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1 **Biallelic *CAVI* null variants induce Congenital Generalized Lipodystrophy with achalasia**

2

3 **Short title: *CAVI*, generalized lipodystrophy and achalasia**

4

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25

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27

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41

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43

44 **Abstract**

45 **Objective:** *CAVI* encodes caveolin-1, a major protein of plasma membrane microdomains
46 called caveolae, involved in several signalling pathways. Caveolin-1 is also located at the
47 adipocyte lipid droplet. Heterozygous pathogenic variants of *CAVI* induce rare heterogeneous
48 disorders including pulmonary arterial hypertension and neonatal progeroid syndrome. Only
49 one patient was previously reported with a *CAVI* homozygous pathogenic variant, associated
50 with congenital generalized lipodystrophy (CGL3). We aimed to further delineate genetic
51 transmission, clinical, metabolic and cellular characteristics of CGL3.

52 **Design/Methods:** In a large consanguineous kindred referred for CGL, we performed next-
53 generation sequencing, as well as clinical, imagery and metabolic investigations. We studied
54 skin fibroblasts from the index case and the previously reported patient with CGL3.

55 **Results:** Four patients, aged 8 months to 18 years, carried a new homozygous p.(His79Glnfs*3)
56 *CAVI* variant. They all displayed generalized lipodystrophy since infancy, insulin resistance,
57 low HDL-cholesterol and/or high triglycerides, but no pulmonary hypertension. Two patients
58 also presented at the age of 15 and 18 years with dysphagia due to achalasia, and one patient
59 had retinitis pigmentosa. Heterozygous parents and relatives (n=9) were asymptomatic, without
60 any metabolic abnormality. Patients' fibroblasts showed a complete loss of caveolae and no
61 protein expression of caveolin-1 and its caveolin-2 and cavin-1 partners. Patients' fibroblasts
62 also displayed insulin resistance, increased oxidative stress and premature senescence.

63 **Conclusions:** The *CAVI* null variant investigated herein leads to an autosomal recessive
64 congenital lipodystrophy syndrome. Loss of caveolin-1 and/or caveolae induces specific
65 manifestations including achalasia which requires specific management. Overlapping
66 phenotypic traits between the different *CAVI*-related diseases require further studies.

67

68 **INTRODUCTION**

69

70 Caveolae are plasma membrane microdomains that act as signalling platforms in several cell
71 types, including adipocytes, smooth muscle cells, endothelial cells and fibroblasts. The integral
72 membrane protein caveolin-1, encoded by the *CAVI* gene, is required for caveolae formation
73 and is the main protein component of caveolae. Caveolin-1 interacts, among others, with the
74 insulin receptor and contributes to the compartmentalization of insulin signaling pathways (1).
75 Caveolin-1 is also a fatty acid-binding protein able to translocate from the plasma membrane
76 to the adipocyte lipid droplets, therefore contributing to the regulation of lipid storage (2,3). In
77 addition, it plays a role in tumor suppression and oxidative stress-induced cellular senescence
78 (4). Loss of caveolin-1 expression in mice induces several defects including progressive
79 lipodystrophy with insulin resistance and hypertriglyceridemia (5,6), cardiomyopathy and
80 pulmonary hypertension (7,8).

81

82 The phenotypic spectrum of the rare *CAVI* genetic defects in humans remains difficult to
83 delineate. A single patient has been previously reported with a homozygous *CAVI* pathogenic
84 variant. This patient was a young woman described with congenital generalized lipodystrophy
85 (CGL), severe insulin-resistant diabetes, and hypertriglyceridemia, referred to as CGL3 (9).
86 Heterozygous *CAVI* null variants, including nonsense and frameshift mutations, have been
87 reported so far in ten patients with different phenotypes, *i.e.* partial lipodystrophy with
88 neurological involvement (10), precocious and severe pulmonary arterial hypertension (11-13)
89 and/or neonatal progeroid syndromes (14-16).

90

91 In the present study, we investigated a large consanguineous kindred referred for CGL and
92 identified a novel homozygous *CAVI* frameshift variant in four affected young patients.

93 Besides variable insulin resistance-associated metabolic abnormalities, esophageal achalasia,
94 leading to severe dysphagia, emerges as a specific comorbidity. One affected patient also
95 displayed atypical retinitis pigmentosa. By studying cultured skin fibroblasts from the index
96 case and from the previously reported patient with a homozygous *CAVI* nonsense variant (9),
97 we show that CGL3 is associated with a complete loss of protein expression of caveolin-1 and
98 its partners caveolin-2 and cavin-1, and with the absence of caveolae at the plasma membrane.
99 Fibroblasts from patients also display insulin resistance, increased oxidative stress and
100 premature senescence.

101

102 **PATIENTS AND METHODS**

103

104 **Patients**

105 This study includes nineteen individuals from a large Turkish consanguineous family
106 investigated at Mersin University, Department of Pediatric Gastroenterology, Hepatology and
107 Nutrition, Turkey. Genetic studies were performed in the Department of Molecular Biology
108 and Genetics, and the disease features were reviewed in the French Reference Center for Rare
109 Diseases of Insulin Secretion and Insulin Sensitivity (PRISIS), both at Assistance-Publique
110 Hôpitaux de Paris, Saint-Antoine Hospital, Paris, France. Clinical and molecular studies, and
111 skin biopsy were performed after full written informed consent, according to the Ethics
112 Committee of Mersin University. The study was approved by a French institutional research
113 ethics board (CPP Ile de France 5). Written informed consent for publication of their clinical
114 details and/or clinical images was obtained from the patients' parents, from patients themselves
115 when aged above 12, and from their relatives.

116

117 **Genetic analyses**

118 Exons and flanking intronic sequences of a panel of 23 genes involved in lipodystrophic and/or
119 insulin resistance syndromes, including *CAVI*, were sequenced from genomic DNA in Patient
120 1, as described (17) (**Figure 1**). Sanger sequencing was performed with the Big Dye Terminator
121 v3.1 sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) after polymerase chain
122 reaction. Data were analyzed on a 3500xL Dx device with the SeqScape v2.7 software (Thermo
123 Fisher Scientific). *CAVI* variants were described based on the longest isoform (NM_001753.4)
124 using Alamut 2.11 (Sophia Genetics, Switzerland) and Human Genome Variation Society
125 guidelines.

126

127 **Fibroblast cultures**

128 Primary fibroblast cultures from Patient 1 were established after skin biopsy. Cultured
129 fibroblasts from the previously reported patient with *CGL3*, carrying the homozygous *CAVI*
130 p.Glu38* pathogenic variant (9), and from two non-obese, non-diabetic, normotensive women
131 who underwent plastic surgery (18) were studied at similar passages. Cells were grown in
132 DMEM low glucose with pyruvate (#31885049; Thermo Fisher Scientific) supplemented with
133 10% fetal bovine serum (D. Dutscher, Bernolsheim, France), 1% penicillin/streptomycin, and
134 2 mM glutamine (Invitrogen, Cergy-Pontoise, France).

