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Case report

Berardinelli-Seip syndrome patient with novel *AGPAT2* splicesite mutation and concomitant development of non-diabetic polyneuropathy

Short title: New *AGPAT2* splicesite mutation with polyneuropathy.

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Abstract

Primary polyneuropathy in the context of Seip-Berardinelli type 1 seipinopathy, or congenital lipodystrophy type 1 (CGL1) is previously unknown. We report the case history of a 27 year old female CGL1 patient presenting with unusual additional development of **non-diabetic** peripheral neuropathy (PN) and learning disabilities in early adolescence. Whole exome sequencing (WES) of the patient genome identifies a novel rare variant homozygous **for a** 52 bp intronic deletion in the *AGPAT2* locus uniquely associated with CGL1 seipinopathies, with no molecular evidence for dual diagnosis. Functional studies using RNA isolated from patient peripheral blood leucocytes show abnormal RNA splicing resulting in

the loss of 25 amino acids from patient AGPAT2 protein coding sequence. Stability and transcription levels for the misspliced *AGPAT2* mRNA in our patient nonetheless remain normal. Any *AGPAT2* protein produced in our patient is therefore likely to be dysfunctional. However, formal linkage of this deletion to the neuropathy observed remains to be shown. The classical clinical presentation of a patient with *AGPAT2*-associated lipodystrophy shows normal cognition and no development of polyneuropathy. Cognitive disabilities and polyneuropathy are features to date associated exclusively with clinical CGL type 2 arising from *BSCL2* gene mutations. This case study suggests that in some genetic contexts, *AGPAT2* mutations can also produce phenotypes with **primary** polyneuropathy.

What is already known on this topic?
Within Berardinelli-Seip syndromes (Congenital Lipodystrophy disorders characterized by total absence of both metabolic and mechanical fat tissue), only CGL type 1 retains mechanical fat and is exclusively associated with *AGPAT2* gene mutations. Polyneuropathies and learning deficiencies are currently unknown in the context of classical CGL1 disease and *AGPAT2* lesions.

What this study adds?
Unusual lifelong 27yr case history of classical CGL1 with mechanical fat retention, featuring developmental polyneuropathy, learning deficiencies, and a new *AGPAT2* intronic deletion. Our results describe a phenotype expansion for CGL1 and suggest certain *AGPAT2* gene lesions cause neuropathy which blurs clinical presentation boundaries for CGL and other fat biology disorders.

Introduction
Berardinelli-Seip syndrome, also known as congenital generalized lipodystrophy (CGL), occurs in approximately 1 in 10 million of the world population and can result from mutation in 4 genes, giving rise to 4 clinically similar but distinguishable subsyndromes affecting fat biology[1]. CGL type 1 (OMIM#608594) is autosomal, recessive and uniquely associated with mutation in the *AGPAT2* gene encoding 1-acylglycerol-3-phosphate-O-acyltransferase 2[2]. This enzyme is integral to phospholipid biosynthesis, triglygeride/fat formation and storage, adipocyte formation and fat metabolism pathways and has multiple molecular interaction partners[1].
Clinical symptoms associated with CGL1 to date involve both metabolic malfunctions and physical malformations present in all forms of CGL. Complete lack of all metabolic body fat (adipose tissue that stores energy) is the central clinical characteristic from birth for all forms. CGL1 patients alone, however, retain mechanical fat (adipose tissue that provides protective padding for joints and points of impact i.e. palms, soles of feet, joints, scalp facial bones). This fat distribution is specific and differentially diagnostic for CGL1[1]. Clinical neuropathy and cognitive deficits are associated with CGL2 and mutations in *BSCL2*, and are rare but not unknown for CGL3 and CGL4 syndromes (associated with *CAV1* and *PTRF* gene mutations respectively[1,2]). Primary neuropathy and cognitive deficit in the context of CGL1 are previously unreported in the absence of diabetic or other secondary disease complications. We present the natural history of a female CGL1 patient, continuously recorded from infancy to adulthood. We further demonstrate this patient carries a previously unknown homozygous intronic deletion variant g.12562_12613del p.(Val197Glufs*32) in the *AGPAT2* gene. Our functional studies show the deletion disrupts normal *AGPAT2* transcriptional processing and

mRNA coding content consistent with a dysfunctional and therefore potentially pathogenic effect for this deletion.

