**Diagnostic challenge in *PLIN1*-associated Familial Partial Lipodystrophy**

Isabelle Jéru1,2\*, Marie-Christine Vantyghem3\*, Elise Bismuth4, Pascale Cervera5, Sara Barraud6, *PLIN1*-Study Group, Martine Auclair1, Camille Vatier1,8, Olivier Lascols1,2, David B. Savage7, Corinne Vigouroux1,2,8

\* These authors equally contributed to this work

1 Sorbonne University, Inserm U938, Saint-Antoine Research Centre, Institute of Cardiometabolism and Nutrition, Paris, France

2 Assistance Publique-Hôpitaux de Paris, Saint-Antoine University Hospital, Department of Molecular Biology and Genetics, Paris, France

3 Department of Endocrinology, Diabetology and Metabolism, Lille University Hospital, and Inserm U1190, European Genomic Institute for Diabetes, F-59000 Lille, France

4 Paris-Diderot University, Sorbonne Paris Cité, and Assistance Publique-Hôpitaux de Paris, Robert Debré University Hospital, Department of Pediatric Endocrinology and Diabetology, Competence Center for Rare Diseases of Insulin Secretion and Insulin Sensitivity (PRISIS), Paris, France

5 Sorbonne University, Inserm U938, Assistance Publique-Hôpitaux de Paris, Saint-Antoine University Hospital, Pathology Department, Paris, France

6 Department of Endocrinology, Diabetes, Nutrition, Reims University Hospital

7 The University of Cambridge Metabolic Research Laboratories, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge, United Kingdom

8 AP-HP, Saint-Antoine University Hospital, National Reference Center for Rare Diseases of Insulin Secretion and Insulin Sensitivity (PRISIS), Department of Endocrinology, Diabetology and Reproductive Endocrinology, Paris, France

*PLIN1*-Study Group includes Fabrice Devemy (Department of Endocrinology, Lens Hospital, France), Wassila Karrouz (Department of Endocrinology, Diabetes and Metabolism, Lille University Hospital, France), Sophie Haye (Endocrinology, Béthune Hospital, France), Sonja Janmaat (AP-HP, Endocrinology, PRISIS, Paris, France), Brigitte Delemer (Endocrinology, Reims University Hospital, France), Hilary Meggison (Endocrinology and Metabolism, Ottawa Hospital, Ottawa Ontario, Canada) and Michael T Geraghty (Pediatrics, Childrens Hospital of Eastern Ontario, University of Ottawa, Ottawa Ontario, Canada).

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**Corresponding author:** Corinne Vigouroux, Sorbonne Université Médecine, Site Saint-Antoine, 27, rue Chaligny, 75571 Paris Cédex 12, France ; E-mail : corinne.vigouroux@inserm.fr

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**Precis**:

We describe novel heterozygous *PLIN1* frameshift variants in the largest cohort of Type 4 Familial Partial Lipodystrophy (FPLD4) reported to date and provide a set of arguments for their pathogenicity.

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**Abstract**

*Context:* Heterozygous frameshift variants in *PLIN1* encoding perilipin-1, a key protein for lipid droplet formation and triglyceride metabolism, have been implicated in familial partial lipodystrophy type 4 (FPLD4), a rare entity with only 6 families reported worldwide. The pathogenicity of other *PLIN1* null variants identified in patients with diabetes and/or hyperinsulinemia was recently questioned based on the absence of lipodystrophy in these individuals and on the elevated frequency of *PLIN1* null variants in the general population.

*Objectives :* To reevaluate the pathogenicity of *PLIN1* frameshift variants in light of new data obtained in the largest series of patients with FPLD4.

*Methods* : We performed histological, and molecular studies in patients referred to our French National Reference Center for Rare Diseases of Insulin Secretion and Insulin Sensitivity for lipodystrophy and/or insulin resistance, and carrying *PLIN1* frameshift variants.