135

136 **Transmission electron microscopy**

137 Cultured fibroblasts were fixed in 2.5% glutaraldehyde, 0.1M cacodylate buffer at 4°C, rinsed
138 in 0.2M cacodylate, post-fixed in 1.5% potassium ferrocyanide 1% osmium tetroxide,
139 dehydrated using graded alcohol series, then embedded in epoxy resin. Semi-fine sections (0.5
140 µm) were stained with toluidine blue. Ultrathin sections (70 nm) were contrasted with
141 UranylLess and lead citrate (Delta Microscopies, France) and examined using an electron
142 microscope (JEOL 2110 HC, Croissy, France) with a 2Kx2K Veleta CCD camera (Olympus,

143 Rungis, France).

144

145 **Western blot**

146 Protein expression studies were performed on whole cell extracts using antibodies described in

147 **Supplemental Table 1.**

148

149 **Cellular response to insulin**

150 To measure their ability to bind insulin, fibroblasts were maintained for 16 hours in serum-free
151 medium supplemented with 0.1% albumin (Sigma-Aldrich, Saint-Louis, MO, USA), then
152 incubated with ¹²⁵I-insulin (0.3 ng/mL, PerkinElmer, Coutaboeuf, France) with or without
153 unlabeled insulin (5.10⁻⁸M) in Hepes buffer at pH 7.65, 15°C, for 5 hours. Radioactivity was
154 measured in a gamma counter (PerkinElmer 2470 Wizard2) and results were normalized to
155 protein content. Insulin effect on glycogen synthesis was evaluated by the incorporation of ¹⁴C-
156 glucose as previously described (19). Results were normalized to the protein content and
157 expressed as a percentage of the basal value.

158

159 **Oxidative Stress and Cellular Senescence**

160 The production of reactive oxygen species (ROS) was assayed by quantifying the oxidation of
161 5-6-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA). The blue
162 staining produced by hydrolysis of X-gal (5-bromo-4-chloro-3-indolyl-β-Dgalactopyranoside)
163 by senescence-activated-β-galactosidase at pH 6.0 was used as a biomarker of cellular
164 senescence, as previously described (20). The ratio of pH 6-to-pH 4 staining of blue X-gal was
165 quantified at 630 nm.

166

167 **Statistical analyses**

168 GraphPad Prism software (GraphPad Software Inc., CA, USA) was used to calculate statistical
169 significance with a threshold at $p < 0.05$. Gaussian distribution was tested with Kolmogorov–
170 Smirnov test. Differences between two groups were assessed by unpaired two-sample t-tests or
171 Mann-Whitney tests and multiple comparisons between more than two groups were conducted
172 by ANOVA with Bonferroni-test or Kruskal-Wallis test for post-hoc analysis. All data are
173 means \pm SEM of at least three independent experiments.

174

175 **RESULTS**

176 **Identification of a novel *CAVI* pathogenic variant responsible for an autosomal recessive** 177 **congenital generalized lipodystrophy syndrome**

178 Patient 1, a 15-year-old girl from a large consanguineous Turkish family was referred for
179 genetic investigation of CGL (**Figure 1**). We identified a novel homozygous *CAVI* variant
180 (NM_001753.4) : c.237_238del, p.(His79Glnfs*3) through sequencing of a panel of 23 genes
181 involved in lipodystrophy and/or insulin resistance syndromes. This variant, confirmed by
182 Sanger sequencing, was absent from the gnomAD and ExAC databases that list genetic variants
183 from the general population, and is classified as pathogenic according to the American College
184 of Medical Genetics and Genomics (ACMG) criteria (21). The deletion of 2 nucleotides in exon
185 3 leads to a shift in the reading frame. If the transcript is expressed, it is predicted to result in
186 the synthesis of an abnormal form of caveolin-1, truncated in its oligomerization domain and
187 deprived from its scaffolding and intra-membrane domains (**Figure 2**). Sequencing of the gene
188 panel did not reveal any other pathogenic variant. Patient 1 was thus diagnosed with CGL3.

189 The same *CAVI* variant was observed in the homozygous state in Patient 2, who was
190 subsequently referred with similar symptoms, and in Patients 3 and 4, diagnosed with CGL
191 after familial investigations. The parents of Patients 1 to 4 were asymptomatic. Genetic
192 investigations, performed in seven of them, revealed that they were heterozygous carriers of

193 the variant. Unaffected siblings of the patients (n=8) were heterozygous for the variant or had
194 a normal genotype.

195 This family thus showed an autosomal recessive transmission of CGL3 due to a novel
196 p.(His79Glnfs*3) *CAVI* pathogenic variant (**Figures 1 and 2**).

197

198 **Phenotype of the disease**

199 **Table 1** summarizes the patients' main phenotypic features.

200

201 **Patient 1**

202 Patient 1 was referred at the age of 15 to Mersin University with difficulty in swallowing liquids
203 and solids. She was born from first-cousin parents of Turkish origin, at term after an uneventful
204 pregnancy, with a birth weight and height of 2800g and 48cm respectively. At examination, her
205 height was 153 cm, BMI was 17.9, Tanner pubertal stage 5. She showed a triangular and
206 acromegaloid face, with a generalized loss of fat sparing palms and soles, a taut and thin mottled
207 skin with visible dermal vessels, extensive acanthosis nigricans in the armpits and groins, a
208 prominent musculature, and enlarged hands and feet (**Figure 3A-C**). She also complained about
209 hirsutism, with secondary amenorrhea since the age of 14. The family noticed her dysmorphic
210 facial and body appearance during early infancy. Although her linear growth was delayed with
211 poor weight gain during infancy, she was described with a voracious appetite. She did not
212 present with any skeletal deformity, joint contractures or muscular functional defect. No
213 cognitive delay was observed and she had normal developmental milestones. Neurological
214 examination was normal. Blood pressure was normal.

215 Laboratory parameters showed hypertriglyceridemia (5.4 mmol/L), low HDL-cholesterol (0.73
216 mmol/L), major fasting hyperinsulinemia (300 mIU/L) with normal glycemia (5 mmol/L) and
217 HbA1c (5.8%). Liver tests and creatine kinase were normal as well as kidney and thyroid

218 function. Androgen levels were in the normal range. Serum luteinizing hormone (LH, 13.9
219 IU/L) was higher than follicle-stimulating hormone (FSH, 5.8 IU/L). Estradiol levels were in
220 the normal range for the follicular phase. Calcemia and parathormone serum and urinary levels
221 were normal, with decreased vitamin D level and bone mineral density (Z score -2.78 at lumbar
222 spine).

223 Generalized lipoatrophy was confirmed by magnetic resonance imaging (MRI) (**Figure 4**), and
224 by very low leptin levels (0.3 ng/mL), also consistent with hyperphagia. MRI also revealed
225 enlarged polycystic ovaries (**Figure 5**). Electrocardiogram and echocardiogram, including
226 estimated right ventricular systolic pressure, were normal. Liver steatosis was diagnosed with
227 ultrasonography. Medical history, physical examination and results of metabolic investigations
228 allowed the diagnosis of CGL. A treatment with metformin and medium-chain triglycerides
229 was initiated.

230 Dysphagia was investigated with several procedures. Barium esophagogram series showed a
231 dilated esophagus with a distal bird's beak deformity (**Figure 6A**), and a severe stenosis of the
232 distal esophagus was confirmed by endoscopy. High resolution esophageal manometry showed
233 the absence of lower esophageal sphincter relaxation with increased integrated relaxation
234 pressure (51 mmHg) and decreased peristalsis, consistent with esophagogastric junction
235 outflow obstruction (22) (**Figure 6 B,C**). Based on clinical, endoscopic, radiological and
236 manometric findings, an early stage of achalasia was diagnosed. Peroral endoscopic myotomy
237 (POEM) was performed leading to a significant relief in dysphagia-related symptoms.