Case History

Table 1 lists clinical symptoms from infancy (3 months) to current age (27 years) in chronological order of emergence.

Family history discloses distant parental consanguinity; identity by descent (IBD) is corroborated by extensive absence of heterozygosity (AOH) totalling 46Mbp, in the patient genome, with an average AOH region size of 321Kbp. The AOH block encompassing the patient AGPAT2 gene is 1.1Mbp (see Methods). Direct ancestors on both sides lived in the same village for many generations. Both parents are clinically asymptomatic for CGL1. One grandfather however presented with lipodystrophy and diabetes mellitus (DM) (Column 4, Table 1).

The patient was born to a 34 year old G5P4->5 mother by spontaneous vaginal delivery. The father was 39 years old. Parents reported an unremarkable prenatal history. Clinical evaluation at patient age of 3 months revealed the presence of numerous dysmorphic and metabolic features associated with CGL (Table 1) including 7 clinical features diagnostic for CGL seipinopathies (1-7, Table 1). Physical examination revealed generalized lipodystophy, large hands and feet, and enlarged tongue (8-10, Table 1), a low anterior hairline and low set ears (11-12, Table 1), hepatomegaly and an umbilical hernia (13, Table 1).

The patient presents with **cardiovascular system** abnormalities including concentric hypertrophic cardiomyopathy, with left heart-ventricular enlargement and thickened intraventricular septum detected using imaging techniques (5, Table 1). Heart muscle contractility was good. Abdominal ultra sound showed a hyperechogenic liver (3, Table 1). Pathology evaluation of liver biopsy specimen showed microvesicular steatosis and intertrabecular fibrosis (3, Table 1).

Metabolic abnormalities present from birth included elevated serum triglycerides (4.01 mmol/l, normal range 0.4-1.8 mmol/l), low HDL-C (0.58 mmol/l, normal range 0.9-2.0 mmol/l)

Oral glucose tolerance test (OGTT) was normal – fasting glucose was 4.7 mmol/l and after 120 min – 4.7 mmol/l, glycated hemoglobin (HbA1c) – 4.82% (normal range 4.5-6.5%). Other abormal laboratory studies included mildly elevated serum alanine aminotransferase (ALAT) (50.2 U/l, normal range 0-41 U/l) and high alkaline phosphatase (ALP) levels (351 U/l, normal range 20-150 U/l) levels. All of these metabolic imbalances except elevated alkaline phosphatase, are progressive conditions (1-6, Table 1).

At age 7 years our patient displayed acanthosis nigricans on the nape of the neck and in axillary and popliteal regions and prominent musculature, due to general absence of metabolic fat tissue and abnormal fat deposition in muscles, (14-17, Table 1). At this age we noted that despite absence of metabolic fat, mechanical fat tissue was maintained. We also found accelerated linear growth velocity, concomitant with accelerated skeletal maturation of 2 years. Serum concentration for growth hormone was low and an MRI scan of the hypophysis was normal. Gigantism was not observed, despite the appearance of acromegaloid features.

At age 14, neurological symptoms (18-22, Table 1) began to emerge. Learning disability (IQ score 88) (18, Table 1) was first noted at this age. We also noted acroparaesthaesiae of both hands (19, Table 1). In addition we found changes in electromyogram (EMG) tracings, mildly reduced neurogenic pattern and decreased motor fibre nerve conduction velocity (NCV) in median, sural and peroneal nerves; also for sensory fibres in median and sural nerves (20-22, Table 1). MRI scans however showed no sign of median nerve compression. Normal serum

calcium phosphorus, magnesium, and parathormone concentrations further excluded hypoparathyroidism.

