenesis of neural anterior arch of C5 and C6 (data not shown). Thyroid ultrasound was normal, but was suggestive of an accessory thymus. Normal laboratory findings were observed for lipid and

glucose metabolism, whereas elevated creatinine kinase levels were detected (225 U/L, normal values: 0–171 U/L).

3.4 | Case 4

This patient was born from a consanguineous union (Figure 2i–l; Tables 1 and 2). Birth measurements were unavailable, but he was thought to have presented with low birth weight, and had to stay in an incubator in the hospital for 10 days after delivery. At 12 months, weakness of the neck muscles with a drooping head and a deviation of the vertebral spine were noted. We examined this patient at 3 years of age (Figure 2i). He presented a growth retardation, with height: 82 cm (–3 SD), weight: 9 kg (–3.5 SD), microcephaly: OFC 46 cm (–3 SD), and typical progeroid features including beaked nose, thin skin, severe caries, and sparse hair. In addition, he also presented axial and proximal muscle weakness with hyporeflexia, lumbar hyperlordosis, scoliosis, and rigid spine (Figure 2k,l). Radiography showed clavicle hypoplasia (Figure 2k) and rounded fingertips were noted, due to acroosteolysis (Figure 2j). EMG was normal but creatine kinase levels were elevated: 390 U/L (normal values: 15–130 U/L).

3.5 | Ancestry and parental clinical phenotypes

The cases had mostly different ancestry, since two unrelated cases were from Algeria while the other two cases were from Turkey and Brazil (Table 1). None of the parents of the four cases we report showed hypertension or diabetes nor displayed signs of myopathy or muscular dystrophy. Specific lipid profiles were not evaluated but their physical examination was unremarkable.

4 | MOLECULAR AND IN VITRO ANALYSES

4.1 | Molecular analyses

Sanger sequencing of the coding regions and intronic boundaries of the *LMNA* gene identified in all four probands a homozygous substitution of cytosine by thymine in exon 9, at position 1583 of the coding sequence, relative to transcript number NM_170707.4: c.1583C>T (Figure S1). At the protein level, this nucleotide change predicts a missense variant p.Thr528Met (p.T528M). All unaffected parents tested were heterozygous carriers (Figure S1). Notably this variant has been identified in 2 out of 214,466 alleles by the Genome Aggregation Database (gnomAD) (Karczewski et al., 2020), and the heterozygous variant is listed as the Variant of Uncertain Significance by ClinVar in the Human Gene Mutation Database (HGMD). According to the American College of Medical Genetics and Genomics (ACMG) criteria, the homozygous variant, can be classified as pathogenic, evidence: PS3, PM1, PM2, PM5, PP1, and PP3 (Richards et al., 2015).

To exclude a possible effect on splicing in Patient 1, we performed RT-PCR analyses using primer encompassing the variant. No

abnormal band size was showed by RT-PCR and electropherograms from Sanger sequencing of the amplicons including exon 9 only confirmed the presence of the homozygous c.1583C>T variant in the patient's cDNA (data not shown).

4.2 | In vitro analyses

Immunoblots performed at passage 8, on proteins extracted from skin fibroblasts of Patients 1 and 2 compared to control or HGPS, revealed the absence of progerin or any other prelamin truncated isoform, whereas lamin A and C levels were unaltered (Figure 3) along with the expression levels of lamin B1 and emerin. Indirect immunofluorescence with specific antibodies confirmed the absence of prelamin A and progerin, in accordance with the lamin A/C antibody staining (data not shown).

Immunofluorescence experiments performed on skin fibroblasts of Patients 1 and 2 at passage 8 showed a high percentage of dysmorphic nuclei (43% and 35% of the cells, respectively) (Figure 4a-l) compared to control fibroblast cell lines (5%) (Figure 4m-t). When stained with antibodies directed against lamins A/C, the patients' nuclei were characterized by irregular shapes with large protrusions, lobulations, or nuclear blebs (Figure 4a-c). Moreover, some of these blebs showed emerin aggregation (Figure 4d-f), particularly within nuclear blebs