238

239 **Patient 2**

240 Patient 2 was an 18-year-old cousin of Patient 1 (**Figure 1**) referred for difficulty in swallowing.
241 Generalized lipodystrophy and mild dysmorphic features were present from early infancy. His
242 symptoms and physical examination were very similar to those of Patient 1 (**Table 1, Figure**

243 **3D**). However, his metabolic abnormalities were milder, with low HDL-cholesterol but normal
244 triglyceridemia. Although glucose tolerance was normal, normal-to-high 2h-postprandial
245 insulin (45.8 mIU/L, N: 4-52.5 at T120 min after oral glucose tolerance test) (23) and C-peptide
246 levels (8.32 µg/L, N: 1.1-4.4) suggested insulin resistance. Blood pressure, cardiac examination
247 including echocardiography were normal, as well as liver, renal and thyroid function, and
248 testosterone and FSH levels. Type 2 achalasia, diagnosed upon radiological, endoscopic and
249 manometric findings (**Figure 6 D-F**), was successfully treated by POEM. Patient 2 also
250 complained of trouble seeing at night for two years. Although neurological examination and
251 ophthalmological fundus analysis were normal, optical coherence tomography (OCT) revealed
252 bilateral loss of ellipsoid line and atrophy of the retinal pigment epithelium, and defective outer
253 limiting membrane in the left eye, suggesting atypical retinitis pigmentosa.

254

255 **Patient 3 and Patient 4**

256 Patient 3, a 10-year-old girl, and Patient 4, an 8-month-old girl, were investigated during the
257 systematic familial screening (**Figure 1**). As Patients 1 and 2, they were born from
258 consanguineous parents, at term after normal pregnancies, with normal birth weight and height.
259 Their linear growth was delayed and they presented with a dysmorphic appearance and
260 generalized lipoatrophy, which were noticed by the family during early infancy (**Figure 3 E,F**).
261 Their physical examination was otherwise normal. They both achieved normal developmental
262 milestones. Laboratory investigations revealed increased levels of triglycerides and insulin, and
263 decreased HDL-cholesterol (**Table 1**).

264

265 **Patients' relatives**

266 Relatives of Patients 1 to 4 (n=15) (**Figure 1**) had no complaint and did not show any sign of
267 lipodystrophy. Their anthropometric measurements and physical examination were normal,

268 except for the older brother of Patient 1 who presented with a mild mental retardation of
269 unknown origin. Their laboratory evaluation including full blood count, fasting glucose and
270 insulin, HbA1c, liver function tests and serum lipids, were normal.

271

272 **Homozygous *CAVI* p.(His79Glnfs*3) and p.(Glu38*) pathogenic variants induce a loss of**
273 **protein expression of caveolin-1, caveolin-2 and cavin-1, and a loss of caveolae formation**
274 **in patients' fibroblasts**

275 We compared cultured fibroblasts from Patient 1 and from the previously reported patient with
276 CGL due to the homozygous p.(Glu38*) *CAVI* pathogenic variant (9), to those from controls.
277 The p.(His79Glnfs*3) and the previously described p.(Glu38*) *CAVI* pathogenic variants
278 predict to interrupt the caveolin-1 aminoacid sequence at the N-terminal part of the protein,
279 proximally to its scaffolding and intra-membrane domains (24) (**Figure 2**). As expected, we
280 show that, similarly to the fibroblasts carrying the homozygous *CAVI* p.(Glu38*) variant (9),
281 cells from Patient 1 with the novel *CAVI* p.(His79Glnfs*3) variant do not exhibit detectable
282 expression of caveolin-1 (**Figure 7A**). We evaluated the protein expression of caveolin-2 and
283 cavin-1, which are binding partners of caveolin-1. We show that caveolin-2 and cavin-1 protein
284 levels are strongly decreased in patients' cells (**Figure 7A**). Notably, pathogenic variants in
285 *CAVIN1* (previously referred to as *PTRF*), encoding cavin-1, lead to a form of CGL previously
286 associated with achalasia (25). As expected from the major role of caveolin-1 and cavin-1 for
287 caveolae formation, electron microscopy shows that caveolae, abundant in control cells, are
288 completely absent in fibroblasts from patients with *CAVI* null variants (**Figure 7B**).

289

290 **Homozygous *CAVI* null variants impair insulin signaling**

291 We then investigated the insulin-mediated activation of proximal signalling intermediates from
292 the metabolic and mitogenic insulin pathways (*i.e.*, IR β , IRS1, AKT, and ERK1/2) and show

293 that it is strongly impaired in fibroblasts from patients with *CAVI* null variants (**Figure 8A**). In
294 addition, the effect of insulin on its distal signalling, as evaluated by glycogen synthesis, is also
295 severely inhibited in patients' cells (**Figure 8B**). Although the total amount of insulin receptors
296 is not significantly decreased in whole protein extracts from patients' cells (**Figure 8A**), the
297 absence of caveolae, which are normally enriched at the plasma membrane with insulin
298 receptors (1), could impair the initiation of the insulin signal. To test this hypothesis, we
299 evaluated the capacity of fibroblasts to bind insulin, and showed that it was decreased by ~ 20%
300 in cells bearing *CAVI* null variants as compared to control cells (**Figure 8C**). These results
301 suggest that the loss of caveolae could contribute, in part, to the insulin resistance observed in
302 cells from patients carrying *CAVI* null pathogenic variants.

303

304 **Homozygous *CAVI* null variants increase oxidative stress and senescence in fibroblasts**

305 Caveolin-1 has been shown to modulate oxidative stress-induced cellular senescence (4), a
306 pathway which has been involved in lipodystrophic diseases (26). We thus aimed to evaluate
307 the effects of *CAVI* pathogenic variants on the production of reactive oxygen species (ROS)
308 and on cellular senescence. Patients' fibroblasts, as compared to control cells, display a major
309 increase in oxidative stress (**Figure 9A**), as well as elevated levels of senescence markers
310 (**Figure 9B**), and increased senescence-activated- β -galactosidase activity (**Figure 9 C,D**).

311

312 **DISCUSSION**

313 We report here a novel homozygous null pathogenic variant in *CAVI* in four patients with
314 CGL3, and show the autosomal recessive transmission of the disease in a large consanguineous
315 family. This study adds important phenotypic data, since CGL3 was previously described in
316 only one patient (9). By revealing the consequences of *CAVI* loss-of-function in patients' cells,
317 this study also highlights the involvement of caveolin-1 and caveolae in cellular insulin

318 response, oxidative stress and cellular senescence, that could contribute to specific clinical
319 manifestations.

320

321 Insulin resistance and related signs, including acanthosis nigricans, prominent veins, muscular
322 hypertrophy, hypertriglyceridemia, low HDL-cholesterol, hirsutism and/or polycystic ovary
323 syndrome, and hepatic steatosis, are consistently associated with lipodystrophy syndromes (27).
324 These manifestations, together with generalized lipoatrophy, were present in the previously
325 described patient with CGL3 (9) as well as, with variable severity, in the affected patients from
326 this study. As previously described in other CGL subtypes, with the exception of CGL2 (9, 28),
327 lipoatrophy only spared mechanical adipose tissue (from palms, plantar and retro-orbital
328 regions) and bone marrow fat. It can be hypothesized that different adipose tissue depots, which
329 have specific developmental origin and gene expression, could be differently impacted by
330 CGL-associated pathogenic variants (29).

331

332 Lipodystrophy-associated insulin resistance was shown to result, at least in part, from defective
333 adipocyte lipid storage and leptin deficiency leading to cellular lipotoxicity (30). Our studies in
334 patients' fibroblasts suggest that deficient caveolin-1 and cavin-1 protein expression and/or the
335 complete absence of caveolae could also directly contribute to insulin resistance.