At age 16, the patient presented with multiple endocrine abnormalities. In addition, at 16 years, a number of clinical features emerged relating to hormonal disturbances (23-30, 32 Table 1). She presented with primary amenorrhoea with clinical and laboratory assay symptoms of hyperandrogenism (clitoral enlargement and free testosterone 11.33 pg/ml, normal range 1.1-6.3 pg/ml, androstenedione 3.60 ng/ml, normal range 0.8-2.4 ng/ml, respectively) were noted (28,30 Table 1). Sonographic evaluation showed atrophic ovaries with no ovarian follicles (28, Table 1). Hirsutism was also first noted (32, Table 1). Hormonal function of the hypophysis was normal.

The patient also presented with diabetes mellitus (DM) (27, Table 1) identified on the basis of an OGTT (0 min – 3.7 mmol/l; 120 min – 11.9 mmol/l) with a high homeostasis model assessment of insulin resistance value (10.39). HbA1c was 4.6%.

Current status: At 27 years, our patient has graduated from college and is employed as a clerk in an office. Paraesthesiae of the hands has not aggravated since first appearance. Diabetes is well-controlled (HbA1c – 5,1%), but dyslipidemia persists despite aggressive therapy (serum triglycerides – 3.8 mmol/l; HDL-C – 0.29 mmol/l). Current medications include metformin (3 g/day), fenofibrate (267 mg/day), rosuvastatin (10 mg/day) and insulin (1.5 UI/kg/day).

Molecular analyses

WES analysis reveals a homozygous 52 bp intronic deletion, g.12562_12613del p.(Val197Glufs*32), affecting the 5'splice site for exon 5 of the *AGPAT2* gene. Bioinformatic prediction software (MutationTaster) indicates the deletion (g.12562_12613del) is of unknown pathogenicity. This variant is absent from the ExAC and 1000G databases. No rare variant alleles in other known disease associated genes were found which could potentially explain the lipodystrophy phenotype or the neuropathy observed in our patient[3].

Sanger sequencing confirmed the *AGPAT2* deletion variant and cosegregation with the disease trait according to Mendelian expectations (Figure 1A), and also showed the expected reference sequence around the deletion at the nucleotide level (Figure 1B). This shows the patient is homozygous for the g.12562_12613del p.(Val197Glufs*32) allele. Also, each clinically asymptomatic parent is heterozygous for the identical variant allele (Figure 1C-D). Standard PCR (Figure 1E, upper image) on genomic template DNA from both patient and parents generates a PCR product shorter (red arrowhead) than the wildtype (grey arrowhead), consistent with a 52 bp genomic deletion. Standard PCR on cDNA templates (lower image) reveals a PCR product shorter by 75 bp for the patient *AGPAT2* mRNA (red arrow) relative to the wildtype control individual mRNA (grey arrowhead). Direct Sanger sequence of the cDNA PCR products shows complete deletion of exon 5 (75 bp), leaving exon 4 joined to exon 6 with frameshifted coding sequence downstream of the join creating a premature translation stop signal. The parents are each heterozygous for the deletion and generate both forms of mRNA, and therefore show both mutated and wildtype PCR products (Figure 1E, mother, father).

To assess mutant *AGPAT2* mRNA expression levels and/or stability we used Real Time PCR (RT PCR) to quantify mutant and WT *AGPAT2* mRNA. Expression levels of AGPAT2 mRNA generated from patient (homozygous for deletion allele) and both parent (heterozygous for deletion allelle) mRNA samples are comparable to those of a healthy control individual (homozygous for wiltdtype allele) (Figure 1F). Stability and/or transcription levels therefore appear unaffected for the mutant mRNA. We conclude *AGPAT2* mRNA expression levels and stability in our patient remain unaffected by this deletion.

Methods:

Genomic DNA samples were isolated from blood leucocytes from each individual. Copy number variations (CNV) were identified using array Comparative Genomic Hybridization (aCGH) and bioinformatic analyses using XHMM[4] and HMZDelFinder[5] algorithms; single nucleotide variation (SNV) was determined by WES analysis[6], and confirmed by Sanger sequencing. Chromosomal regions demonstrating absence of heterozygosity (AOH) were detected by analyzing B-allele frequency data obtained from WES[6] by running BafCalculator[7] (https://github.com/BCM-Lupskilab/BafCalculator). *AGPAT2* expression was measured by quantitative real-time polymerase chain reaction (RT PCR) (TaqMan Gene Expression Assay for *AGPAT2* gene (Life Technologies, Grand Island, NY, USA), on blood lymphocyte mRNA isolated using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA). Level of *AGPAT2* expression is corrected to the mRNA level of housekeeping genes *GAPDH* and *TBN*. Expression data reflect means of three independent experiments each performed in triplicate.

