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Repeat Expansions in *PLIN4* Cause Autosomal Dominant Vacuolar Myopathy With Sarcolemmal Features

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ABSTRACT

Objective: We aim to describe and characterize two unrelated Spanish families suffering from an autosomal dominant autophagic vacuolar myopathy caused by repeat expansions in *PLIN4*.

Methods: We evaluated the clinical phenotype and muscle imaging, and performed a genetic workup that included exome sequencing, muscle RNAseq, and long-read genome sequencing. Muscle pathology was assessed by means of histochemistry, electron microscopy, *PLIN4*, p62, LC3, and NBR1 immunofluorescence and/or western blotting. Detailed characterization of autophagic vacuoles was performed.

Results: Patients presented around the age of 30 with mild proximal weakness followed by prominent distal weakness in lower legs, eventually spreading to other muscle groups. Muscle biopsies showed unique pathological features characterized by numerous rimmed vacuoles that displayed sarcolemmal features and were located beneath the sarcolemma and within the cytoplasm. Ultrastructural studies showed autophagic vacuoles, replications, and loops of the basal lamina and tubulofilamentous sarcolemmal inclusions. p62 and NBR1 co-localized with *PLIN4* at the sarcolemma and vacuoles. LC3 immunoreactivity and other

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lysosomal markers were increased at the vacuoles. Targeted long-read sequencing of *PLIN4* in affected individuals revealed a single expanded allele of 39×99 bp repeats in family 1 and of 37×99 bp repeats in family 2.

Interpretation: We characterize two new families suffering from an autosomal dominant myopathy carrying repeat expansions in *PLIN4*. Subsarcolemmal p62 expression is a powerful although nonspecific marker of this disease. No correlation between the size of the expansion and clinical severity can be clearly established. *PLIN4* expansions should be considered in the diagnosis of autosomal dominant vacuolar myopathies, especially when sarcolemmal features are present.

1 | Introduction

Distal myopathies are a group of inherited muscle disorders clinically characterized by progressive muscular weakness and atrophy initially involving the hands, forearm, lower legs, or feet. These highly heterogeneous conditions are classified on the basis of age at onset, distribution of muscle weakness, histopathological features, and mode of inheritance. Autosomal dominant, autosomal recessive, and rare X-linked forms have been reported [1]. The first description of a distal myopathy was by Lisa Welander in 1914 in a cohort of Swedish and Finnish patients affected by adult-onset, distal upper-limb weakness and wasting slowly progressing over time, mainly affecting finger extensor muscles. The causative gene, named *TIA1*, was not identified until 2013 [2]. Since the description by Welander, more than 29 different forms of distal myopathy have been identified, and the number of genetic distal myopathies is expected to grow with the use of next-generation sequencing technologies [3].

In 2004, Di Blasi et al. described a large Italian kindred affected by an autosomal dominant distal myopathy with rimmed vacuoles displaying a characteristic expression of ubiquitin at the cell surface and lysosomal markers in the cytoplasm [4]. By linkage analysis, the disease was initially mapped to a locus in 19p13.3 with a LOD score of > 3. Despite an exhaustive genetic workup, the causative gene in this family remained unsolved for decades. In 2020, by using a multiomic approach combining laser microdissection and mass spectrometry of vacuolated compared to nonvacuolated myofibers, Ruggieri and colleagues identified increased abundance of perilipin 4 at the site of the vacuoles and subsarcolemmal regions of patients' muscle biopsies. DNA analysis of the gene *PLIN4* using long-read sequencing (Oxford Nanopore Technology, ONT) revealed a heterozygous expansion in exon 3 of 40 repeats of the well-conserved 99-nucleotide or 99-mer repeat sequence (40×99 bp) encoding the amphipathic domain of perilipin 4, compared to the normal 31×99 bp sequence present in healthy subjects [5].

More recently, other groups in China described three additional families and one sporadic case with an autosomal dominant vacuolar myopathy that harbor the same or other 99-mer repeat expansions (40×, 50× and 39×99bp, respectively) in *PLIN4*, presenting with proximal rather than distal weakness [6, 7]. In all cases, muscle pathology was distinctive, showing autophagy and ubiquitinated-protein markers (p62, FK2, NBR1) in the subsarcolemmal area and at the site of the vacuoles, which perfectly co-localized with perilipin 4, especially in type I myofibers. Additional immunohistochemical studies performed by Wang et al. described diffuse MHC-I expression and membrane attack

complex (MAC) deposition in isolated myofibers of three available biopsies. Interestingly, despite perilipin 4 being known to localize to lipid droplets (LD), all the reports of muscle pathology in *PLIN4* myopathy do not show intracellular lipid deposition [5–7].

Perilipins are a family of proteins that coat the surface of LD and are tissue-specifically expressed. *PLIN4* is known to localize to LD in human and yeast cells, and endogenous *PLIN4* has been detected mainly, but not exclusively, in adipocytes at the cell periphery and LD [8–10]. The 99-mer sequence expanded in the disease is highly conserved, and the encoded amphipathic helix or domain of perilipin 4 is exceptional in terms of its length and repetitiveness compared to other perilipins. Its main role in adipose tissue is storing triacylglycerol and controlling lipolysis by responding to hormonal signals [9, 11, 12]. In human skeletal muscle, the most highly expressed perilipin is *PLIN4*, which mainly localizes to the subsarcolemmal region of type 1 myofibers corresponding to subsarcolemmal LD binding [13].

Here, we aim to report and characterize in detail the clinical, pathological, and genetic features of two unrelated Spanish families suffering from an autosomal dominant myopathy carrying different 99-mer repeat expansions in *PLIN4*. In addition, we performed a literature review of all the published families to date. After a deep muscle pathology evaluation, we characterize the disease as a member of autophagic vacuolar myopathies with sarcolemmal features (AVSF).

2 | Methods

2.1 | Patients

We studied three patients from two unrelated Spanish families suffering from an autosomal dominant predominantly distal myopathy with rimmed vacuoles. We performed pedigree analysis (Figure 1), neurological examination including muscle strength evaluation according to the Medical Research Council (MRC) grading scale, serum creatine kinase (CK) levels, cardiac evaluation including EKG, 24-h Holter monitoring and cardiac ultrasound, respiratory function tests, sensory and motor nerve conduction studies and electromyography, and genetic workup in the probands of both families and additional family members. Muscle biopsy was obtained from individuals F1 III:4 and F2 IV:1. Clinical data from seven additional individuals (F1 I:2, II:3, II:4; and F2 II:1, II:4, II:5, and III:5) were retrospectively collected from medical reports or from information obtained from their relatives. The study was approved by the ethics committees of the participating institutions. Sample collection was performed after written

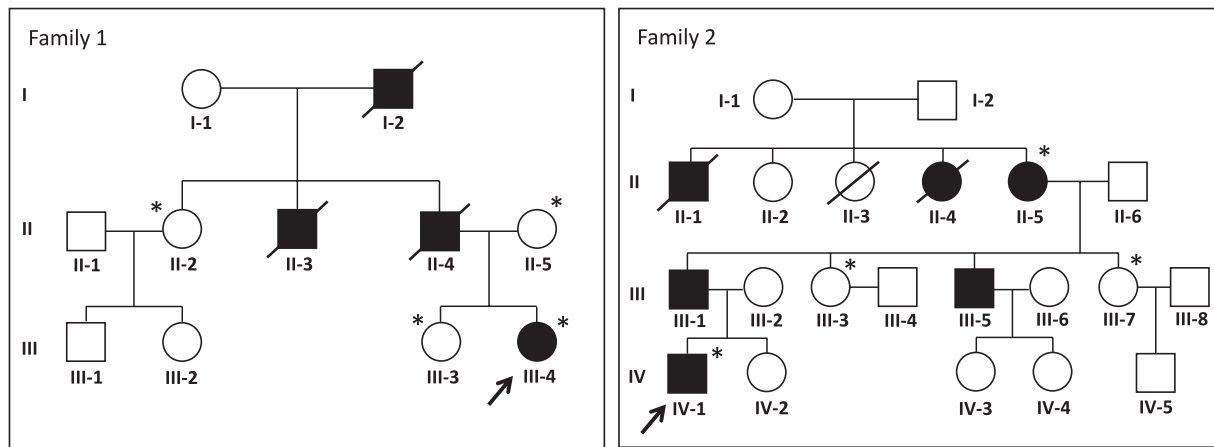


FIGURE 1 | Pedigree of Family 1 and 2. Affected individuals are shown in black. Asterisks indicate individuals that have been genetically tested.

informed consent from the patients according to the declaration of Helsinki.

2.2 | Muscle MRI Studies

Whole-body muscle MRI was performed in individuals F1 III:4 and F2 IV:1 (4 and 7 years after disease onset, respectively). In addition, pelvic and lower-limb muscle MRI was performed in individual F2 III:1. T1-weighted (T1W) and short-tau inversion recovery (STIR) sequences were recorded.