336

337 The CGL3 phenotype was previously associated with short stature, hypocalcemia, osteopenia
338 and megaesophagus (9), but whether these signs were due to the homozygous *CAVI* null variant
339 remained elusive. We show that achalasia, diagnosed at age 15 and 18 in two affected patients,
340 should be considered as a main complication of CGL3 requiring careful investigations and
341 specific management. Endoscopic myotomy was successful in the two affected patients, and
342 avoided invasive surgical procedures. Three affected patients from this study had short stature,

343 and one patient (Patient 3) had a height percentile under 25th, contrasting with the accelerated
344 growth frequently described in patients with CGL1 and CGL2, due to *AGPAT2* or *BSCL2*
345 pathogenic variants, respectively (27). Whether this could be due to the severe impairment of
346 insulin-activated mitogenic signalling pathways linked to the loss of caveolae requires further
347 studies. The four patients did not present with any clinical bone or joint abnormality. However,
348 we did observe osteopenia in two of the investigated patients, with a Z-score below -2.5 at the
349 lumbar spine level, contrasting with the increased bone density usually observed in patients
350 with CGL1 or CGL2 (27,31). Interestingly, osteopenia was confirmed by DEXA at the age of
351 21 in the previously described patient with CGL3, with a lumbar spine Z-score of -3 (Dr Chong
352 Kim, personal communication). The decreased bone mass observed in Patient 1 and Patient 2
353 was not explained by vitamin D nor sex steroid hormone levels. In addition, in contrast to
354 hypocalcemia with hypercalciuria observed in the previously reported patient (9), and in
355 caveolin-1 knockout mice (32), serum and urinary calcium were normal in patients from this
356 study. Detailed investigations of calcium homeostasis remains to be performed in CGL3.

357

358 The biallelic pathogenic variants responsible for CGL3, located in exon 2 and proximal exon 3
359 of *CAVI*, lead to similar phenotypes associated with a loss of protein expression of caveolin-1
360 and of its partner caveolin-2 (9, this study). Our results show that these variants also strongly
361 inhibit cavin-1 protein expression, and lead to a complete loss of caveolae at the plasma
362 membrane of patients' fibroblasts. Heterozygous subjects were asymptomatic, suggesting that
363 half amount of functional caveolin-1 is sufficient to avoid any specific pathological
364 consequences. In contrast, *CAVI* variants affecting the C-terminal domain of the protein, were
365 reported as pathogenic in the heterozygous state and lead to rare but heterogeneous diseases
366 (**Figure 2**) that may result from different pathophysiological mechanisms. Functional studies
367 of the *CAVI* p.(Leu159Serfs*22) variant, responsible for autosomal dominant pulmonary

368 arterial hypertension, showed that the mRNA and protein expression of the mutated allele is
369 preserved, and that the resulting abnormal protein could act *via* a dominant-negative effect on
370 the trafficking of caveolin-1 to the plasma membrane (11-13). The *CAVI* p.(Phe160*) variant,
371 reported in one patient with a neonatal complex progeroid syndrome associated with pulmonary
372 artery hypertension and lipodystrophy, was also shown to be transcribed, without increased
373 RNA degradation (14,15). In accordance, caveolae were present at the cell surface of the
374 patient's fibroblasts, but caveolin-1 oligomers displayed decreased stability and weakened
375 interactions with cavin-1 (14-16).

376

377 Cavin-1, encoded by the *CAVIN1* gene, is a caveolin-1-interacting protein, which, like caveolin-
378 1, stabilizes caveolae structures. Cavin-1 is located at adipocyte lipid droplets, and contributes
379 to the adipocyte differentiation process (2,3,18,33). Biallelic *CAVIN1* pathogenic variants are
380 responsible for CGL4, a form of CGL also characterized by skeletal and/or cardiac muscular
381 dystrophy (30,31), with achalasia in some patients (25,34,35). Achalasia is due to the
382 dysfunction of nitrergic inhibitory motor neurones that innervate the circular smooth muscle of
383 the distal esophagus, resulting in impaired relaxation of lower esophageal sphincter (36). We
384 and others have previously shown that CGL4-related *CAVIN1* pathogenic variants severely
385 impair cavin-1 and caveolin expression, as well as caveolae formation (18,35,37). Since
386 caveolae have been shown to regulate calcium homeostasis and excitation-contraction coupling
387 in smooth muscle (38,39), their deficiency could promote the development of uncontrolled
388 esophageal contractions, that may evolve to achalasia. Oxidative stress could also contribute to
389 achalasia, as reported in the Triple A (alacrima – achalasia – adrenal insufficiency) syndrome
390 (OMIM 231550) (40). In addition, oxidative stress and/or premature senescence could
391 participate in the pathophysiology of lipodystrophy (26,27,30,41).

392

393 Patient 2 from this study was diagnosed with atypical retinitis pigmentosa. Interestingly,
394 caveolin-1 has been shown to modulate retinal neuroprotective signalling (42,43), and retinitis
395 pigmentosa was reported in two patients with a syndrome of partial lipodystrophy, congenital
396 cataracts, and neurological abnormalities, due to the heterozygous *CAVI* p.(Lys135Argfs*4)
397 variant (10). However, ophthalmological fundus examination did not show any sign of retinitis
398 pigmentosa in the previously described patient with *CGL3*, investigated at the age of 26 (9, and
399 Dr Chong Kim, personal communication). Whether this sign could be due to *CGL3*-associated
400 *CAVI* variants remains hypothetical. Dysmorphic features and/or mottled skin were observed
401 in the affected patients from this study and in the two patients reported with neonatal premature
402 ageing syndrome due to *de novo CAVI* variants (14,15) (**Figure 2**), but no other progeroid sign
403 was observed in patients with *CGL3*. Although right heart catheterization was not performed,
404 clinical examination and echocardiography did not reveal any sign of pulmonary arterial
405 hypertension in patients with *CGL3*.

406

407 Other phenotypic studies are required to better delineate the clinical relationships between the
408 different *CAVI*-related diseases, due to loss-of-function or dominant negative mechanisms.
409 However, in addition to careful metabolic monitoring, we suggest that gastrointestinal, cardiac,
410 ophthalmological and neurological evaluation should also be included in the follow-up of
411 patients with *CGL3*.

412

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423

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430

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564

565

566 **FIGURE LEGENDS**

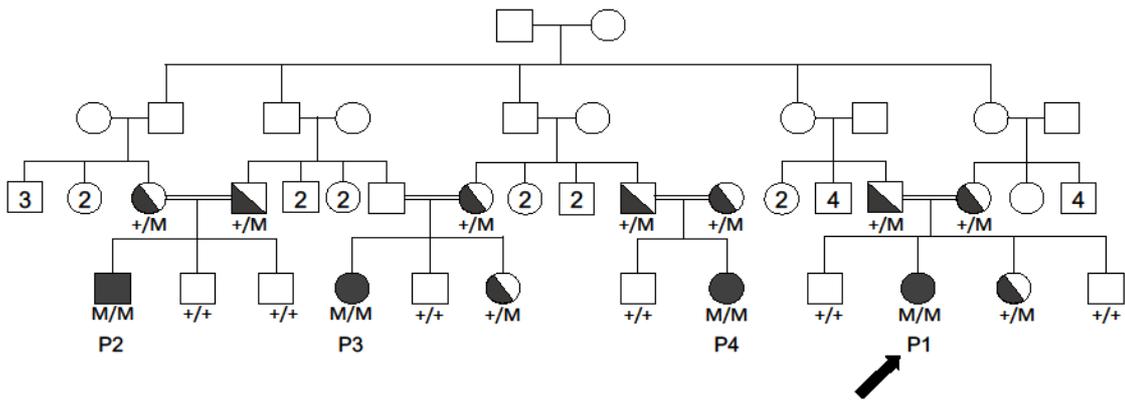
567 **Figure 1. Genealogical tree of the studied family**

568 Patients 1 to 4, diagnosed with congenital generalized lipodystrophy and homozygous *CAVI*

569 p.(His79Glnfs*3) variant (M/M) are depicted by filled symbols. Half-filled symbols

570 (heterozygous subjects, +/M), and patients with normal genotype (+/+) were not affected by the

571 disease. Patient 1 (P1, arrow) is the index case.