Primers and probes:

gDNA PCR

F: CTCACTGGCTTCCTGAGATGG; R: GGTCCATCCGTGTGAAGTCT

cDNA PCR

F: GGGAGAACCTCAAAGTGTGG; R: GGTCTTGGAGATGTGGAGGA

RT PCR

TaqMan Gene Expression Assay, Thermofisher labelled probes cat no. HS00944961.

Study approval: Bioethics Committee for Institute of Mother and Child, Warsaw. Informed consent was obtained from the patient and her parents.

Discussion

The detailed, lifelong clinical case history reveals findings diagnostic for Berardinelli-Seip syndrome from infancy. Childhood mechanical fat distribution differentially diagnostic for CGL1, in this case however, was followed by development of polyneuropathy and cognitive disability in early adolescence, symptoms not previously reported in CGL1 patients. Intellectual disability is typical for CGL2 and rare in other forms of CGL. Primary neuropathy in the absence of diabetes mellitus complications leading to neural pathology, is associated with CGL2 but is to date, not reported for CGL1. Polyneuropathy is associated with a range of lipodystrophic disorders (AKINCI ref here), but in CGL1 patients, the neuropathy reported to date arises from diabetic complications or other secondary conditions. In our patient, laboratory test evidence of diabetes was only found 2 years after initial development of neuropathy. The polyneuropathy observed here is therefore unlikely to be a diabetic complication. (EMG/NCV) results (see 20-21 Table 1) suggest demyelinating neuropathy similar to that caused by duplications in the *PMP22* gene responsible for Charcot-Marie Tooth type 1A syndrome. However, no *PMP22* duplication was detected, nor were any recessive CMT genes found to map within AOH intervals. The possibility that elevated patient triglyceride levels contribute to the clinical manifestation of peripheral neuropathy however, cannot be excluded.

All forms of CGL involve complete lack of metabolic fat (body fat) from birth and the majority show early presentation of severe hypertriglyceridemia, hepatic steatosis, hepatosplenomegaly, acanthosis nigricans and insulin resistance, generally leading to diabetes in early adolescence. Enlargement of liver tissue and slightly enlarged hands and feet are also typical. Myocardiopathies arise in approximately 25% of individuals. The emergence at age 16 in our patient, of multiple symptoms related to hormonal disturbances after puberty (eg. polycystic ovarian syndrome and hyperinsulinemia) is also typical for all Berardinelli-Seip syndromes including CGL1. Our patient presents with all these CGL-associated symptoms by

early-mid adolescence, with concomitant emergence of neuropathological symptoms and learning disability in early adolescence. In addition, umbilical hernia, present in our patient at 3 months, was found by one study to be associated only with *BSCL2* mutations[9]. The clinical picture therefore suggests CGL2, despite the normal mechanical fat distribution differentially diagnostic for CGL1[1].

Our genomic investigation nonetheless confirms a previously unknown, single exon, homozygous 52bp deletion in *AGPAT2;* a gene uniquely associated with CGL1 seipinopathy. Given the inability to identify any other known disease genes that might explain the unusual phenotypic features (i.e. polyneuropathy, cognitive deficiency) associated with known bona fide *AGPAT2*-related CGL1 clinical manifestations in this patient – we suggest a potential phenotypic expansion. Whether this new mutation causes the neuropathology observed however, remains unresolved. Our molecular analyses show the intronic 5' splice site deletion eliminates 25 codons of protein coding sequence and generates a frameshift resulting in a premature translation stop codon. There is no evidence for mutant *AGPAT2* mRNA instability in the blood cell studies. Structure and function of any AGPAT2 protein in our patient however would likely be impaired.