*Results:* We identified two heterozygous *PLIN1* frameshift variants segregating with the phenotype in 9 patients from 4 unrelated families. The FPLD4 stereotypical signs include postpubertal partial lipoatrophy of variable severity, muscular hypertrophy, acromegaloid features, PCOS and/or hirsutism, metabolic complications (hypertriglyceridemia, liver steatosis, insulin resistance, and diabetes), and disorganised subcutaneous fat lobules with fibrosis, and macrophage infiltration.

*Conclusions:* These data suggest that some FPLD4-associated *PLIN1* variants are indeed deleterious. Thus the evidence for the pathogenicity of each variant ought to be carefully considered prior to genetic counseling, especially given the importance of early diagnosis for optimal disease management. In this regard, we recommend detailed familial investigation, adipose tissue-focused examination, and follow-up of metabolic evolution.

**MAIN TEXT**

**INTRODUCTION**

Familial partial lipodystrophy syndromes (FPLD) are rare diseases characterized by a limited capacity of peripheral fat to store triglycerides, which drives metabolic abnormalities including insulin resistance, hypertriglyceridemia, liver steatosis, and polycystic ovary syndrome (PCOS) (1). Heterozygous frameshift variants in the *PLIN1* gene encoding perilipin-1 have been identified in 6 families (2-4), thereby defining the FPLD4 subtype.

Perilipin-1 is a structural lipid droplet protein which facilitates triglyceride storage or initiates lipolysis, depending on hormonal stimuli. We showed that several FPLD4-associated *PLIN1* frameshift variants disrupt the ability of perilipin-1 to inhibit basal lipolysis in adipocytes (2, 3, 5). However, the pathogenicity of other heterozygous *PLIN1* null variants, identified in patients referred for Maturity Onset Diabetes of the Young, hyperinsulinic hypoglycemia, or type 2 diabetes, has recently been questioned by Laver et al. (6).

Since proper interpretation of *PLIN1* variants is crucial for genetic counselling, the aim of this study was to discuss the pathogenicity of *PLIN1* null variants in the light of new genotype-phenotype data obtained in the largest series of patients with FPLD4 reported to date.

**SUBJECTs and Methods**

Sequencing of a panel of lipodystrophy genes (*AGPAT2, AKT2, BSCL2, CAV1, CIDEC, LIPE, LMNA, PLIN1, POLD1, PPARG, PTRF,* and *ZMPSTE24*) was performed in 237 independent index cases, investigated in our French National Reference Network for Rare Diseases of Insulin Secretion and Insulin Sensitivity, for manifestations evocative of a lipodystrophic syndrome. Capture (SeqCapEZ enrichment protocol, Roche NimbleGen) was followed by massively parallel sequencing on a MiSeq platform (Illumina Inc, San Diego, CA, USA). Data were analysed using the Sophia Genetics DDM pipeline®. *PLIN1* frameshift variants were confirmed by Sanger sequencing. Affected relatives were identified after familial investigations. Abdominal subcutaneous adipose tissue biopsies were obtained from two patients. Western blot and histological analyses were performed as previously described (2). Written informed consents were collected according to legal procedures for molecular and histological investigations, and publication of photographs. This study was approved by the Comité de Protection des Personnes Ile-de-France 5 (Paris, France).

**RESULTS**

### Molecular diagnosis

A heterozygous *PLIN1* frameshift variant was identified in 4 index cases and 5 affected relatives (Figure 1A). Patients from family D and L carried the c.1191\_1192del deletion, previously shown to be expressed as an abnormal p.(Val398Glyfs\*166) elongated form of perilipin-1, leading to constitutive activation of basal lipolysis (2, 5). In family E and C, we identified a novel 4bp-duplication in exon 8 (c.1202\_1205dup) leading to the synthesis of a p.(Pro403Argfs\*164) mutant protein, whose expression in adipose tissue was confirmed by Western Blot (Figure 2). These variants were not found in public databases (Exome Aggregation Consortium - ExAC, Genome Aggregation Database - gnomAD) and co-segregated with the disease within each family (Figure 1A). In all patients, we did not find any other molecular defect in known lipodystrophy genes.