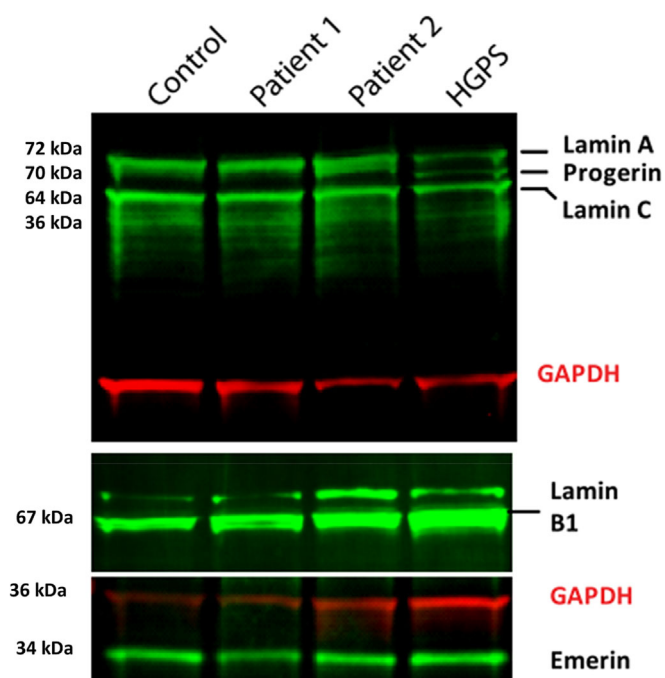


FIGURE 3 Western blot analysis. Total protein extracts were obtained from fibroblast cultures of Control (lane 1), Patient 1 (lane 2), Patient 2 (lane 3) as well as a HGPS patient (lane 4), at passage 8. Lamins A/C, lamin B1, and emerin are shown in green and GAPDH, as a loading control protein, in red. HGPS protein extracts (lane 4) show progerin accumulation migrating between lamins A and C. The double band visible on patient 2's lane under lamin A band is a frequent western blot artifact.

(Figure 4d,f) with parallel loss of lamin B1 staining (Figure 4g-i). Typical honeycomb staining patterns were frequently evidenced using lamin A specific antibodies (Figure 4j-l). We further explored LAP2 α and H3K9me3 (trimethylation of lysine 9 in Histone H3) distribution (Figure 5a,b, respectively). Interestingly, we evidenced in some nuclei the accumulation of LAP2 α in blebs, also characterized by lower lamin A specific staining (Figure 5a). Abnormal staining was also observed for H3K9me3 (Figure 5b): in some nuclei, an accumulation of this repressive histone mark was observed in blebs, as noted for LAP2 α or emerin. A generally lower H3K9me3 staining intensity was observed, compared to controls, recalling the low H3K9me3 expression observed in HGPS nuclei (Figure 5b). Globally, when large nuclear envelope protrusions are present (i.e., in about 35%–46% of the cells), systematically the immunofluorescence stainings of LAP2 α and H3K9me3 are reinforced into them, while Lamin A shows a honeycomb staining pattern and lamin B is absent, as shown in Figures 4 and 5. On the other hand, emerin staining is reinforced especially in small nuclear blebs, as shown in Figure 4, which are observed more rarely, in about 5% of the cells.