Precisely how this would affect physiological pathways involving AGPAT2 is unknown. The AGPAT2 protein is located in the membrane of the endoplasmic reticulum, and is primarily involved in triglyceride and phospholipid biosynthesis, with multiple interaction partners involved in lipid biosynthesis/degradation and related pathways including acyl chain remodelling of phosphatidylethanolamine (PE)[10], adipose droplet formation, lipid signalling and ER and mitochondrial membrane transport pathways[11,12]. Many CMT neuropathy genes involve this transport biology, hinting at possible overlapping molecular bases[12] for the polyneuropathy observed in this patient. Disruption of post-translational protein-protein interactions central to lipid homeostasis and of related pathway function, such as cholesterol metabolism, are highly probable and likely to have fundamental physiological effects. Other mutations leading to similar disequilibrium of lipid homeostasis, phospholipid degradation and remodelling in ER and mitochondrial membranes for example, have been linked to neural degeneration and epileptic seizures in flies and worms, with similar phenotypes for mutations in human gene counterparts[13]. Disruption of phospholipid homeostasis is also linked to α-synuclein protein aggregation implicated in Parkinson Disease pathology[11,14]. Disrupted cholesterol metabolism, also, is linked to protein aggregation leading to mitochondrial distribution defects and neurodegenerative disease[15,16].

We remark that known *AGPAT2* regulatory circular RNA (circRNAs; non-coding post-transcriptional splicing products) expression levels are high in normal foetal tissues, including adrenal tissue, which regulates circulating hormonal levels in the developing foetus[17,18]. Recent elucidation of fundamental functions for circular regulatory RNA in eukaryotic gene expression programs[19] highlights the potential for future investigations into defective RNA processing during foetal development as a possible contributor to genetic disorders.

Finally, an epidemiological note: *AGPAT2* mutations predominate in American CGL cases of African descent. The vast majority of European seipinopathies arise from mutations in *BSCL2*[20]. The newly identified *AGPAT2* deletion in our patient is located within a large block (approxmately 1.1Mb) of AOH (chromosome sequence with both alleles identical at nucleotide sequence level) (Figure 1, C-D). Blocks of AOH arise when parentage is related, as is the case for this patient. The rare deletion in *AGPAT2* we identify appears to have arisen in an individual of a small European village and accumulated in the local population over generations.

In short, this work documents the first report of primary polyneuropathy within the classical clinical CGL1 syndrome exhibiting differentially diagnostic mechanical fat retention, establishing a potential phenotypic expansion for CGL1 disease. We further identify a new, recessive intronic splice site deletion in the CGL1-associated *AGPAT2* locus, resulting in an apparently translatable truncated mRNA species with missense coding. Precisely how the splicing defect identified affects AGPAT2 protein physiology or noncoding transcriptional regulatory RNA functions remains undefined. This however, is the case for all *AGPAT2* mutations linked to CGL1 phenotype: the mechanism of action has not been defined for any[12].

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J.R.L. has stock ownership in 23andMe and is a paid consultant for Regeneron. J.R.L. is a coinventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases, and bacterial genomic fingerprinting. The Department of Molecular and Human Genetics at Baylor College of Medicine derives revenue from the chromosomal microarray analysis (CMA) and clinical exome sequencing offered at Baylor Genetics (MGL; http://www.bcm.edu/geneticlabs/). The other co-authors declare that they have no conflict of interest. Study support: National Science Centre, Poland 2015/19/B/NZ2/01824. Also funded in part by the US National Human Genome Research Institute (NHGRI)/ National Heart Lung and Blood Institute (NHLBI) grant number UM1HG006542 to the Baylor-Hopkins Center for Mendelian Genomics (BHCMG).