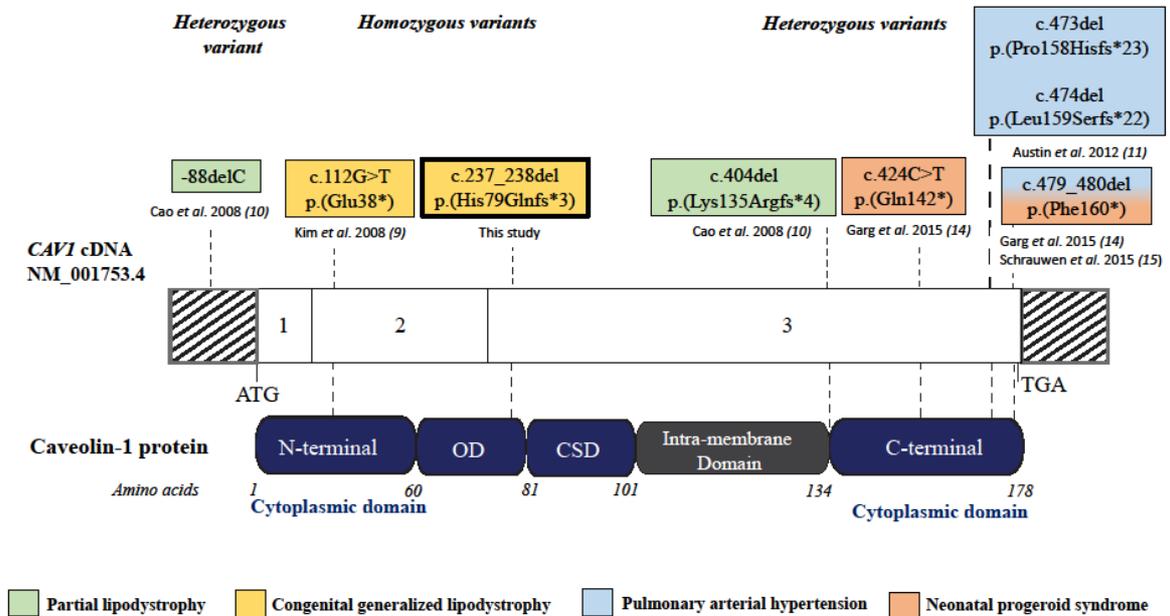


572

573

574 **Figure 2. Schematic representation of *CAVI* gene and caveolin-1 protein**

575 *CAVI* pathogenic variants (NM_001753.4) identified in this study or previously reported (9-
 576 11, 14, 15) are indicated, with the corresponding phenotypes. OD: oligomerisation domain,
 577 CSD: caveolin-scaffolding domain

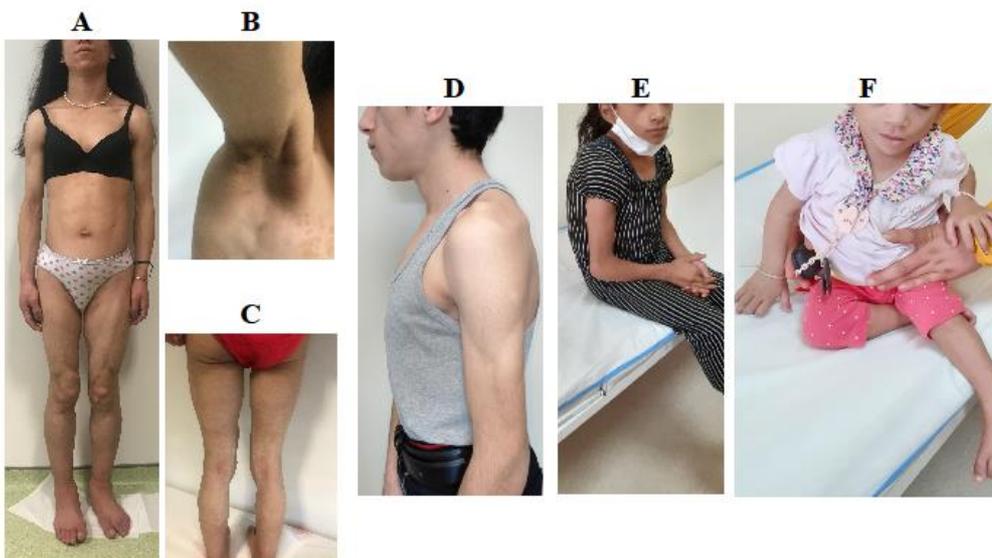


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579

580 **Figure 3. Photographs from patients with Congenital Generalized Lipodystrophy due to**
581 **the novel *CAVI* p.(His79Glnfs*3) homozygous pathogenic variant**

582 Photographs from Patient 1 (A-C), Patient 2 (D), Patient 3 (E), and Patient 4 (F) are shown.
583 Triangular face with empty cheeks, and generalized lipoatrophy are observed in all patients.
584 Muscular hypertrophy is visible in Patients 1-3. Photographs from Patient 1 also show mottled
585 skin (A-C), acromegaloid features (A), and acanthosis nigricans (B).



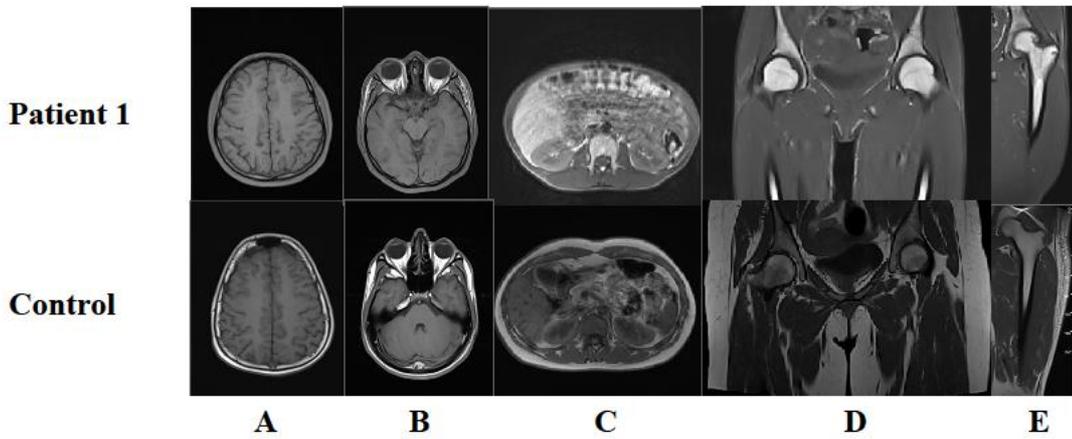
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587

588 **Figure 4. Magnetic resonance imaging of Patient 1 showing generalized lipoatrophy**

589 Magnetic resonance T1-weighted images without fat suppression of Patient 1 and of an age,
590 sex, and ethnically-matched control subject are shown.