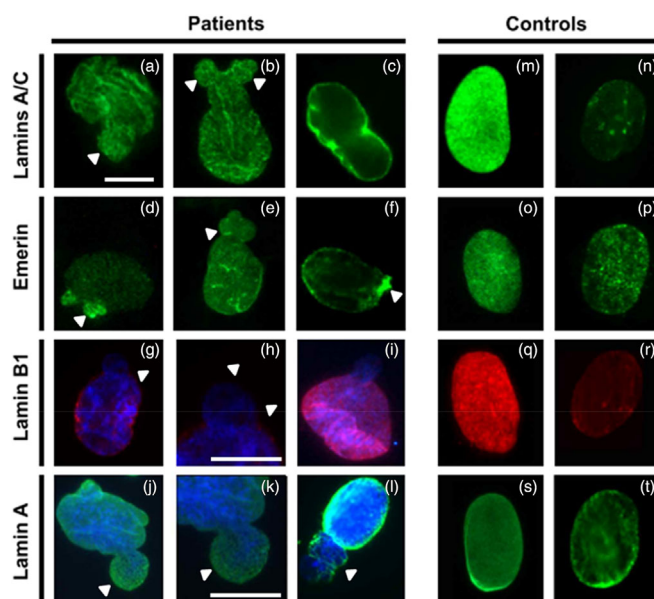


FIGURE 4 Indirect immunofluorescence of lamins and emerin. Illustrative nuclei issued from the fibroblast cell lines of Patients 1 and 2 are presented on the left panel and compared with control nuclei issued from two fibroblast cell lines on the right panel, using lamin A/C, emerin, lamin B1, and lamin A specific antibodies. Lamin A/C (a–c, m, n) and emerin (d–f, o, p) staining are in green. Lamin B1 staining (g, h, q, r) is in red, and lamin A specific staining is in green (j–l, s, t). Lamin B1 and lamin A indirect immunofluorescence is counterstained with DAPI, evidencing abnormal protein localization in nuclear blebs. Arrowheads indicate blebs (a, b, d, e, g, h, j–l); in some blebs intense staining can be observed, suggesting protein aggregation (d, f). Lamin A specific antibody (j–l, s, t) evidenced the typical honeycomb pattern that can be observed in nuclear blebs of patients affected with laminopathies with antibodies directed against lamins A and C (Novelli et al., 2002); (h) and (k) are zoomed portions of (g) and (j) respectively, highlighting nuclear deformations and altered ultrastructure. Scale bar: 5 μ m.

Specific anti-progerin antibodies confirmed the absence of this aberrant prelamin A derivative in both patients (Figure 5b). The same results were obtained with specific anti-(wild-type)-prelamin A antibodies (data not shown).

5 | DISCUSSION

We report four unrelated patients born to consanguineous parents, presenting with a strikingly similar clinical phenotype, combining progeroid features with severe congenital muscular dystrophy resulting from the identical homozygous *LMNA* missense variant. The p.Thr528Met is localized in a very conserved region within the C-terminal globular domain of A-type lamins, as discussed by (Verstraeten et al., 2006). All patients presented very similar clinical features, characterized by proximal muscle weakness and severe scoliosis, in addition to typical features of lamin-linked premature aging syndromes such as micrognathia, beaked nose, distal acroosteolysis of phalanges and clavicles, prominent superficial vessels with thin skin, sparse hair, and dental overcrowding with caries.

Growth retardation was developed either prenatally or in the first years of life in all children. Muscular dystrophy was either congenital or developed in most cases between 10 and 15 months of age, while progeroid features became apparent later, in most cases after 24 months of age.

The clinical features of our patients carrying the homozygous *LMNA* p.Thr528Met variant can be compared to those of three categories of previously described patients: (i) patients carrying *LMNA* missense variants, affected with both myopathy and progeroid features; (ii) patients carrying the identical missense variant on only one allele, either in the heterozygous or compound heterozygous state; and (iii) patients carrying other *LMNA* missense variants affecting the same residue (Thr528).

- To date, to the best of our knowledge, the association of myopathy and progeroid clinical features is caused by the following *LMNA* missense variants: homozygous p.Thr101Ile (Garg et al., 2009), heterozygous p.Ser143Phe (Kirschner et al., 2005; Madej-Pilarczyk et al., 2008), homozygous p.Arg436Cys (Madej-Pilarczyk et al., 2009), homozygous p.Arg471Cys (Zirn et al., 2008), the compound heterozygous p.Arg527His/p.Val440Met (Lombardi et al., 2007), as well as the homozygous p.Arg527His (Luo et al., 2014).

The phenotype of the patients we report is similar to those previously described with an early onset myopathy, before 10 months in most cases (see Table 1), fast progression of muscle weakness, and rigid spine. However, severe myopathy leading to dramatical scoliosis is very specific and homogeneous in the case we report. Interestingly, a similar extremely severe scoliosis was reported in one of two brothers carrying a homozygous missense variant affecting the