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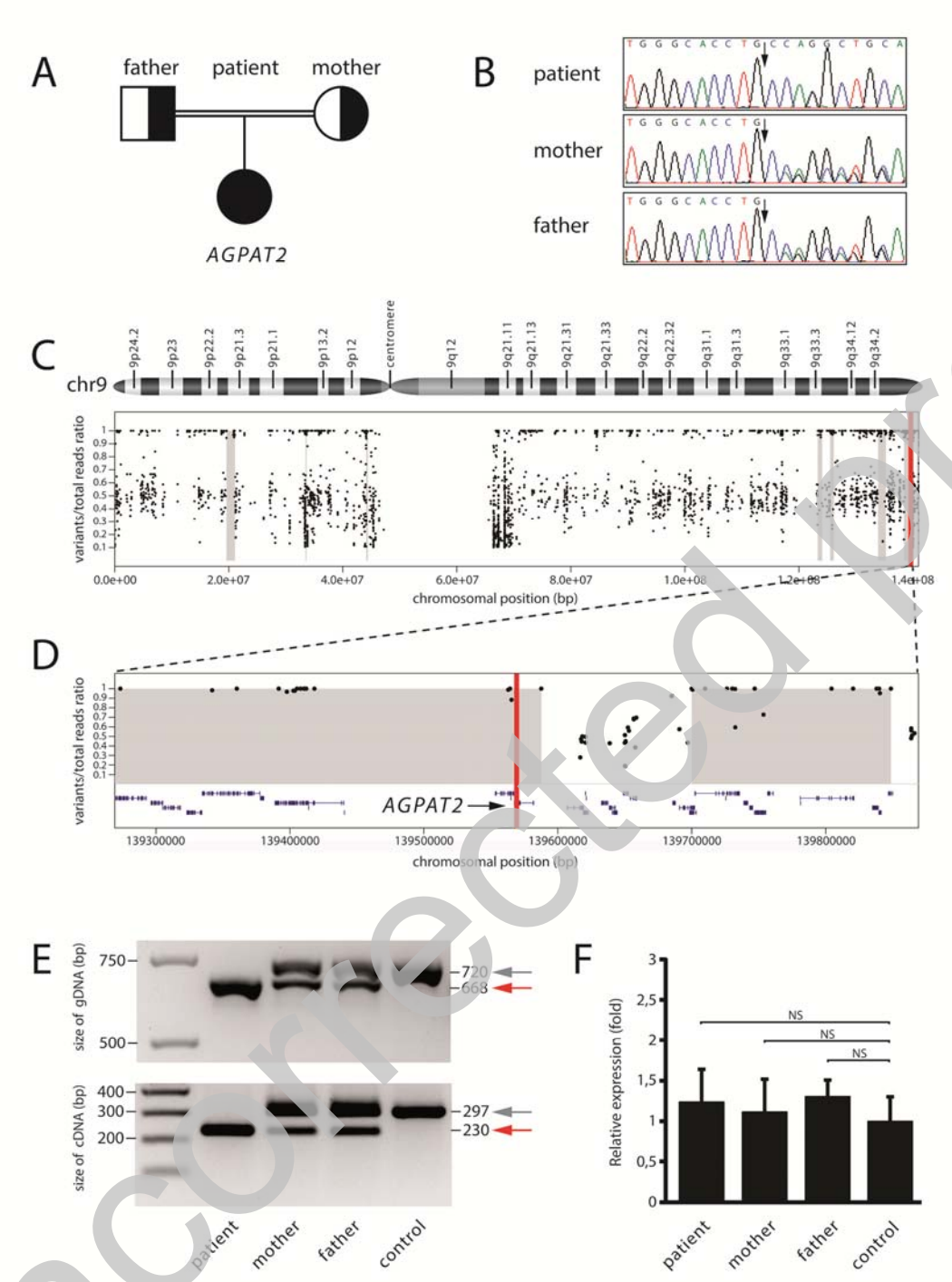