2.3 | Muscle Biopsy

Muscle biopsies were obtained after written informed consent from the biceps brachii from individual F1 III:4 and from the quadriceps muscle from individuals F2 III:1 and F2 IV:1. The samples were immediately frozen in liquid nitrogen-chilled isopentane. Cryostat sections 8- μ m thick were processed for routine histological and histochemical techniques and for acid phosphatase and acetylcholinesterase (AChE) activity, as well as for Alizarin red, the latter used to detect calcium deposition. For comparative purposes, samples from two patients affected by autophagic vacuolar myopathies (one patient suffering from late onset Pompe disease, and one patient affected from colchicine myopathy) and a healthy control were processed in parallel.

2.4 | Immunohistochemistry

For immunohistochemical analysis, 6- μ m thick cryostat sections were fixed with acetone for 5 min, washed with PBS, and incubated with a blocking solution (UltraCruz Blocking Reagent, Santa Cruz Biotech, Dallas, TX, USA) for 30 min and then incubated with antibodies against dystrophin (Leica Biosystems, Nussloch, Germany), caveolin-3 (BD Bioscience, Franklin Lakes, NJ, USA), p62 (Enzo Biochem, Farmingdale, NY, USA), laminin α 2 (Bio-Rad, Hercules, CA, USA), lysosomal-associated membrane protein-2 (LAMP-2) (Abcam, Cambridge, UK), MAC (C5b-9) (Dako, Glostrup, Denmark), and MHC class I (Dako, Glostrup, Denmark). After washing, the sections were processed with a DAB substrate kit, peroxidase (Vector Laboratories,

Newark, CA, USA). Antibodies used in the present study are summarized in Table S1.

2.5 | Single and Double-Labeling Immunofluorescence and Confocal Microscopy

Serial cryostat 6- μ m thick sections were fixed in acetone, and double-label immunofluorescence was performed as previously described [14]. Briefly, after incubation with a blocking solution, the sections were incubated overnight at 4°C with a combination of two primary antibodies as follows: (a) guinea pig anti-PLIN4 antibody (Abcam, Cambridge, UK) and rabbit anti-p62 antibody; (b) guinea pig anti-PLIN4 antibody and mouse anti-NBR1 (Santa Cruz Biotech, Dallas, TX, USA). In addition, single LC3 (Cell Signalling, Danvers, MA, USA) immunofluorescence was performed. After washing, the sections were incubated with Alexa 488 goat anti-mouse and Alexa 594 (Invitrogen, Waltham, MA, USA) anti-guinea pig (Life Technologies, Waltham, MA, USA). The sections were mounted in a medium containing polyvinyl alcohol (Fluoprep, BioMerieux, France), sealed, and dried overnight at 4°C. Sections were examined with a Leica TCS-SL confocal microscope. Sections incubated only with the secondary antibodies were used as controls.

2.6 | Electron Microscopy

A small sample of muscle was fixed in 2% glutaraldehyde, post fixed with 1% osmium tetroxide, and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with a JEOL 1011 electron microscope.

2.7 | Western Blot (WB)

Briefly, frozen muscle samples were homogenized with 19 volumes of treatment buffer containing 0.125 mol/L Tris/HCL buffer pH 6.4, 10% glycerol, 4% SDS, 4 mol/L urea, 10% mercaptoethanol, and 0.001% bromophenol blue. 30 mL of samples were loaded in the gel (equivalent to 200 mg of noncollagen protein). WB was performed using a guinea pig monoclonal antibody to PLIN4 (Origene Technologies Inc., Rockville, MD, USA).

IRDyeH800 conjugate goat anti-guinea pig was used as a secondary antibody (Li-cor, Lincoln, NE, USA). Fluorescent bands corresponding to *PLIN4* were acquired and analyzed using an Odyssey Infrared Imaging System (Li-cor). Myosin present in the gel after transfer was stained with Gel code (ThermoFisher) and used as a loading control.

2.8 | Exome Sequencing

DNA was extracted from peripheral blood and whole exome sequencing (WES) was performed in the proband from family 1 (F1 III:4). Briefly, DNA library was prepared using Kapa reagents (Roche, including HyperExome probes targeting the whole exome) and sequenced in a NextSeq500 (Illumina, CA, USA). Fastq files were analyzed using standard procedures: BWA alignment to GRCh37/hg19 [15], GATK variant calling [15] and ExomeDepth for copy number variants (CNVs), and variant annotation using ANNOVAR [16]. Data filtering to obtain candidate variants included assessment of population frequencies (<0.05 MAF in gnomAD v2.1.1) and impact on the protein. Variants were classified according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guidelines [17]. *PLIN4* expansion was visually assessed with Integrative Genomics Viewer among other samples sequenced within the same batch.

2.9 | Long-Read Sequencing

Genomic DNA was extracted from patient blood samples using QIAasympphony DNA Kit (QIAGEN). Deidentified samples were transferred to the Garvan Institute of Medical Research for long-read ONT sequencing and analysis. Prior to ONT library preparation, the DNA was sheared to ~20 kb fragment size using Covaris G-tubes and visualized, post-shearing, on an Agilent TapeStation. Sequencing libraries were prepared from ~1.5 to 5 µg of HMW DNA, using native library prep kits (SQK-LSK109 or SQK-LSK110), according to the manufacturer's instructions. For whole-genome sequencing runs, each library was loaded onto a FLO-PRO002 (R9.4.1) flow cell and run on an ONT PromethION device. For targeted sequencing runs, each library was loaded onto a FLO-MIN106D (R9.4.1) flow cell and run on an ONT MinION device with live target selection/rejection executed by the *ReadFish* software package [18]. Detailed descriptions of software and hardware configurations used for ReadFish/ReadUntil experiments are provided in our recent publication that demonstrates the suitability of this approach for profiling tandem repeats [19]. Samples were run for a maximum duration of 72 h, with nuclease flushes and library reloading performed at approximately 24- and 48-h timepoints for targeted sequencing runs to maximize sequencing yield.

2.10 | Bioinformatics Analysis

Raw ONT sequencing data was converted and archived in SLOW5 format using *slow5tools* (v0.3.0) [20]. Data were base-called using *Guppy* (4.4.1) and reads with mean quality <7 were excluded from further analysis. Resulting FASTQ files were aligned to the hg38 reference genome using *minimap2*

(v2.14-r883) [21]. Alignments to the *PLIN4* locus were phased into maternal and paternal haplotypes using *Longshot* (v0.4.1) [22], converted back to FASTQ format, and assembled separately *de novo* using *Flye* (v2.8.1-b1676) [23], and sequence-polished using *Racon* (v1.4.0) [24], yielding two contigs representing the two *PLIN4* haplotypes in a given individual. *Tandem Repeat Finder* (4.09) [25] was used to identify and measure the $n \times 99$ bp tandem repeat sequence within *PLIN4* exon 5 [note: this is the fifth annotated exon, and the fourth coding exon in the *PLIN4* canonical transcript ENST00000301286.5 (NM_001367868.2), and is referred to as exon 3 by Ruggieri et al. [5]]. In control populations, the “normal” allele contains $29 \times$ or 31×99 bp repeat motifs mapped to the human reference genome (hg38) [5, 26].

2.11 | RNA Sequencing

Total RNA was extracted from 30 mg of muscle biopsy from individual F1 III:4 using the Animal Tissue RNA Purification Kit (Norgen Biotek, Canada) according to the manufacturer's recommendations. RNA integrity was assessed with the Agilent RNA 6000 Nano kit (Agilent Technologies, CA, USA), and only samples with RIN >7 were subsequently processed. Libraries were prepared using Illumina Stranded Total RNA Prep with Ribo-Zero Plus (Illumina, CA, USA) and sequenced on a NovaSeq 6000 (Illumina, CA, USA) (2×150 bp) with an intended 100 million reads per sample. FastQC [27] and Fastp [28] were used on raw fastq data, and RNAseq reads were then mapped to GRCh37/hg19 with HISAT2 [29]. Gene expression data was analyzed using OUTRIDER [30], FRASER [31] and FRASER 2.0 [32] within a cohort of 96 muscle biopsies, including healthy controls and patients with other muscular diseases.

3 | Results

3.1 | Clinical Features

3.1.1 | Family 1

The proband of Family 1 (F1 III:4, Figure 1) was a 41-year-old woman born from nonconsanguineous parents. She met her early motor developmental milestones at an appropriate age. During her childhood, she wore special footwear due to her high-arched feet; otherwise, neurological examination at 8 years of age revealed no additional abnormalities. At the age of 37, she first noticed difficulties climbing upstairs and rising up from the floor. Very soon afterwards, she noticed weakness in her hands and finger extensors. These symptoms slowly progressed during the following years.