### Disease phenotype

The clinical and biological features of the 9 family members investigated herein and carrying a *PLIN1* variant recapitulated the FPLD4 cardinal signs (2), *i.e.* lipoatrophy, muscular hypertrophy, facial acromegaloid features, insulin resistance-related ovarian dysfunction, and metabolic complications (hyperinsulinemia or insulin-resistant diabetes, hypertriglyceridemia, liver steatosis) (Table 1). Index cases were initially referred for ovarian hyperandrogenism with lipodystrophy (patient D-II3), suspicion of acromegaly (patients L and E-II2), or early-onset non-autoimmune diabetes (patient C-II1). The disease manifestations were only detected thanks to family studies in several individuals (D-I2, C-I2, C-II2).

Lipoatrophy mainly affected trunk, limbs and femorogluteal regions and was associated with muscular hypertrophy predominantly in the calves (Figure 1B). It could be mild, especially in young patients (E-III1, C-II2). Cervicofacial fat accumulation was observed in three patients (E-I1, C-I2, C-II1). Low serum leptin levels and a decrease in total fat mass, as assessed by dual energy X-ray absorptiometry, were consistent with lipoatrophy. Abdominal subcutaneous adipose tissue, studied in patients E-I1 and E-II2, displayed dizorganised fat lobules of heterogeneous size, with macrophage infiltrates, increased fi­brosis and vascularization, consistent with previous findings (2) (Figure 2). Seven patients showed a facial acromegaloid appearance with enlarged hands and feet. All investigated patients presented with hyperinsulinemia or diabetes, frequently accompanied by acanthosis nigricans. Five women among 7 had PCOS and/or hirsutism and oligomenorrhea. Hypertriglyceridemia was present in all investigated adult patients. No history of acute pancreatitis was reported. All examined patients had liver steatosis. Patient L-II1 suffered from major complications, including neuropathy, hypertension and myocardial infarction with rhythm disturbances, which required the implantation of a cardioverter defibrillator.

**discussion**

The increasing use of next generation sequencing in clinical practice highlights the need for accurate interpretation of variants. When large population exome data became available, the pathogenicity of several genes involved in Mendelian disorders was questioned (7). This issue was recently raised for *PLIN1* by Laver et al. (6). Considering the importance of early genetic counselling for appropriate disease management (1), the clues arguing for and against a pathogenic effect of *PLIN1* null variants should be weighed up carefully (Table 2).

On one hand, the allele frequency of *PLIN1* null variants in the general population, estimated at about 4.10-4 in public databases, is higher than the prevalence of all forms of FPLD, estimated at about 3.10-6 (8). Even if FPLD remains underdiagnosed, this suggests that certain *PLIN1* null variants are not pathogenic. On the other hand, we observed a marked enrichement of *PLIN1* frameshift variants in patients with FPLD. Indeed, the allele frequency of *PLIN1* frameshift variants was estimated at 1.9% in one of our previous studies (3 heterozygotes among 78 independent patients with clinically ascertained FPLD) (2), and at about 0.8% in the present study (4 positive probands for 237 tested patients).

Strikingly, FPLD4-associated *PLIN1* variants co-segregated with the disease within the 8 families so far available ((2-4), and current study). If these variants were all polymorphisms, the probability to see such a segregation by chance in the 13 informative affected relatives would be extremely low ((1/2)13, *i.e.* 1.10-4).

The pathogenicity of *PLIN1* null variants was also questioned since patients reported by Laver et al. did not have overt lipoatrophy (6). As acknowledged by the authors, it is difficult to exclude lipodystrophy in young patients and the lack of adipose tissue-focused examination in large cohort studies hamper the recognition of subtle lipodystrophic morphotypes. In this regard, one study underlined that FPLD remains underdiagnosed and could affect more than 3% of patients investigated for metabolic syndrome (9). Accordingly, lipodystrophy was diagnosed a posteriori in several patients investigated herein, thanks to the familial investigations.