Figure 1. (**A**) Patient pedigree for homozygous *AGPAT2* deletion mutation c.589-55_589-4del p.(Val197Glufs*32). **(B)** Sanger sequence confirmation of biparental inheritance for the mutation. Black arrow shows point of deletion. (**C**) Genomic context of *AGPAT2* mutation. Cartoon shows Chromosome 9 organization. Grey blocks denote regions with absence of heterozygosity (AOH). Scattered dots indicate SNV for proband along chromosome 9. Absence of SNV in centromere-adjacent areas reflects lack of reference sequence for these region. **(D)** Detail for AOH region surrounding the *AGPAT2* mutation (vertical red line at around 13 958 000 base pair). (**E**) PCR products for *AGPAT2* in patient (mutation homozygous), each parent (mutation heterozygous) and control individual (wildtype

homozygous). Red arrowhead, reduced *AGPAT2* product size reflecting deletion mutation. Grey arrowhead, normal size wildtype *AGPAT2* product. Genomic gDNA template, upper image; complementary cDNA template lower image. (**F**) RT PCR products for *AGPAT2* mRNA expression levels in patient, parents and control are comparable. NS: nonsignificant.

Table 1. *Developmental timeline for clinical emergence of Seip-Berardinelli syndrome and neuropathology features in our patient.*

#	FEATURES	HPO no.	Age at emergence	Familial features (Grandfather)
1	Congenital generalized lipodystrophy **x**	HP:0009059	+++ 3 m ●	+++ ▲
2	Hypertriglyceridemia	HP:0002155	+++ 3 m ●	Nd
3	Hepatomegaly **x**	HP:0002240	++ 3 m ●	Nd
4	Hepatic steatosis **x**	HP:0001397	++ 3 m ●	Nd
5	Concentric hypertrophic cardiomiopathy **x**	HP:0005157	++ 3 m ●	Nd
6	Elevated hepatic transaminases (ALAT)	HP:0002910	+ 3 m ●	Nd
7	Elevated alkaline phosphatase	HP:0003155	+ 3 m	Nd
8	Large hands **x**	HP:0001176	+ 3 m	Nd
9	Large feet **x**	HP:0001833	+ 3 m	Nd
10	Increased size of tongue	HP:0000158	+ 3m	Nd
11	Low anterior hairline	HP:0000294	+ 3m	Nd
12	Lowset ears	HP:0000369	+ 3m	Nd
13	Umbilical hernia **x**	HP:0001537	+ 3m	Nd
14	Acanthosis nigricans **x**	HP:0000956	+++ 7 y ●	+++ ▲
15	Accelerated skeletal maturation **x**	HP:0005616	+7y	Nd
16	Accelerated linear growth **x**	HP:0000098	+7y	Nd
17	Abnormality of the musculature **x**	HP:0003011	+ 7 y	Nd
18	Intelectual disability	HP:0001256	+ 14 y	Nd
19	Acroparesthesia (hands)	HP:0031006	+ 14 y	Nd
20	Electromyography (EMG): neuropathic changes	HP:0003445	+ 14 y	Nd
21	Decreased motor nerve conduction velocity (NCV)	HP:0003431	+ 14 y (median nerve and peroneal nerves)	Nd
22	Decreased sensory NCV	HP:0003448	+ 14 y (median and sural nerves)	Nd
23	Amenorrhea x	HP:0000141	+++ 16 y	Na
24	Polycystic ovarian syndrome **x**	HP:0000147	+++ 16 y	Na
25	Hypoplasia of the ovary	HP:0008724	+++16 y	Na
26	Hyperinsulinemia x	HP:0000842	+++ 16 y	Nd
27	Noninsulin-dependent diabetes mellitus **x**	HP:0005978	++ 16 y ●	++ ▲
28	Increased circulating androgen level	HP:0030348	++ 16 y	Nd
29	Labial hypertrophy	HP:0000065	++ 16 y	Na
30	Clitoromegaly **x**	HP:0008665	++ 16 y	Na
31	Weakness of the intrinsic hand muscles (asymmetric)	HP:0009005	+ 16 y	Nd
32	Hirsutism **x**	HP:0001007	+ 16 y	Nd

Seip-Berardinelli symptoms are shown on white background, **primary** neuropathologies are shaded grey.; HPO no., Human Phenotype Ontology database identification number for

phenotypic abnormality. ; EMG, electromyogram; NCV, nerve conduction study. Symbols: +++, strong presentation; ++, medium presentation; +, mild presentation; nd, no data; na, not applicable; y, years; m, months; ●, progressive condition; ▲, age at emergence unknown.; **x**-diagnostic for CGL1.