Her father (F1 II:4) presented at the age of 28 with difficulties climbing stairs and rising his arms. His muscle weakness rapidly progressed to involve proximal and distal muscles of all limbs, and 4 years later, he was no longer able to walk and required a wheelchair. Based on the results of the EMG examination, he was misdiagnosed with distal spinal muscular atrophy and died at age 39 due to a viral infection. The paternal uncle (F1 II:3) and grandfather (F1 I:2) of the proband were also affected by a progressive muscle weakness, and they both died at early ages, 41 and 39 years old respectively. No evidence of cardiac or

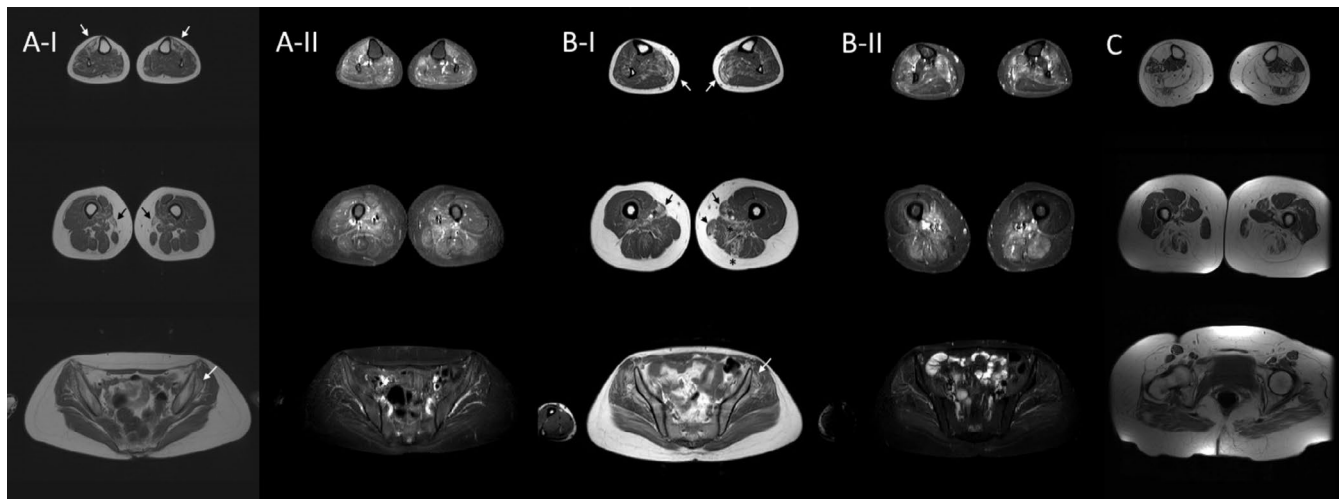


FIGURE 2 | Lower-limb and pelvic MRI from three patients (F1 III:4, F2 IV:1 and F2 III:1). A-I, B-I and C show T1-weighted images from each patient. A-II and B-II show STIR images from individuals F1 III:4 and F2 IV:1. While patient F1 III:4 (A-I) had almost exclusive fatty infiltration of tibialis anterior muscles (arrows) and mild involvement in the thighs and pelvis, the other patients from F2 had a predominant involvement of the posterior leg compartment, initially affecting gastrocnemius medialis (arrows in B-I) and soleus muscles, and mild to severe infiltration of hamstrings and adductors muscles with preserved quadriceps muscles. Arrows in thigh images show selective involvement of sartorius muscles in two of the patients. Note in B-I additional involvement of left gracilis muscle (arrowhead), adductor major and semitendinosus muscles (asterisks). In pelvic images, note early involvement of gluteus minimus muscles in the probands (white arrows in A-I and B-I). STIR sequences showed diffuse muscle edema in legs and thighs.

respiratory involvement could be found in their respective clinical records.

On the proband's first evaluation, her muscle strength according to MRC was 4/5 in elbow flexion, 2/5 in wrist and fingers extension, 4/5 in fingers abduction and adduction, 4/5 in hip flexion, 4/5 in leg extension, 3/5 in foot dorsiflexion, and 4+/5 in foot plantar flexion. The rest of the muscle groups were preserved. Deep tendon reflexes were abolished, except for the tricipital, which was preserved. Atrophy was noted on leg and forearm muscles, both in the anterior and posterior compartments. Sensation in all its modalities was intact.

Serum CK levels were normal. Sensory and motor nerve conduction studies showed normal values. Needle electromyography revealed abundant spontaneous activity at rest and a mixed neurogenic-myogenic pattern. Whole-body muscle MRI revealed very mild atrophy of paravertebral muscles, mild atrophy of glutei minimi muscles, and severe atrophy of sartorius muscles bilaterally with relative sparing of the rest of the thigh muscles. At the mid-leg level, there was selective involvement of the anterior compartment and very mild atrophy of the soleus and medial gastrocnemius. STIR sequences revealed diffuse edema in thigh and leg muscles (Figure 2A). Complete cardiac evaluation including EKG, 24-h Holter monitoring, cardiac ultrasound, and cardiac MRI revealed no abnormalities. Respiratory function tests including FVC in sitting and supine position and polysomnography were normal.

3.1.2 | Family 2

The proband (F2 IV:1, Figure 1) was a 27-year-old male at the time of first evaluation. He had a normal motor development. He reported his first symptoms at the age of 24, at which time

he was a handball elite athlete. He started noticing that he got tired more easily than before, and mild weakness appeared when performing low-intensity exercise. The neurological exam at first examination showed minimal right-winged scapula with muscle strength 5–/5 in left hip flexion, 4+/5 in left hip abduction, and 4/5 in left knee flexion. Strength was 5/5 in the rest of the muscle groups, and the remaining exam did not reveal additional findings.

At last follow-up visit at 31 years of age, examination showed strength 4/5 in bilateral shoulder abduction and arm external rotation, 4/5 in finger extension, 3/5 in right and 2/5 in left hip flexion, 4/5 in hip extension, and 4/5 in left knee flexion. The rest of the muscle groups had normal strength. He had developed bilateral asymmetric scapular winging, atrophy in pectoralis and both quadriceps muscles. Deep tendon reflexes were globally diminished. He had elevated serum CK levels up to 1200 IU/L. Muscle MRI in the pelvic girdle showed moderate fatty infiltration in glutei minimi muscles, which were more affected than glutei medii. At the thigh level, there was mild to moderate atrophy of hamstrings and adductors muscles, left gracilis and both sartorii. Leg imaging showed moderate atrophy of posterior leg muscles, more prominent in medial gastrocnemii. Increased STIR signal was observed in hamstrings, hip adductors, and vastus medialis muscles, while at the leg level hyperintensity was present in tibialis anterior, medial gastrocnemii, and solei muscles bilaterally (Figure 2B).

Family history revealed muscle weakness in five additional family members spanning a total of three generations and following an autosomal dominant inheritance pattern (Figure 1).

The proband's father (F2 III:1) was 55 years old when first evaluated. He presented with a 20-year history of progressive weakness initially affecting his distal lower extremity muscles, later

also involving his upper extremities and respiratory muscles. He was on nocturnal ventilatory support 10 years after symptom onset. He had been wheelchair-bound since 42 years of age and at the time of his last evaluation was only able to perform slight movements with his fingers. He did not present ptosis or ophthalmoparesis, nor facial weakness. His muscle strength was 4/5 in neck flexors and extensors, 2/5 in finger flexors and extensors, and 0/5 in the rest of the muscle groups. Muscle tendon reflexes were globally abolished. Contractures were noted in both hands. T1-weighted muscle MRI images showed severe fatty infiltration of pelvic muscles and the posterior compartment of his thighs. Unlike in his son and patient F1 III:4, sartorius muscles were relatively spared. There was also severe symmetric involvement of leg muscles, with exclusive sparing of tibialis posterior, peronei muscles, and lateral gastrocnemii (Figure 2C). A cardiological assessment was performed with echocardiography and 24-h Holter monitoring, both without abnormal findings.

The proband's grandmother (F2 II:5) developed distal upper-limb weakness at 48 years of age and was ambulant until the 8th decade, at which time she also required ventilatory support. In the same generation, a brother and a sister were also affected (II:1, II:4) with a mild late-onset distal weakness and were still ambulant until their deaths around the ages of 78 and 85, respectively. The last affected family member was an uncle of the proband (F2 III:5), who presented at 27 years of age with fatigue and proximal upper-limb weakness. He was wheelchair-bound since the age of 35 and also required ventilatory support.

A summary of clinical data of the patients here described and the previously reported families is provided in Table 1.