591 Brain/cranial (A,B) and abdomen (C) axial images, and pelvis coronal images (D,E) of Patient
592 1 show that lipoatrophy is generalized, sparing only periorbital and bone marrow regions.
593 Hepatic steatosis and muscular hypertrophy are also visible.



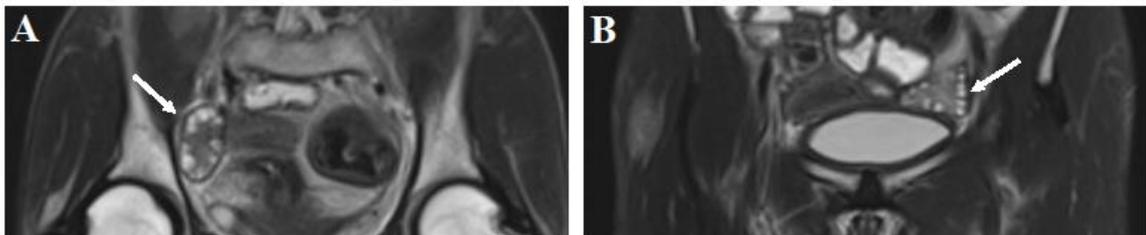
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596 **Figure 5. Magnetic resonance imaging of Patient 1 showing enlarged polycystic ovaries**

597 Magnetic resonance T1-weighted coronal images of Patient 1, showing polycystic right (A) and

598 left (B) ovaries of enlarged size (20 x 15 x 39 mm and 37 x 35 x 30 mm, respectively) (arrows).



599

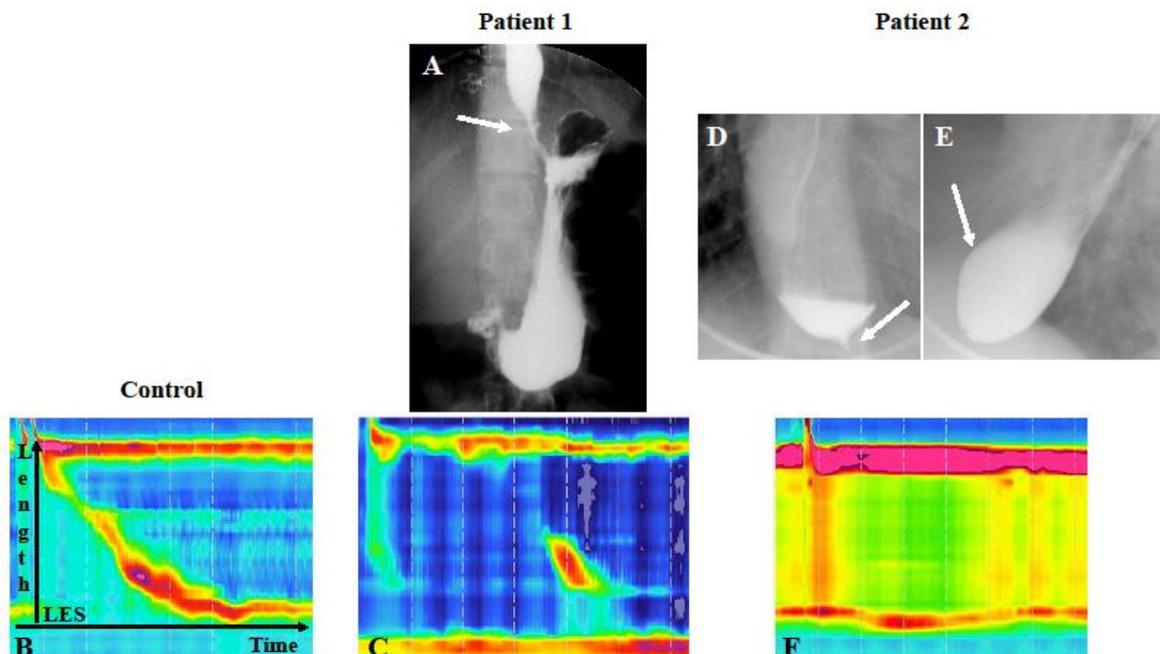
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601 **Figure 6. Barium esophagogram series and high-resolution manometry showing achalasia**
602 **in Patient 1 and Patient 2**

603 Barium esophagogram series in Patient 1 (A) and Patient 2 (D, E). Arrows show the bird's
604 beak deformity at the distal part of esophagus (A, D) with dilated esophageal body (E).

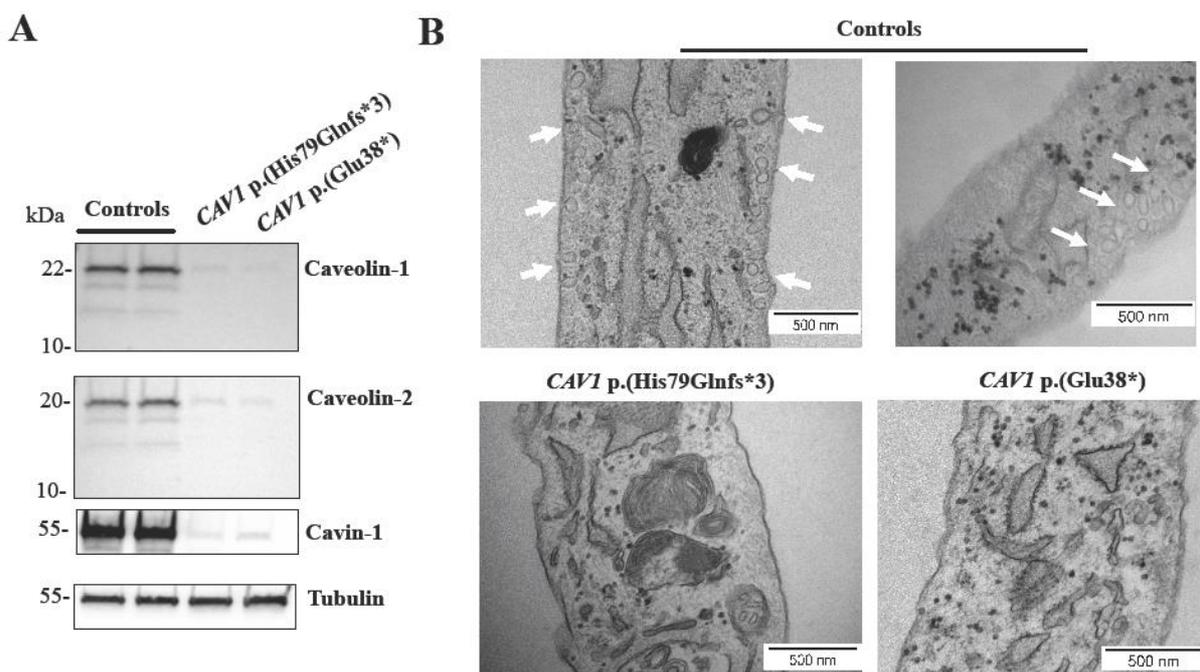
605 High-resolution manometry, showing a pressure topography during a swallow, in a control
606 subject (B), Patient 1 (C) and Patient 2 (F). The horizontal axis refers to time, and the vertical
607 axis to length along the esophagus, with the lower esophageal sphincter (LES) depicted below.