Four FPLD4-associated frameshift variants lead to the synthesis of aberrant perilipin-1 isoforms ((2, 3), and current study). Expression in adipocyte models of three of these mutant forms of perilipin-1 decreases the size of lipid droplets and increases basal lipolysis (2, 3, 5). Notably, all but one FPLD4-associated *PLIN1* variants (p.(Val398Glyfs\*166), p.(Tyr401Leufs\*165), p.(Pro403Argfs\*164), p.(Leu404Alafs\*158)) alter the interaction domain of perilipin-1 with ABHD5 (amino acids 380-427). Consistently, the p.(Val398Glyfs\*166) and p.(Leu404Alafs\*158) mutants fail to interact with ABHD5, leading to constitutive activation of adipocyte triglyceride lipase (5). The p.(Pro439Valfs\*125) mutant, which is located in the vicinity of this binding domain, fails to inhibit basal lipolysis by an alternate mechanism (3). It would be interesting to determine the functional consequences of *PLIN1* null variants identified in the general population or in MODY.

At the present time, the classification of genetic variants follows the guidelines of the American College of Medicals Genetics based on a five-class score (10). According to these criteria, FPLD4-associated *PLIN1* frameshift variants disrupting protein function should be classified as “pathogenic”. Laver et al suggested that *PLIN1* null variants should not be reported as causative of lipodystrophy. Our study provide a set of arguments supporting the pathogenicity of several frameshift variants. In our opinion, it is important to evaluate each of these variants carefully for genetic counselling. To this purpose, we would recommend detailed familial investigation, adipose tissue-focused examination, and careful follow-up of metabolic evolution in patients carrying such variants.

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**FIGURE LEGENDS**

**Figure 1.**

**A. Molecular and clinical investigations in patients with heterozygous *PLIN1* null variants.** Arrows indicate index cases. Genealogical trees show a co-segregation of *PLIN1* variants with the disease phenotype. The nomenclature of *PLIN1* variants is based on RefSeq accession numbers NM\_002666.5 and NP\_002657.3.

**B. Morphotype of patient E-II2 with partial lipodystrophy and acromegaloid features.** Subcutaneous lipoatrophy of upper and lower limbs is observed with muscle hypertrophy (arrows). As compared with patients with FPLD2 (*LMNA*-linked FPLD), the neck is less broadened and breast and subcutaneous abdominal fat are not affected by lipoatrophy.

Acromegaloid features are composed of face infiltration, slightly enlarged nose, deep wrinkles, thick lips and hands, and enlarged feet.

**Figure 2. Study of subcutaneous adipose tissue from patients**

A- Perilipin-1 expression in abdominal subcutaneous adipose tissue from patients E-II2 and E-I1, as compared to controls and previously described patients with FPLD4 (2). Western blot on whole cell extracts was performed using antibodies directed against the N-terminal and C-terminal parts of wild-type perilipin-1, as previously described (2). The mutant isoform was recognized by the N-terminal antibody as an additional band (arrow) just above the 62-kD molecular-weight (MW) marker, which was not detected by the C-terminal antibody. Tubulin (antibody T5168, Sigma-Aldrich) was used as a loading control.

B- Consequences on perilipin-1 protein of FPLD4-associated *PLIN1* frameshift variants studied in A.

C- Histology and immunohistology analyses of abdominal subcutaneous adipose tissue from patients E-I1 and E-II-2, as compared to controls. Adipose tissue samples from patients display disorganised fat lobules of heterogeneous size, with increased ­fibrosis (Sirius Red (SR) staining 16-35% of the total sample surface, versus 0.2-3% in controls), increased vascularization (density of CD34 staining 0.13-0.44% in adipose lobules from patients, versus 0.001-0.004% in controls) and increased macrophage infiltration with crown-like structures (assessed by CD163 and CD68 staining, density per 10-5 m2 : 1.9-2.2 and 3.6-4.3 in patients versus 0.0 and 0.0-0.01 in controls, respectively).

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