3.2 | Myopathological Findings

The muscle biopsies from the three studied patients were highly similar and showed increased variability in myofibre size, increased number of internal nuclei, and the presence of numerous rimmed vacuoles in a high proportion of myofibers, particularly of type I. The vacuoles were often but not always located at the periphery of the myofibre (Figure 3A–D). There was mild endomysial tissue proliferation. Necrosis or myophagocytosis was not observed. Oxidative activity was absent at the site of the vacuoles and in central myofibre regions as observed with NADH, SDH, and COX reactions (Figure 3E,F). The vacuoles showed prominent acid phosphatase (Figure 3G) and acetylcholinesterase activity (Figure 3H). Alizarin red stained strongly positive in the subsarcolemmal region of some muscle fibers demonstrating focal calcium deposition (Figure 3I). Immunohistochemistry for sarcolemmal proteins including dystrophin (Figure 4A), laminin α 2 (Figure S1), and caveolin-3 showed the vacuoles are surrounded by membrane. Interestingly, the sarcolemma of many fibers appeared irregularly thickened due to the particular subsarcolemmic localization of many vacuoles. p62 was robustly expressed at the myofibre membrane and at the site of the vacuoles (Figure 4B). Moreover, LAMP-2 (Figure 4C) and LC3 (Figure 4D) immunoreactivity was increased at the site of the vacuoles. MHC class I showed variable expression at the sarcolemma and within the cytoplasm of myofibers (Figure 4E), whereas membrane MAC deposition was seen in scattered fibers (Figure 4F). Double labeling immunofluorescence and confocal

microscopy revealed increased PLIN4 co-localizing with p62 at the sarcolemma and within the cytoplasm in the majority of myofibers. Moreover, PLIN4 co-localized with the autophagy-related protein NBR1 (Figure 5).

Comparison of histochemical and immunohistochemical characteristics of PLIN4 myopathy with other autophagic vacuolar myopathies revealed very similar features compared to X-linked myopathy with excessive autophagy and colchicine myopathy (Table S2).

3.3 | Ultrastructural Analysis Revealed Membrane-Bounded Autophagic Vacuoles and Redundant Basal Lamina

Ultrastructural analysis showed abundant subsarcolemmal and cytoplasmic autophagic vacuoles containing myelin bodies, degraded organelles, and electron-dense granular material (Figure 6A,B). Large numbers of vacuoles were membrane-limited and often appeared abutting the extracellular space (Figure 6C,D). The basal lamina formed multiple layers and loops in the extracellular space (arrows in A and D). Collections of tubulo-filaments of 18 nm were seen within the sarcoplasm (asterisks in Figure 6D,E). Proliferation of sarcoplasmic reticulum was observed in some myofibers (Figure 6F).

3.4 | Western Blot (WB) Analysis Revealed Increased PLIN4 Protein Expression

WB analysis of PLIN4 was performed in the proband from Family 1 (F1 III:4) and individual F2 IV:1, and in two healthy controls. WB from both patients showed a normal band of 134 kDa and an extra band of higher molecular weight (Figure 7A) corresponding to protein produced from the expanded allele.

3.5 | Whole Exome Sequencing and RNA Sequencing

Alignment of the WES from patient F1 III:4 revealed an increase in coverage of exon 5 of *PLIN4* in the patient's DNA. This was not observed in any other sample sequenced in the same batch (Figure S2), suggesting the presence of an expanded allele in the patient. RNAseq data from the muscle sample revealed normal mRNA expression of *PLIN4* compared to a cohort of 96 muscle samples (data not shown), suggesting that the expanded allele is not overexpressed. Moreover, we did not observe any effect on *PLIN4* mRNA splicing.

3.6 | Identification of a *PLIN4* Expanded Tandem Repeat in Affected Individuals After Long-Read Sequencing

We performed either whole genome or targeted ONT sequencing to genotype *PLIN4* in three affected and five unaffected individuals from the two families (Figure 7B,C, Figure S3). In each case, alignments to *PLIN4* were phased into maternal and paternal haplotypes, which were then separately assembled.

TABLE 1 | Summary of clinical data of the patients here described and the previously reported families. Asterisks indicate the index patients from Family 1 (F1) and 2 (F2). The size of the *PLIN4* expansion in our affected cases is reported in the genetically tested individuals.

Patient	Gender	Age of onset/ age at last examination		Age at death	Initial symptoms	Weakness progression	Scapular winging	Loss of ambulation at age		CK levels	Respiratory failure	EMG	Size of 99bp expansion in <i>PLIN4</i> (n × 99bp)
								Ambulant					
F1, I:2	M	uk	39		Distal LL and UP weakness	Proximal and distal 4 limbs, and axial	No	No	uk	Normal	uk		
F1, II:3	M	uk	41		Distal LL and UP weakness	Proximal and distal 4 limbs, and axial	No	No	uk	uk	uk		
F1, II:4	M	28/uk	39		Proximal LL and UL weakness	Distal > proximal 4 limbs, and axial	No	No	32	uk	uk	Mixed myogenic- neurogenic features with spontaneous activity at rest	
F1, III:4 *	F	37/42	Alive		Proximal LL weakness	Distal > proximal 4 limbs	No	Yes	No	Normal	No	Mixed myogenic- neurogenic with spontaneous activity	39×
F2, II:1	M	6th decade/uk	78		UL weakness	Proximal and distal 4 limbs	uk	Yes (with a cane)	No	Normal	No	Myogenic features	
F2, II:4	F	uk	85		uk	Proximal and distal 4 limbs	uk	Yes (with a cane)	No	uk	No		
F2, II:5	F	48/80	Alive		Asymmetric distal UL weakness	Proximal and distal 4 limbs, and axial	No	No	74	Normal	Yes, VS at age 74. Age 73: FVC 79% sitting, 66% supine	Mixed myogenic- neurogenic features with spontaneous activity	37×
F2, III:1	M	31/59	Alive		Distal anterior LL weakness	Proximal and distal 4 limbs, and axial	No	No	42	Normal	Yes, VS at age 49. Age 49: FVC 43%, FEV1 68%, FEV1/FVC 67%, MVV 38%	Mixed features	

(Continues)

TABLE 1 | (Continued)

Patient	Gender	Age of onset/ age at last examination	Age at death	Initial symptoms	Weakness progression	Scapular winging	Ambulant	Loss of ambulation at age	CK levels	Respiratory failure	EMG	Size of 99bp expansion in <i>PLIN4</i> (n × 99bp)
F2, III:5	M	27/58	Alive	Proximal UL weakness	Proximal and distal 4 limbs, and axial	No	No	35	740 U/L	Yes, VS at age 48. Age 53: FVC 12%, FEV1 14%, FEV1/FVC 92%	Myogenic with spontaneous activity, abundant CRD	
F2, IV:1*	M	24/32	Alive	Fatigue, proximal LL weakness in climbing stairs	Proximal and distal 4 limbs	Yes, asymmetric	Yes	No	x5 (1260 U/L)	Yes, mild. Age 30: FVC 102% (–3% supine), FEV1 111%, FEV1/ FVC 88%. MIP 34%, MEP 34%		37×
L. Maggi, 2023/A. Ruggieri, 2020/C. Di Blasi, 2004 (F1: 22pt)	13M, 9F	Range: 30–66/39–73	65 to 94	Distal LL > UL weakness mainly in foot dorsiflexors and hand extensors (*Only 2/14 patients with proximal weakness at onset)	60% proximal weakness at progression Neck flexors weakness (6/13) 40% wheelchair-bound at 17 years from onset Ankle contractures at end stage (7/15)	Scapular weakness without scapular swinging (at least four reported)	Some	58–85	Normal or slightly↑ (x2) (except patient IV:22 with CK 988 U/L)	Only 2 pt. III:20 invasive VS (tracheostomy) at 71 years, and IV:21 low FVC (Other patients had a “respiratory” cause of death at 65–94 years)	Myogenic with spontaneous activity at rest +CRD (also in 2 asymptomatic pt)	40×
K. Yang, 2022 (F1: 13pt)	8M, 5F	45–65/49–82	uk	Proximal LL weakness	Slow progression of proximal LL > UL weakness Mild distal weakness (finger extensors in UL)	uk	All except III	80 (III)	Normal or slightly↑ (max. 668 U/L)	No	Myogenic with spontaneous activity at rest +CRD/ myotonic discharges (also in 3 asymptomatic pt)	40×

(Continues)

TABLE 1 | (Continued)

Patient	Gender	Age of onset/ age at last examination	Age at death	Initial symptoms	Weakness progression	Scapular winging	Ambulant	Loss of ambulation at age	CK levels	Respiratory failure	EMG	Size of 99bp expansion in <i>PLIN4</i> (<i>n</i> × 99bp)
K.Yang, 2022 (F2: 6pt)	3M, 3F	25–26/41 and 35	uk	Proximal LL weakness	Earlier onset, greater and faster decline than in F1. Moderate distal weakness over time (finger extensors, foot dorsiflexors)	uk	Some	39 (III4)	378–406 U/L	No	Myogenic	50×
Q.Wang, 2022 (F1: 10 pt)	10M, 0F	Range: 40s to 60s/uk	All alive	Proximal LL weakness	Proximal > Distal LL > UL	Some (at least 1 reported: II7)	Some	uk	267– 822 U/L	No	Myogenic CRD (even 1 asymptomatic pt)	39×
Q.Wang, 2022 (sporadic case)	M	49/60	All alive	Weakness in climbing upstairs	Proximal > DistalLower > Upper limbsNeck flexion weakness	No	No	uk	256 U/L	Yes (VS unknown)	Myogenic CRD	39×

Abbreviations: CK, creatine kinase; CRD, complex repetitive discharges; EMG, electromyography; F, female; Fn, family; FVC, forced vital capacity; LL, lower limbs; M, male; MEP, maximal expiratory pressure; MIP, maximal inspiratory pressure; MVV, maximal voluntary ventilation; pt, patients; uk, unknown; UL, upper limbs; VS, ventilatory support.