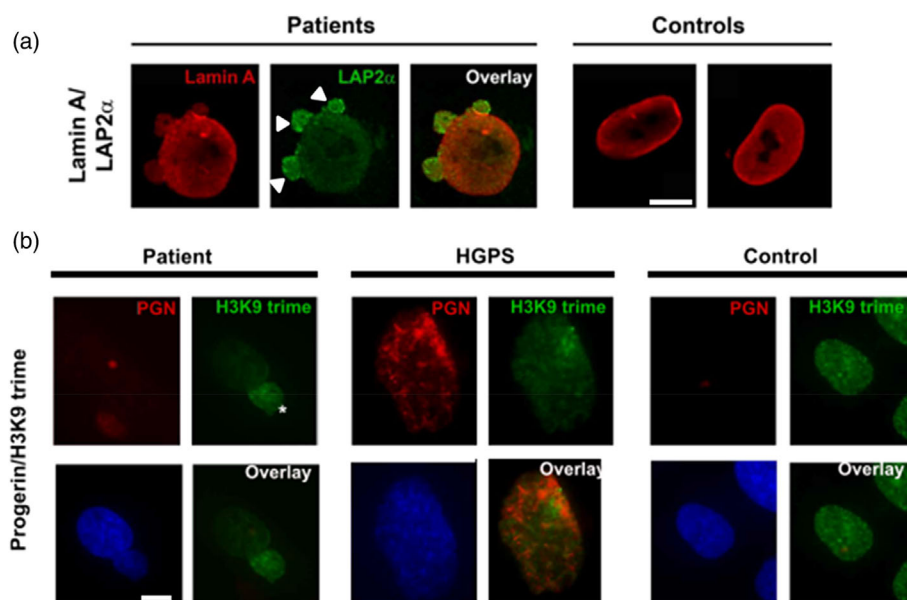


FIGURE 5 Immunocytochemistry of additional nuclear proteins on patients' fibroblasts. The antibodies used are indicated on each picture: double staining of lamin A and LAP2 α (a), double staining of H3K9me3 (H3K9 trimethylation) and progerin (b). (a) Lamin A is stained in red and LAP2 α in green. An overlay is shown. Arrowheads indicate abnormal LAP2 α accumulation in nuclear blebs. Patients' nuclei are shown on the left panel and compared to control nuclei on the right panel. (b) Patients 1 and 2 nuclei on the left panel were compared to HGPS nuclei (middle) as positive control for progerin antibody and to a normal control (right panel). The asterisk on one patient's nucleus highlights the abnormal accumulation of H3K9me3 observed in a bleb, resulting in very low staining in the remaining nucleus. H3K9me3 staining both on patient and HGPS nuclei were lower and more focalized on one nuclear area than that observed on control nuclei. Progerin-specific antibodies showed absence of this protein in patients and controls compared to HGPS. Scale bar: 5 μ m.

adjacent residue in exon 9: p.Arg527Cys (Liang et al., 2009). This patient was described as having atypical HGPS and muscular atrophy. The same homozygous p.Arg527Cys variant was described by Luo et al. (2014) in three children affected with MADA-associated progeria but only the youngest son of 10 months showed more severe lesions, elevated CPK, and possibility of myogenic damage at the electromyogram for the extremities. On the other hand, a patient carrying the identical homozygous variant described by Agarwal et al. (2008) did not show similar muscular nor skeletal abnormalities but rather an atypical progeria phenotype.

The muscular dystrophy presented by the girl described by Zirn et al. (2008), carrying the p.Arg471Cys homozygous mutation, was slowly progressive with asymmetrical contractures. However, the lipodystrophy phenotype was partial, and the radiological findings were osteopenia and coxa valga (Zirn et al., 2008). A similar case of a girl carrying the p.Ser143Phe heterozygous mutation with proximal myopathy, rigid spine without contractures was described (Kirschner et al., 2005). She also presented progeroid features, micrognathia, acroosteolysis, lipodystrophy, incomplete alopecia, and severe growth retardation. In 2008, a 6-year-old girl affected with myopathy and progeria caused by the same heterozygous LMNA variant was reported (Madej-Pilarczyk et al., 2008). Clinically, the patient presented with proximal muscle weakness with elevated CPK; elbows, knees, Achilles tendons, wrists and finger contractures, atrial fibrillation, and progeroid features with micrognathia, prominent eyes, beaked nose, sparse gray hair, dystrophic nails, and numerous freckles, together with lipoatrophy, clavicle resorption, and coxa valga. The same author reported in 2009 a homozygous variant of p.Arg436Cys in a 2-year-old girl with progeroid syndrome with scleroderma-like skin changes, proximal muscle wasting, and axial muscle weakness resulting in poor head control, creatine kinase and EMG were not performed (Madej-Pilarczyk et al., 2009). Furthermore, the p.Thr10Ile homozygous variant was reported by Garg et al. (2009) in an 8-year-old child with progeroid syndrome associated with muscle wasting and poor exercise tolerance without any CPK and EMG information.