608 The pressure magnitude, encoded in color (blue, green, yellow, red from low to high pressure),
609 is shown along the horizontal line. In a control subject, the pressure topography graph during a
610 swallow shows a normal distal propagation of esophageal peristalsis, with LES relaxation over
611 time (B). Weak peristalsis in Patient 1 defines esophagogastric junction outflow obstruction
612 (C). A continuous high pressure without LES relaxation allows the diagnosis of Type 2
613 achalasia in Patient 2 (F).



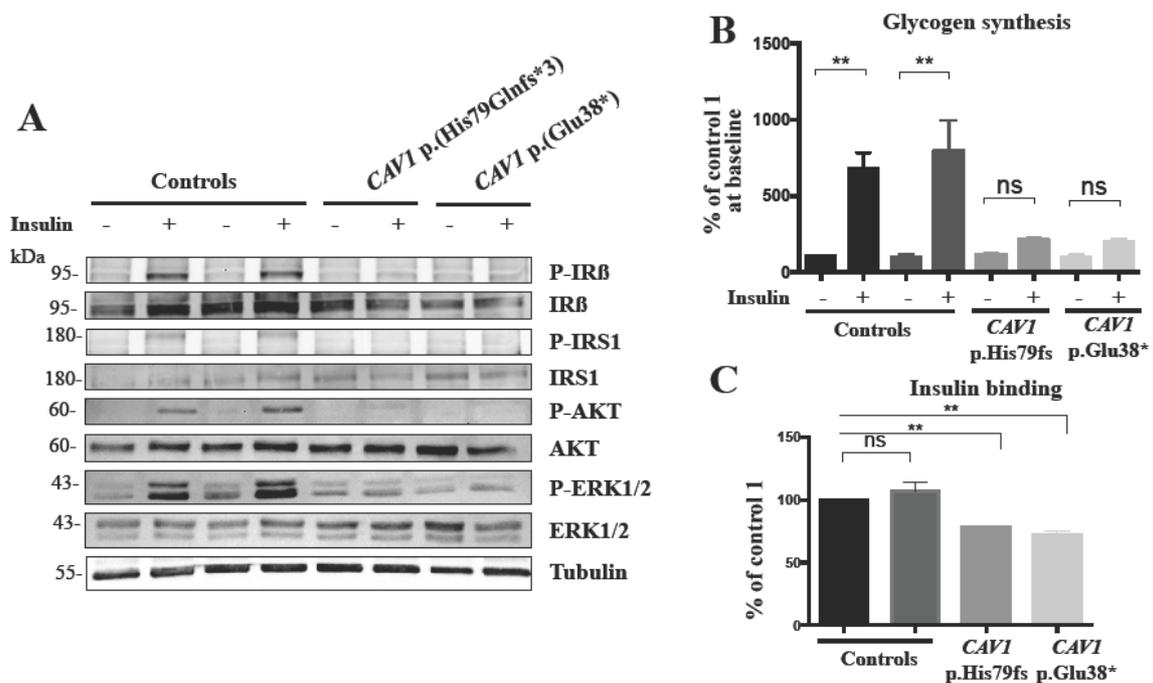
615 **Figure 7. Homozygous *CAVI* p.(His79Glnfs*3) and p.(Glu38*) pathogenic variants induce**
616 **a loss of protein expression of caveolin-1, caveolin-2 and cavin-1, and a loss of caveolae**
617 **formation in patients' fibroblasts**

618 (A) Protein expression of caveolin-1, caveolin-2 and cavin-1 in fibroblasts from controls, from
619 Patient 1, carrying the *CAVI* p.(His79Glnfs*3) homozygous pathogenic variant, and from the
620 patient previously described with a homozygous *CAVI* p.(Glu38*) variant responsible for
621 CGL3 (9). Representative images of Western blots performed in triplicate are shown. Tubulin
622 is used as an index of the cellular protein level. Numbers on the left correspond to the expected
623 protein molecular weight. (B) Representative images of electron microscopy showing caveolae
624 at the plasma membrane of control fibroblasts (arrows), which were completely absent in
625 patients' cells.



626
627 **Figure 8. Homozygous *CAVI* null variants impair insulin signaling in patients' fibroblasts**
628 (A) Insulin-mediated activation of insulin receptor β -subunit ($IR\beta$), insulin receptor substrate-
629 1 (IRS1), protein kinase B (AKT/PKB), and extracellular-regulated kinase (ERK)1/2 was
630 evaluated by Western blot in fibroblasts from controls, from Patient 1, carrying the *CAVI*
631 p.(His79Glnfs*3) homozygous pathogenic variant, and from the patient previously described

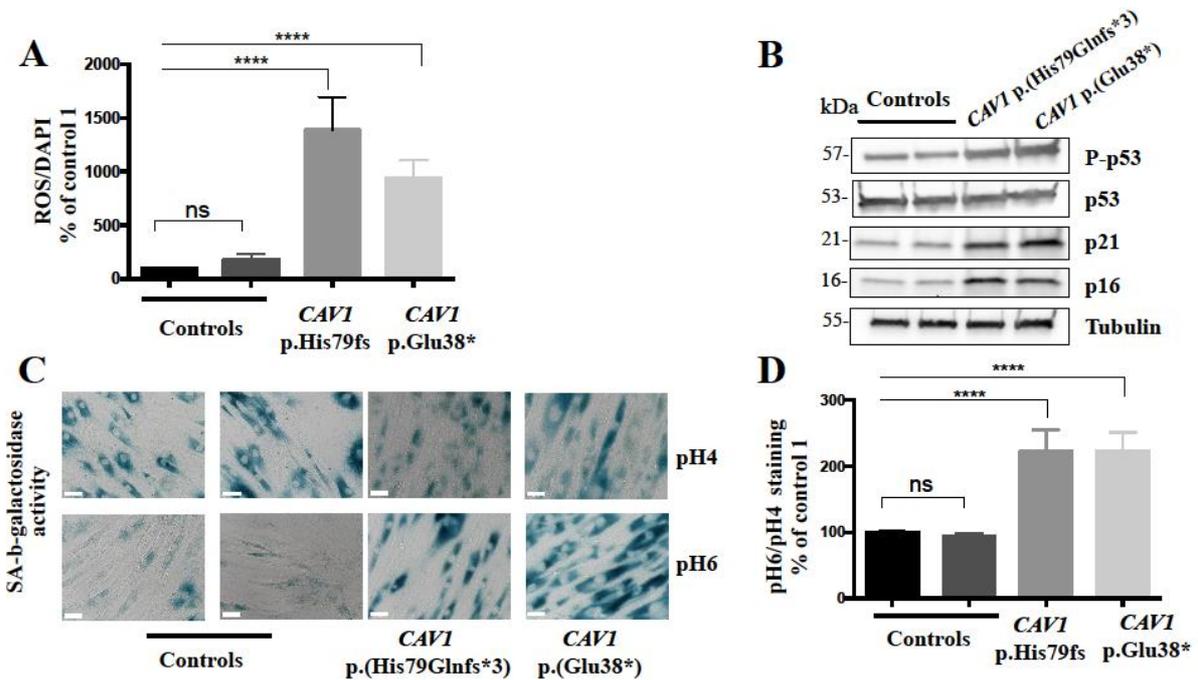
632 with a homozygous *CAVI* p.(Glu38*) variant responsible for CGL3 (9). Cells were maintained
 633 for 24h in a serum-free medium, then incubated or not with 50 nmol/L human insulin (#I9278,
 634 Sigma-Aldrich) for 8 min, and the total protein expression of signaling intermediates as well as
 635 their phosphorylated activated forms were evaluated. Representative images of Western blots
 636 performed in triplicate are shown. Tubulin is used as an index of the cellular protein level.
 637 Numbers on the left correspond to the expected protein molecular weight. (B) The effect of
 638 insulin on glycogen synthesis and (C) the ability of cells to bind insulin to its receptor, were
 639 evaluated as described in Methods. Results are expressed as the percentage of the first control
 640 without insulin. **: p <0.01, ns: not significant.