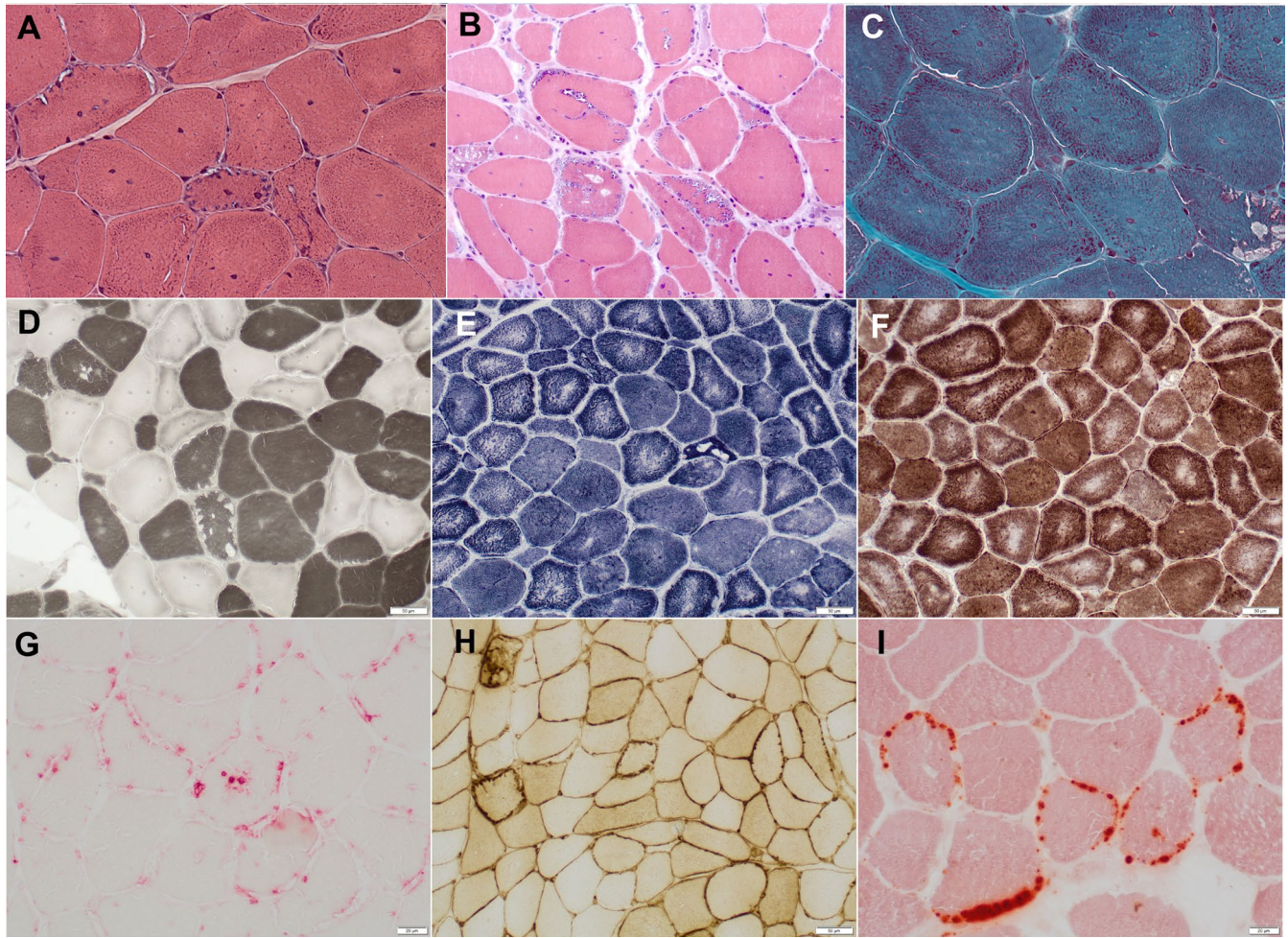


FIGURE 3 | Biopsy findings from family probands show abundant subsarcolemmal and sarcoplasmic autophagic vacuoles, and focal calcium deposition. Hematoxylin–eosin (A, B) and modified Gomori stains (C) from patients F1 III:4 and F2 IV:1 show increased number of internal nuclei, variable increase of endomysial connective tissue, fiber splitting, and presence of abundant cytoplasmic rimmed vacuoles preferentially located at the cell periphery (subsarcolemmal). (D) ATPase at pH 4,3 shows absence of ATPase activity at the sites occupied by the vacuoles. Scale bar: 50 μ m. (E, F) NADH and COX reactions show many central areas devoid of oxidative activity. Scale bars: 50 μ m. (G) Acid phosphatase activity is increased at the site of the vacuoles. Scale bar: 20 μ m. (H) Cholinesterase histochemistry showing a fiber with increased activity at the site of cytoplasmic vacuoles, and several fibers with increased activity at the periphery, some of them showing an irregular lining due to the presence of subsarcolemmal vacuoles. Scale bar: 50 μ m. (I) Focal calcium deposition at the periphery of some myofibers is observed with Alizarin red stain. Scale bar: 20 μ m.

Unaffected individuals in both families harbored two copies of a 29 $\times$ 99 bp tandem repeat within *PLIN4* exon 5 (Figure 7B,C), which encodes the wildtype protein's amphipathic domain. Affected individuals in both families were distinguished by large expansions within the *PLIN4* tandem repeat (Figure 7C). The proband in Family 1 harbored a single expanded allele of 39 $\times$ 99 bp repeat copies in addition to a single “normal” allele of 29 $\times$ 99 bp copies (Figure 7C). The two affected individuals sequenced from Family 2 (II-5 and IV-1) each harbored a single expanded allele of 37 $\times$ 99 bp repeat copies (Figure 7C), indicating that this expanded allele was stably inherited through three generations. The observed repeat expansions encode additional 330 and 264 amino acids, respectively, within the *PLIN4* amphipathic domain, compared to “normal” alleles. The observed repeat lengths in affected individuals were similar, but not identical, to the family reported by Ruggieri et al. [5], in which affected individuals harbored *PLIN4* tandem repeats 40 $\times$ 99 bp in length. After the publication by Wang et al. of a family carrying a 39 $\times$ 99 bp repeat expansion [7], the expanded allele of 37 $\times$ 99

bp copies from Family 2 is here newly reported to cause skeletal myopathy.

4 | Discussion

We report herein two families suffering from an autosomal-dominant vacuolar myopathy with predominantly distal involvement, caused by monoallelic repeat expansions in the amphipathic domain of *PLIN4*. The size of the repeat expansion was 39 $\times$ 99 bp in Family 1 and 37 $\times$ 99 bp in Family 2. The disease in these two families is characterized by adult-onset muscle weakness manifesting with very mild proximal weakness in lower limbs at the beginning, followed soon thereafter by prominent distal involvement of muscles of four limbs, mainly forearm and finger extensors and the anterior compartment of the legs. Affected individuals do not present facial, bulbar, or ocular weakness. Serum CK levels range from normal to moderately increased. So far, cardiac involvement has been ruled out