Finally, Lombardi et al. (2007) reported a 27-year-old Italian woman harboring compound heterozygosity for p.Arg527His/p.Val440Met and showing an overlapping phenotype between MAD-A and limb-girdle muscular dystrophy, electromyographic findings myopathic pattern, creatine kinase was normal and biopsy was not performed (Lombardi et al., 2007).

2. Several patients carrying the p.Thr528Met variant on one allele were described: a patient affected with progeroid syndrome carrying the p.Thr528Met /p.Met540Thr compound heterozygous variants (Verstraeten et al., 2006), and several patients affected with Dunnigan-type familial partial lipodystrophy (FPLD) carrying the p.Thr528Met/p.Ser583Leu compound heterozygous variants (Savage et al., 2004), as well as their relatives/parents carrying the heterozygous p.T528M variant. Moreover, heterozygous carriers affected by FPLD2 were recently described (Araújo-Vilar et al., 2021; Tu et al., 2016) (Table 1).

Verstraeten et al. (2006) reported the heterozygous p.Thr528Met LMNA variant combined with the p.Met540Thr variant in a young child with overlapping features between MAD and HGPS, without prelamin A accumulation. In this case, both parents, including the one carrying the heterozygous p.Thr528Met variant, were asymptomatic, even if, on indirect immunofluorescence studies, a considerable percentage of their fibroblasts' nuclei were dysmorphic (Verstraeten et al., 2006).

In the publication by Savage et al. (2004), while, on one hand, the carriers of the compound heterozygous mutations were invariably affected with typical FPLD, on the other hand, the two reported carriers of the heterozygous p.Thr528Met variant variably presented with increased triglycerides, diabetes, hypertension, and acanthosis nigricans. Recently, the heterozygous p.Thr528Met LMNA variant was reported in two and five sisters affected by typical FPLD2. Western blot analyses showed the absence of prelamin A accumulation (Araújo-Vilar et al., 2021; Tu et al., 2016).

In our case series, none of the eight parents of the patients we report, all carriers of the heterozygous p.Thr528Met variant, presented with hypertension, diabetes, myopathy or muscular dystrophy, lipodystrophy or acanthosis nigricans, while their lipid profiles were not evaluated.

3. Finally, other missense variants affecting Threonine at position 528 have been shown to lead to the development of laminopathic phenotypes: the p.Thr528Lys (Bonne et al., 2000) as well as the p.Thr528Arg (Vytopil et al., 2003) heterozygous variants lead to typical striated muscle dystrophy phenotypes: Emery-Dreifuss muscular dystrophy/Limb-girdle muscular dystrophy (EDMD/LGMD) or dilated cardiomyopathy (DCM).

To our knowledge, we report for the first time the homozygous p.Thr528Met LMNA variant, which causes in four patients an astonishingly homogeneous and severe APS phenotype, characterized by severe muscular dystrophy, rigid spine, and major scoliosis.

Interestingly, the missense p.Thr528Met variant is localized in the C-terminal globular domain involved in lamin A/C interaction with both LAP2 α and emerin proteins (Dechat et al., 2000; Sakaki et al., 2001). This could explain the mislocalization of both proteins observed in some nuclei. Moreover, both LAP2 α and emerin variant are known to cause striated muscle disease (Bonne et al., 1999; Taylor et al., 2005). Their misdistribution in nuclei could thus explain at least in part the myopathic features observed in our patients. Additionally, both analyzed patient-derived primary fibroblasts additionally showed abnormal staining of H3K9me3, including low staining or abnormal aggregates in blebs, recalling the low staining pattern observed in HGPS patients (Columbaro et al., 2005). However to date, H3K9me3 staining has not been evaluated specifically in patients' muscle cells.