641
 642 **Figure 9. Homozygous *CAVI* null variants promote oxidative stress and senescence in**
 643 **patients' fibroblasts**

644 (A) Reactive oxygen species (ROS) production was assessed by oxidation of 5-6-
 645 chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) in fibroblasts from
 646 controls, from Patient 1, carrying the *CAVI* p.(His79Glnfs*3) homozygous pathogenic variant,
 647 and from the patient previously described with a homozygous *CAVI* p.(Glu38*) variant

648 responsible for CGL3 (9). Results are normalized to DNA content measured by DAPI, and are
 649 expressed as the percentage of the first control. (B) Cellular senescence was evaluated by the
 650 protein expression levels of the cell cycle arrest and senescence protein markers p16, p21, and
 651 phospho-p53 as compared to total p53. Representative images of Western blots performed in
 652 triplicate are shown. Tubulin is used as an index of the cellular protein level. Numbers on the
 653 left correspond to the expected protein molecular weight. (C-D) Senescence-associated β -
 654 galactosidase activity (SA- β -gal) was assessed by X-gal staining at pH6 compared to non-
 655 specific staining at pH4. Representative immunofluorescence images from triplicate
 656 experiments are shown. Scale bar is 100 μ m. The ratio of pH 6.0/pH 4.0 staining, which
 657 represents SA- β -galactosidase activity, is expressed as the percentage of the first control.
 658 ****: $p < 0.0001$, ns : not significant.



659

660

Table 1. Main phenotypic features of Patients 1 to 4

| | Patient 1 | Patient 2 | Patient 3 | Patient 4 |
|--|---|--|---------------------------------|-------------------------------|
| Age at evaluation | 15 years | 18 years | 10 years | 8 months |
| Gender | Female | Male | Female | Female |
| Height (centile) (mid-parental target height in postpubertal patients) | 153 cm (< 10 th) (160 cm) | 155 cm (< 3 rd) (170 cm) | 134 cm (< 25 th) | 62 cm (< 3 rd) |
| Weight (kg) (centile) | 42 kg (10 th) | 49 (3 th) | 30.5 (40 th) | 8.6 (75 th) |
| Body Mass Index (kg/m ²) (centile) | 17.9 (25 th) | 20.4 (25 th) | 17 (50 th) | 22.4 (ND) |
| Lipodystrophy onset | Early infancy | Early infancy | Early infancy | Early infancy |
| Generalized lipoatrophy sparing palms and soles | + | + | + | + |
| Failure to thrive in infancy | + | + | + | + |
| Increased appetite | + | - | - | - |
| Triangular face | + | + | + | + |
| Muscular hypertrophy | + | + | + | - |
| Acanthosis nigricans | + | + | - | - |
| Hirsutism | + | NA | - | - |
| Polycystic ovaries | + | NA | - | - |
| Liver steatosis (ultrasonography) | + | - | - | - |
| Blood pressure, cardiac examination and electrocardiogram | Normal | Normal | Normal | Normal |
| Echocardiography | Normal | Normal | ND | ND |
| Estimated right ventricular systolic pressure (N: 12-57 mmHg) | 25 | 27 | ND | ND |
| Megaesophagus | + | + | - | - |
| Muscular strength and neurological examination | Normal | Normal | Normal | Normal |
| Ophthalmological examination | Normal | Atypical retinitis pigmentosa | ND | ND |
| Fasting glucose (N: 3.5-5.6 mmol/L) | 5.0 | 4.8 | 5.3 | 4.7 |
| Fasting insulin (N: 2-9 mIU/L) | 300 | 8.1 | 9.6 | 9.4 |
| HbA1c (N: 4.8-6%) | 5.8 | 5.3 | 5.4 | 5.1 |
| Triglycerides (N: 0.3-1.5 mmol/L) | 5.4 | 0.9 | 3.4 | 2.3 |
| Total cholesterol (N: 3-5.1 mmol/L) | 4.0 | 3.8 | 3.1 | 4.2 |
| HDL-chol (N: 0.9-1.8 mmol/L) | 0.73 | 0.68 | 0.75 | 0.65 |

| | | | | |
|--|--------------|--------------|-------------|--------|
| Serum and urinary calcium | Normal | Normal | Normal | Normal |
| Urinary calcium/creatinine ratio (N < 0.14) | 0.055 | 0.053 | 0.044 | 0.048 |
| PTH (N : 15-65 pg/mL) | 39.9 | 41.3 | ND | ND |
| 25-OH-Vitamin D (N: 50-200 nmol/L) | 28.2 | 23 | 34.4 | 52.4 |
| Bone mineral density (Lumbar spine Z-score) | -2.78 | -3.85 | ND | ND |

663

664 The listed signs are indicated as present (+) or absent (-) in each patient.

665 NA, not applicable; ND, not determined

666 Height, weight and BMI centiles are determined according to CDC (Centers for Disease Control and Prevention, USA); Mid-parental target height is calculated according to Tanner *et al.* (44)

668

669 **Supplemental Table 1**
670 **Antibodies used in Western Blot studies**
671

| Targeted protein | Source | Catalogue reference | Company | Dilution used |
|--|--------|---------------------|--|---------------|
| Primary antibodies | | | | |
| Caveolin-1 (N-terminal part) | rabbit | sc-894 | Santa Cruz Biotechnology (Dallas, TX, USA) | 1:500 |
| Caveolin-2 | mouse | #610684 | BD Biosciences (Franklin Lakes, NJ, USA) | 1:1000 |
| Cavin-1 | rabbit | ab135655 | Abcam (Cambridge, UK) | 1:1000 |
| Insulin receptor β (IR β) | rabbit | #3025 | Cell Signaling Technology (Danvers, MA, USA) | 1:1000 |
| Insulin receptor substrate 1 (IRS1) | rabbit | #17509-1-AP | Protein Tech (Rosemont, IL, USA) | 1:1000 |
| Phosphotyrosine residues | mouse | sc-7020 | Santa Cruz Biotechnology | 1:500 |
| Total AKT | rabbit | #9272 | Cell Signaling Technology | 1:1000 |
| Phospho-Ser473-AKT (P-AKT) | rabbit | #9271 | Cell Signaling Technology | 1:1000 |
| Total extracellular signal-regulated kinase 1/2 (ERK1/2) | rabbit | #9102 | Cell Signaling Technology | 1:1000 |
| Phospho-Thr202/Tyr204-ERK 1/2 (P-ERK) | rabbit | #9101 | Cell Signaling Technology | 1:1000 |
| p53 | mouse | #ab1101 | Abcam | 1:1000 |
| Phospho-p53 | rabbit | #ab38497 | Abcam | 1:1000 |
| p21 | rabbit | #10355-1-AP | Protein Tech | 1:1000 |
| p16 | rabbit | #10883-1-AP | Protein Tech | 1:1000 |
| Tubulin | mouse | #T5168 | Sigma-Aldrich (Saint-Louis, MO, USA) | 1:1000 |
| Secondary antibodies | | | | |
| Rabbit IgG, horseradish peroxidase (HRP)-linked | goat | #7074 | Cell Signaling Technology | 1:3000 |
| Mouse IgG, HRP-linked | horse | #7076 | Cell Signaling Technology | 1:3000 |

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