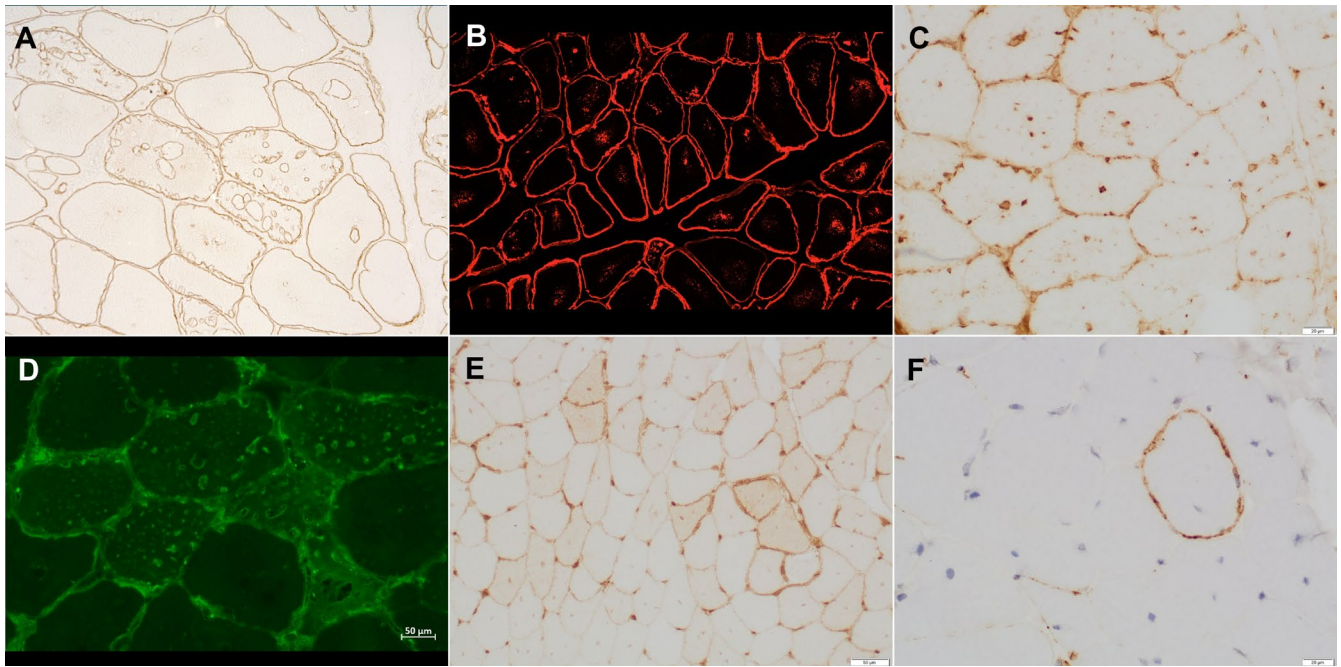


FIGURE 4 | Immunohistochemistry and immunofluorescence features in *PLIN4*-related myopathy. (A) Immunohistochemistry for carboxydystrophin shows abundant membrane-bounded vacuoles. (B) p62 immunofluorescence shows strong staining in the sarcolemma of all fibers and in central areas corresponding to the vacuoles. (C) Lysosomal associated protein LAMP-2 is overexpressed at the site of the vacuoles. Scale bar: 20 μ m. (D) Strong LC3 immunoreaction inside the myofibers. (E) MHC class I is upregulated at the sarcolemma and cytoplasm of many myofibers, while membrane attack complex (MAC) in (F) is strongly deposited in the sarcolemma of isolated myofibers. Scale bars: 50 μ m in E, 20 μ m in F.

in all the reported families, including ours. By contrast, three patients in our families had severe respiratory involvement at late-stage disease. Nevertheless, only three cases were reported before to have restrictive ventilation abnormalities [7, 33]. Notably, we did not find clinical signs of myotonia nor myotonic discharges on electromyography, unlike some previous reports [33]. Interestingly, we observed mixed neurogenic–myogenic features on electromyography in most of the patients, meaning the presence of spontaneous activity at rest and a combination of small and large motor unit potentials. These findings are often misinterpreted as indicative of a neurogenic disease, particularly in patients with distal weakness. In fact, in patient F1 II:4, the EMG findings led to initially misdiagnose him as having a distal hereditary motor neuropathy, a common misdiagnosis in distal myopathies. Indeed, other myopathies, such as inclusion body myositis, myofibrillar myopathies, and many of the distal myopathies with rimmed vacuoles, commonly have mixed patterns on EMG and can be misdiagnosed as neurogenic entities in the absence of a muscle biopsy. These difficulties in EMG interpretation must be borne in mind by clinicians to guide the final diagnosis in distal or rapidly progressing myopathies.

In line with what has been reported, we found a high phenotypic variability among affected individuals even within a family, including mildly affected ones who remained ambulant at advanced ages, especially female patients, in contrast to other patients who were wheelchair-bound or deceased around 40 years of age. Our observations indicate that the age of symptom onset or disease severity is not related to the size of the 99-mer expansion in *PLIN4*, except in one reported family carrying a 50×99 bp tandem repeat whose affected individuals had an earlier onset and faster progression [6] (see Table 1 for details).

In regard to gender differences, male patients in our families were generally affected earlier and more severely than females. This difference cannot be established with certainty based on the published families to date. However, it may be noted that Yang et al. reported three female carriers who remained asymptomatic above 50 years old, and in Wang’s publication, all the clinically affected individuals were males [6, 7]. Further investigations and additional families will need to be identified before a conclusion can be reached regarding this association of age-of-onset and severity with gender.

Muscle imaging did not show a common pattern of involvement between families. While the proband in Family 1 had a distal leg involvement almost exclusive of tibialis anterior, the two available MRI scans in Family 2 showed a predominant involvement of the calves and posterior thigh compartments. These last findings are similar to those reported by Ruggieri et al. [33], but differ from other reported families due to prominent fatty infiltration of vasti muscles in their patients [6, 7]. Interestingly, despite the discrepancy between anterior and posterior leg involvement observed in our families, muscle imaging in both probands showed preferential infiltration of sartorius muscles, a feature also reported in Chinese patients [6, 7]. Overall, we do not identify a specific MRI pattern of involvement in *PLIN4* myopathy, but the most constant feature seems to be the calf-predominant involvement of the legs.

Tandem repeat expansions such as those causing *PLIN4* myopathy are difficult to identify with conventional methods such as gene panels or exome sequencing [34] and, therefore, a complex genetic approach in specialized centers is often required. In this situation, additional complementary tests, including muscle