A study by Goldman and colleagues has reported novel insight into the pathophysiological significance of the characteristic nuclear blebs that are observed in patients affected with progeria or related disorders (Bercht Pflieger et al., 2015). They carried out an analysis of the structural and functional consequences of progeria-associated

nuclear blebs in dermal fibroblasts from a progeria patient carrying the p.Ser143Phe variant in *LMNA*. As observed in our patients, these blebs form microdomains that are devoid of major structural components of the nuclear envelope/lamina including in particular B-type lamins and are enriched in A-type lamins. Using laser capture microdissection and comparative genomic hybridization analyses, the authors showed that these domains were devoid of centromeric heterochromatin and gene-poor regions of chromosomes, while they were enriched in gene-rich chromosomal regions. These results suggested that the p.Ser143Phe variant, leading to both a myopathic and a progeroid phenotype, as previously mentioned, has a severe impact not only on the structure of the nuclear lamina but also on the organization of interphase chromatin domains and transcription. Compared to these findings, we observe in our patients' nuclear blebs rather an accumulation of a repressive histone mark: H3K9me3, suggesting that different *LMNA* missense variants may lead to different epigenetic modifications and correlated gene expression patterns, contributing to the observed phenotypic diversity in laminopathies.

In this respect, it would be interesting to compare nuclear abnormalities, aberrant protein stainings, and gene expression patterns, in patients presenting with myopathic features associated or not with premature aging features due to alterations in this lamin A/C region.

In addition, in the three-dimensional structure context of the C-terminal Ig-like domain (411–553) of A-type lamins (Dhe-Paganon et al., 2002; Krimm et al., 2002), residues Arg471 (Cao & Hegele, 2003; Zirn et al., 2008), Arg527 (Lombardi et al., 2007; Novelli et al., 2002), Thr528 (this report), Met540 (Verstraeten et al., 2006), and Lys542 (Plasilova et al., 2004) define a recessive progeroid syndrome hot spot mutation, unrelated to farnesylated prelamin A precursor accumulation (Barthélémy et al., 2015; Verstraeten et al., 2006).

Taken together, we present four patients harboring the same homozygous *LMNA* variant who present with an extremely homogeneous phenotype at the clinical and cellular level, as evidenced on two patients' fibroblast cell lines. The pathophysiology of APS, compared to classical HGPS, is still largely unexplored and no therapeutic target has been identified yet: to date, healthcare for patients only relies on symptomatic treatments. The scientific literature may be confusing since patients affected with MAD or APS have also been classified as HGPS or progeria-like (Liang et al., 2009; Plasilova et al., 2004; Verstraeten et al., 2006), which, instead, in our opinion, is a definition that should be restricted to the classical disease (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003) or to closely related diseases (HGPS-like diseases) due to the accumulation of progerin or other prelamin A toxic isoforms (Barthélémy et al., 2015). Our study confirms that progeroid features can occur without progerin or prelamin A accumulation in APS and adds to the growing phenotypic spectrum of overlapping and variably severe laminopathies, with a highly specific genotype/phenotype relationship for the homozygous p.-The528Met *LMNA* variant, extrapolated from the four cases we report. Furthermore, this study highlights possible molecular anomalies involved in the disease pathophysiology, hopefully further assisting the identification of therapeutic targets in atypical progeria syndromes.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Annachiara De Sandre-Giovannoli, Patrice Roll, Claire Navarro, Nicolas Lévy; Performed the clinical evaluations: Abdelkrim Saadi, Arnold Munnich, Malika Chaouch, Ozge Ozalp, Charles Marques Lourenco, Athmane Chaouch, Meryem Benahmed, Lamia Ali Pacha; Performed the molecular analyses: Annachiara De Sandre-Giovannoli, Christian Kubisch, Davor Lessel; Performed the functional experiments on fibroblast cell lines: Patrice Roll, Claire Navarro, Racha Fayek, Nathalie Da Silva; Analyzed the data: Annachiara De Sandre-Giovannoli, Claire Navarro, Patrice Roll, Nicolas Lévy, Abdelkrim Saadi; Wrote the manuscript: Annachiara De Sandre-Giovannoli, Abdelkrim Saadi, Claire Navarro, Patrice Roll; Critically reviewed the manuscript: Annachiara De Sandre-Giovannoli, Nicolas Lévy, Arnold Munnich, Malika Chaouch, Christian Kubisch, Davor Lessel.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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