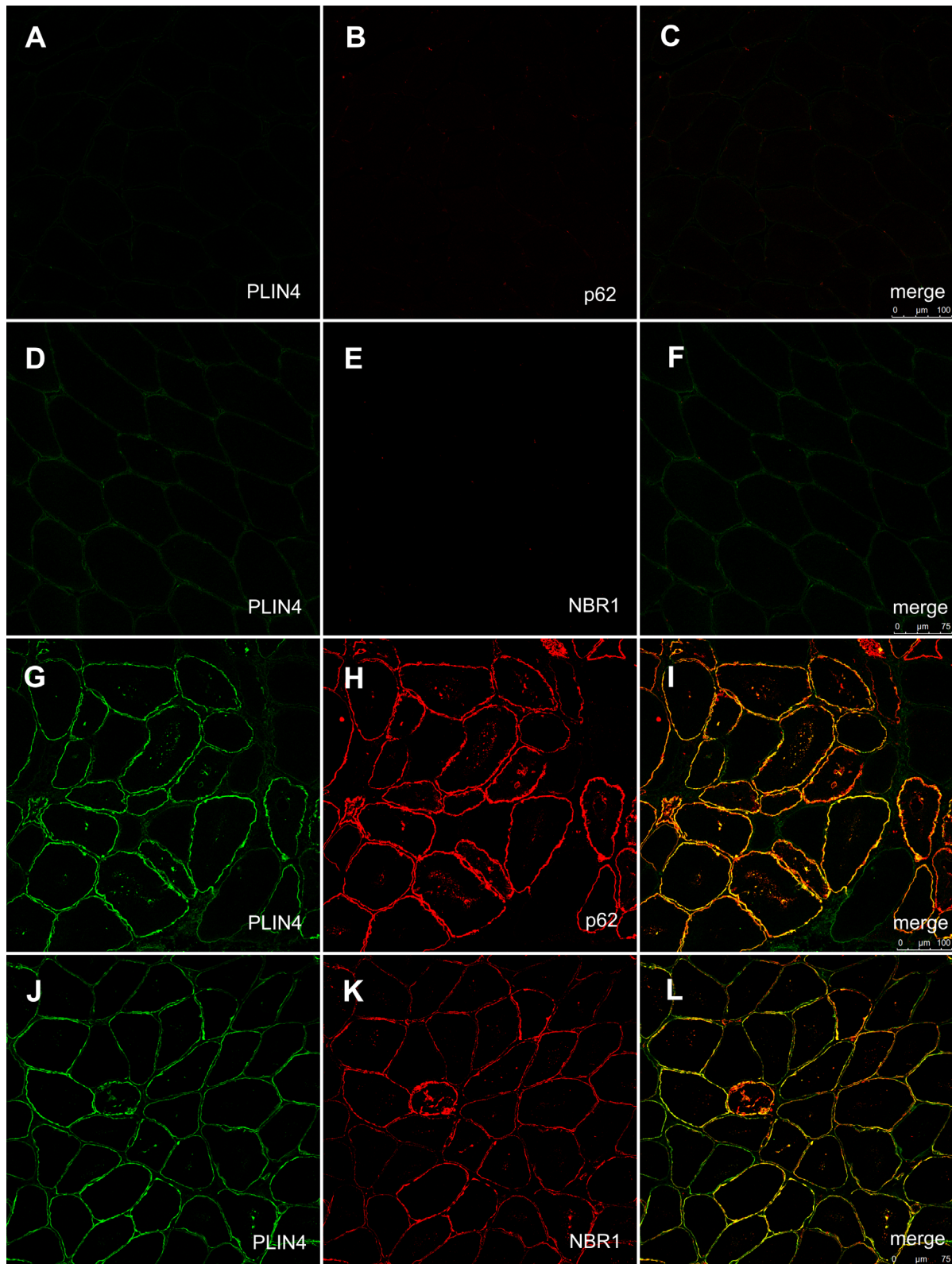


FIGURE 5 | Double labeling immunofluorescence and confocal microscopy show increased PLIN4 in patient muscle co-localizing with p62 and NBR1 at the sarcolemma and within the cytoplasm in the majority of myofibers (G to L). In contrast, control muscle (A to F) shows the absence of p62 and NBR1, and very faint subsarcolemmal PLIN4 expression corresponding to normal expression in healthy muscle. G to I: Patient F2 IV:1 muscle. J to L: Patient F1 III:4 muscle. Immunofluorescence against PLIN4 (A, D, G, J), p62 (B, H), NBR1 (E, K), and merged images (C, F, I, L) are shown.

biopsy, are crucial to guide the molecular diagnosis. In fact, the myopathological features observed in our families were so characteristic that they prompted us to test these families for *PLIN4*

expansions. Prior to performing long-read sequencing, a long-range PCR assay following the protocol described in the publication by Ruggieri et al. was performed. However, we failed

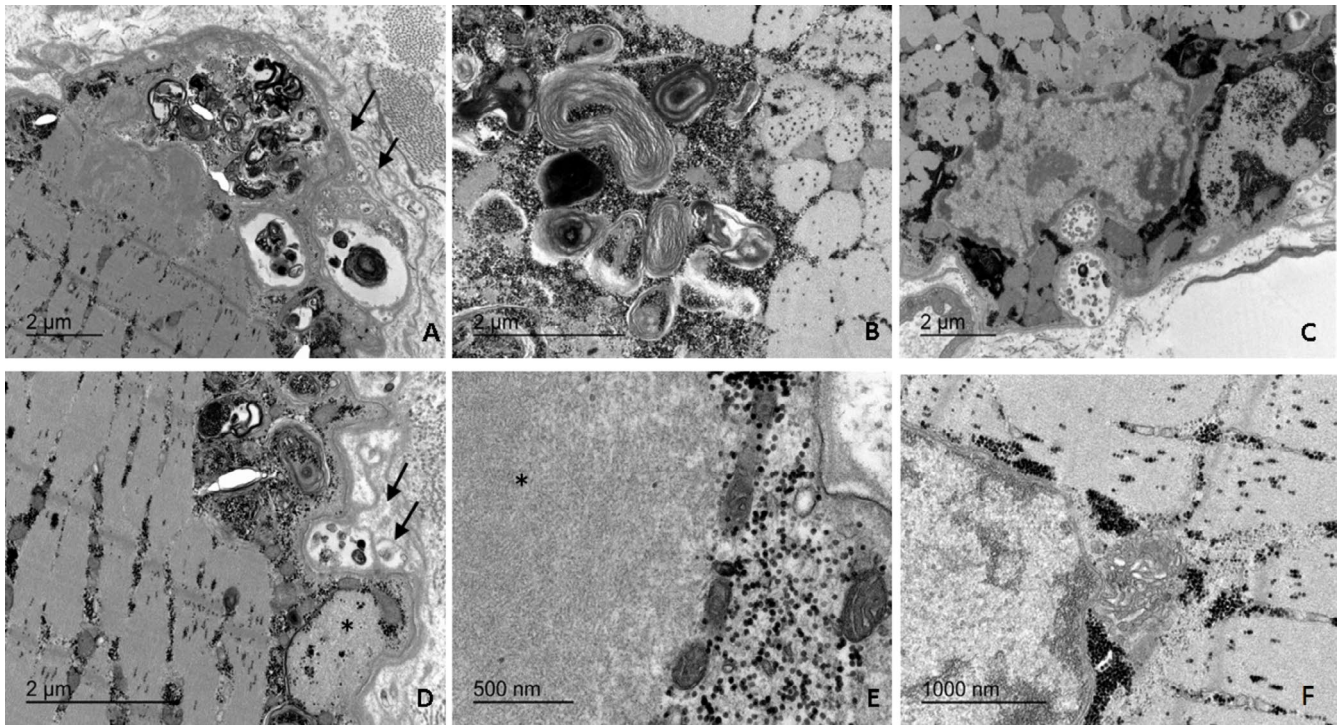


FIGURE 6 | Electron microscopy shows membrane-limited autophagic vacuoles and basal lamina redundancy. Ultrastructural analysis showed large numbers of autophagic vacuoles containing myelin bodies, degraded organelles, and electron-dense and granular material (A to D). The sarcomere structure was disrupted in some regions next to the vacuoles (A). The vacuoles were membrane-limited and often appeared abutting the extracellular space (C and D). Multiple layers of the basal lamina and loops were observed (arrows in A and D). Some myofibers showed collections of tubulo-filaments of 18 nm within the sarcoplasm (asterisks in D and E). Proliferation of sarcoplasmic reticulum was observed in some myofibers (F).

to sequence the expanded fragment probably due to the well-known technical difficulties in amplifying repeat expansions.

The characteristic myopathological features in the muscle biopsies in our patients included abundant rimmed vacuoles located at the subsarcolemmal region and within the sarcoplasm. Importantly, p62 showed a unique pattern of expression in the sarcolemma of myofibers, and at the site of the vacuoles, a constant feature observed in all PLIN4 myopathy patients reported to date [5–7, 33]. Indeed, this sarcolemma labeling of p62 has been considered as highly distinctive of the disease, but it is worth noting that it has also been described in some neuropathic entities [35]. Yet, it has never been described in other vacuolar myopathies.

Results of our histochemical and immunohistochemical analyses demonstrate the vacuoles in PLIN4 myopathy are labeled with sarcolemmal markers (caveolin-3, dystrophin, laminin $\alpha 2$) and display acetylcholinesterase activity, which allows classifying this myopathy within the group of autophagic vacuolar myopathies with sarcolemmal features (AVSF). Remarkably, those sarcolemmal markers, especially dystrophin, laminin $\alpha 2$ and acetylcholinesterase, show an irregular thickened lining of the sarcolemma of several fibers due to the particular subsarcolemmal localization of many vacuoles, which together with the p62 pattern seems to be very distinctive of this disease. Similar to other autophagic vacuolar myopathies including Pompe disease, X-linked myopathy with excessive autophagy (XMEA), Danon disease, and toxic myopathies caused by colchicine or chloroquine, markers of lysosomal dysfunction including acid

phosphatase, LAMP-2, and LC3 are overexpressed in vacuolated myofibers in PLIN4 myopathy. Taken together, these findings indicate that PLIN4 myopathy belongs to the group of autophagic vacuolar myopathies resulting from primary and nonprimary lysosomal dysfunction [36–44].

Additional histopathological analyses revealed calcium deposition along the cell surface of some fibers and peripheral vacuoles, a feature described in XMEA [45–47] and observed in colchicine myopathy (author observation). Finally, MHC class I overexpression and MAC deposition in isolated myofibers were observed in the three muscle samples we assessed. This finding has been previously reported in PLIN4 myopathy [7], as well as in XMEA and colchicine myopathy [43, 47, 48] and has been considered by some authors [7] the result of secondary muscle inflammation. Taken together, the histopathological features of PLIN4 myopathy—autophagic vacuoles with sarcolemmal features, MHC-I and MAC overexpression, calcium deposition, and basal lamina replication—are highly reminiscent of XMEA. Therefore, XMEA constitutes the major differential diagnosis for PLIN4 myopathy from a morphological point of view when studying a hereditary vacuolar myopathy.

Studies performed by Di Blasi et al., in 2004, long before the identification of the gene defect [4], demonstrated that ubiquitin and subunits of the proteasomal complex increase in myofibers, suggesting that a primary abnormality in the autophagy-lysosomal pathway, which led to a secondary ubiquitin-proteasome system (UPS) activation, was responsible for the disease. This fact could explain the presence of muscle inflammatory markers observed in the disease,

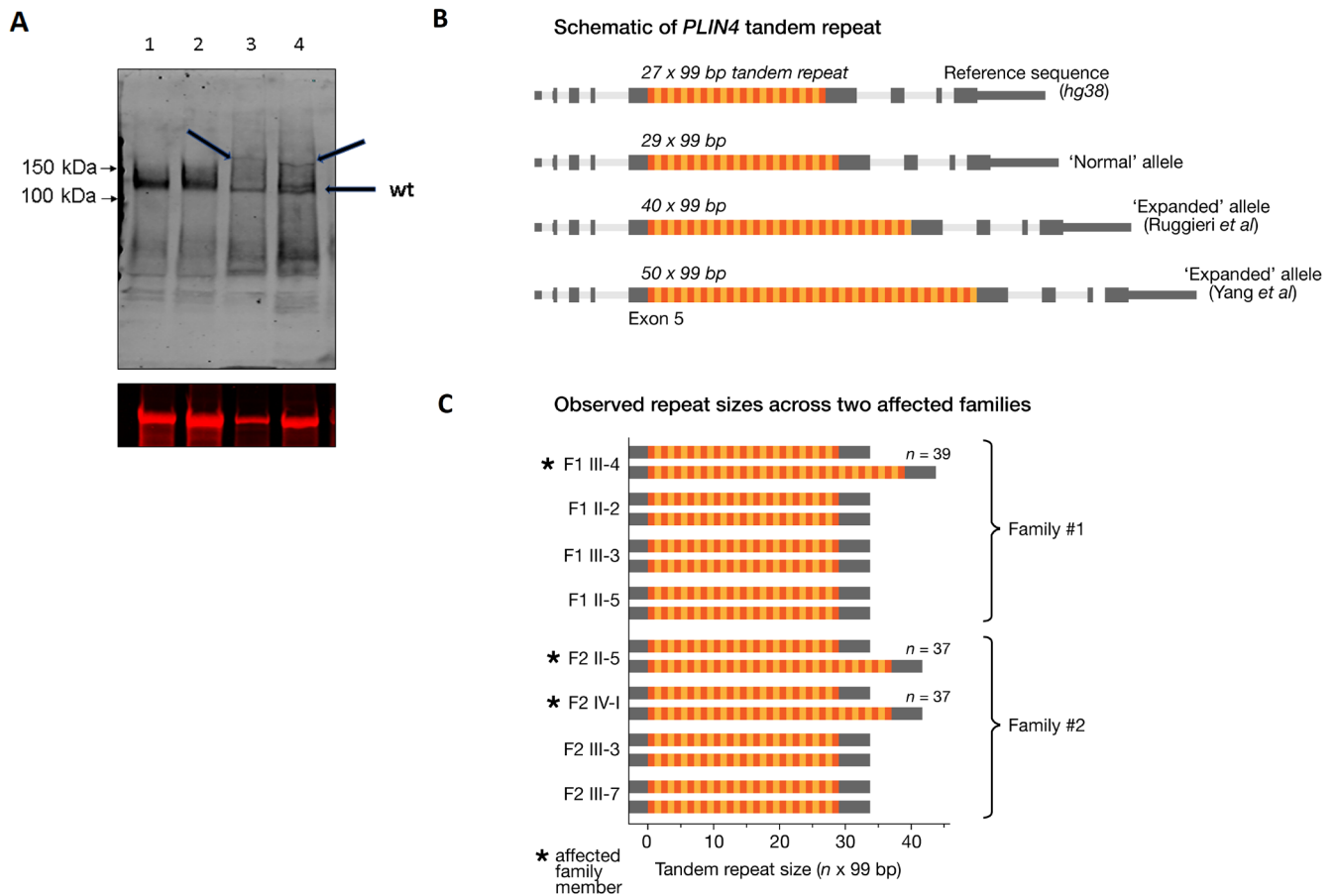


FIGURE 7 | Genomic and protein studies in *PLIN4*-related myopathy families demonstrate the presence of expanded alleles in affected individuals. (A) Western blot (WB) analysis for *PLIN4* in two healthy controls (lane 1 and 2) and in the probands from Family 1 (lane 3) and Family 2 (lane 4). WB from both patients show a normal band of 134 kDa (“wt” as wildtype) and an extra band of higher molecular weight (oblique arrows). Note that the 134 kDa band is weaker in patients compared to controls, and that the extra bands are clearly visible in the patients but not in the controls. Protein loading control is shown below in red. (B, C) Genotyping of the *PLIN4* exon 5 tandem repeat in the two affected families using targeted ONT sequencing. (B) Schematic illustration of $n \times 99$ bp tandem repeat which encodes the amphipathic domain of *PLIN4*. 27 $\times$ 99 bp repeat copies are present in the human reference genome (hg38) sequence. Alleles of 29 $\times$ 99 bp repeat copies are common in the normal population. Expanded alleles of 39 $\times$, 40 $\times$ and 50 $\times$ 99 bp were previously reported to cause myopathy. (C) Observed sizes of *PLIN4*-exon 5 tandem repeat alleles among eight individuals from two unrelated families. Asterisks denote clinically affected individuals.

as UPS-mediated degradation of defective proteins generates peptides presented by MHC-I to T cells upon autophagy impairment [49]. UPS activation would therefore predispose individuals to the presence of MHC-I overexpression at the sarcolemma that we observe in a nonimmune mediated myopathy such as *PLIN4* or even XMEA, but it cannot explain complement activation.

In skeletal muscle, perilipin 4 is normally located beneath the sarcolemma where it is thought to bind to lipid droplets (LD), but its role in muscle tissue is not completely understood [13]. In *PLIN4* myopathy, there is an increase of *PLIN4* at the sarcolemma that perfectly co-localizes with markers of aggrephagy such as FK2, NBR1, and p62/SQSTM1. Interestingly, a complex experimental in vitro study focusing on the amphipathic helix of *PLIN4* showed that there is a direct correlation between the targeting to LD and the length of the amphipathic domain, and that *PLIN4* tends in normal conditions to interact with monolayer (not bilayer) membranes [10]. It was demonstrated that mutant *PLIN4* proteins with more hydrophobicity show

increased binding to LD before invading other cellular membranes, the latter not seen with the wildtype protein. These hydrophobic mutants drastically changed their binding to bilayer cellular membranes, especially in conditions of increased membrane charge or membrane packing defects. Altogether, this evidence could suggest that the tandem repeat expansion leading to *PLIN4* myopathy may cause changes in the binding specificity of *PLIN4* to LD and may induce the abnormal targeting to the cellular membrane that is observed in this muscle disease. This would explain the increase of *PLIN4* observed at the sarcolemma, likely as a result of protein misfolding and aggregation. Its colocalization with ubiquitin, p62, FK2, and NBR1, which form an autophagy receptor complex closely related to LC3, suggests continuous activation of aggrephagy, the degradation process of protein aggregates initiated with autophagosome formation that ultimately fuse with lysosomes [49]. This process leads to the accumulation of autophagic vacuoles. Whether the lack of proper binding to LD would not predispose to lipid accumulation needs to be clarified.

5 | Conclusions

In conclusion, we report two unrelated families carrying different size expansions of the *PLIN4* amphipathic domain suffering from an autosomal dominant autophagic vacuolar predominantly distal myopathy. Vacuoles show sarcolemmal features similar to those observed in lysosomal disorders and AVSF myopathies. No correlation between the size of the expansion and clinical severity could be established. Of note, female patients seem to be less severely affected. We suggest that *PLIN4* expansions lead to abnormal perilipin 4 aggregation and misfolding in the sarcolemma, which in turn causes accumulation of aggregating vesicles that exceed the autophagic capacity within myofibers. *PLIN4* expansions should be considered in the diagnosis of autophagic vacuolar myopathies when conventional NGS technologies fail to identify any candidate variant. Subsarcolemmal p62 expression is a good marker of the disease and helps guide the molecular diagnosis.

Author Contributions

M.O., E.G., P.G. designed the study. L.L., G.M., R.C.-V., R.A., A.V., I.L., and M.O. collected clinical data and biological samples. L.L., R.B., B.S.-M., C.N., E.G., and M.O. performed myopathological analysis. I.S., L.G.-Q., A.S.-C., B.R.-S., P.G., and I.W.D. performed the genetic testing. L.L., I.S., A.S.-C., I.W.D., E.G., and M.O. drafted the manuscript. L.L. and M.O. wrote the final manuscript. L.L., I.S., I.W.D., and M.O. prepared figures and tables. G.R. contributed to data analysis and interpretation. All authors read and approved the final manuscript. M.O. supervised the project.

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Ethics Statement

All patients gave informed consent to participate. Sample collection was obtained after written informed consent from the patients in the participating institutions. The local ethics committee, “Comitè d'Ètica d'Investigació Mèdica (CEIm) Sant Pau Campus Salut Barcelona”, approved the study under project FIS P121/01621.

Conflicts of Interest

I.W.D. manages a fee-for-service sequencing facility at the Garvan Institute of Medical Research that is a customer of Oxford Nanopore Technologies but has no further financial relationship. The authors declare no other competing financial or nonfinancial interests.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding authors upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.