

1 **Germline loss-of-function *PAM* variants are enriched in subjects with pituitary**
2 **hypersecretion**

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65 **Author contributions**

66 GT and CAS conceived the study. GT, LCHR, AFD, RKD, GF, AM, BAE, REM, EA, and

67 CAS formulated study hypotheses and conceived and designed the experiments. GT,

68 LCHR, GF, AM, JRI, TL, CT (Camilo Toro), EA, FRF, CT, BAE, and REM performed the

69 experiments. GT, LCHR, AFD, GF, EA, FRF, CT (Camilo Toro), CT (Christina Tatsi), BAE,

70 and REM analyzed the data. GT, AFD, EA, BAE, and REM constructed the figures. GT

71 wrote the original manuscript draft. All authors reviewed and edited the manuscript.

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91 **Abstract**

92 Pituitary adenomas (PAs) are common, usually benign tumors of the anterior pituitary gland
93 which, for the most part, have no known genetic cause. PAs are associated with major
94 clinical effects due to hormonal dysregulation and tumoral impingement on vital brain
95 structures. Following the identification of a loss-of-function variant (p.Arg703Gln) in the *PAM*
96 gene in a family with pituitary gigantism, we investigated 299 individuals with sporadic PAs
97 and 17 familial isolated pituitary adenomas kindreds for *PAM* variants. *PAM* encodes a
98 multifunctional protein responsible for the essential C-terminal amidation of secreted
99 peptides.

100 Genetic screening was performed by germline and tumor sequencing and germline copy
101 number variation (CNV) analysis. No germline CNVs or somatic single nucleotide variants
102 (SNVs) were identified. We detected seven likely pathogenic heterozygous missense,
103 truncating, and regulatory SNVs. These SNVs were found in sporadic subjects with GH
104 excess (p.Gly552Arg and p.Phe759Ser), pediatric Cushing disease (c.-133T>C and
105 p.His778fs), or with different types of PAs (c.-361G>A, p.Ser539Trp, and p.Asp563Gly). The
106 SNVs were functionally tested *in vitro* for protein expression and trafficking by Western
107 blotting, for splicing by minigene assays, and for amidation activity in cell lysates and serum
108 samples. These analyses confirmed a deleterious effect on protein expression and/or
109 function. By interrogating 200,000 exomes from the UK Biobank, we confirmed a significant
110 association of the *PAM* gene and rare *PAM* SNVs to diagnoses linked to pituitary gland
111 hyperfunction.

112 Identification of *PAM* as a candidate gene associated with pituitary hypersecretion opens the
113 possibility of developing novel therapeutics based on altering *PAM* function.

114

115 **Introduction**

116 The anterior pituitary gland plays a critical role in the dynamic control of major hormonal
117 systems, including growth, fertility, and stress responses. Anterior pituitary adenomas (PAs),
118 also called pituitary neuroendocrine tumors (PitNETs) [1], can be comprised of any of the
119 secretory cell subtypes, such as lactotropes that secrete prolactin, somatotropes (growth
120 hormone (GH)), corticotropes (adrenocorticotrophic hormone (ACTH)), gonadotropes (follicle
121 stimulating hormone (FSH) and luteinizing hormone (LH)), and thyrotropes (thyroid
122 stimulating hormone (TSH)) [2]. Although they are usually benign lesions, PAs can have a
123 major impact through hormonal dysregulation and direct mass effects or invasion of brain
124 structures (optic chiasm, cavernous sinus). Epidemiologically, PAs are one of the most
125 frequent intracranial tumor types, and lead to clinically apparent disease with a frequency of
126 approximately 1 per 1,000 in the general population [3, 4]. Approximately 95% of PAs occur
127 sporadically. Hereditary PAs are often distinguished by a more severe clinical presentation,
128 such as an earlier age at onset, aggressive growth, larger size, and greater resistance to
129 treatment, and they might coexist with other syndromic components. Therefore, identification
130 of germline genetic causes can have implications for earlier identification and treatment of
131 affected individuals via genetic and clinical screening [5].

132

133 Germline changes like single nucleotide variants (SNVs) and/or copy number variants
134 (CNVs) in several genes have been implicated in familial isolated pituitary adenomas - FIPA
135 (*AIP* and *GPR101* genes) [6, 7], familial syndromic pituitary adenomas (*MEN1*, *CDKN1B*,
136 *PRKAR1A*, *PRKACB*, *SDHx*, *MAX*, *NF1*, *DICER1*, *TSC2*, among others) and sporadic PAs
137 (*AIP*, *GPR101*, *CABLES1*). Recurrent somatic SNVs are found most frequently in *GNAS* and
138 *USP8*, in sporadic GH and ACTH-secreting PAs, respectively, and rarely in other genes [8].
139 Despite these advances in establishing gene-disease links, the etiology of the overwhelming
140 majority of inherited and sporadic pituitary adenomas remains unknown. Hence,
141 identification and characterization of novel pathological genetic and genomic variants in PA
142 cohorts is medically significant.

143

144 In a large, international cohort of individuals with PAs, we identified germline loss-of-function
145 (LOF) variants of *PAM* (peptidylglycine α -amidating monooxygenase, MIM: 170270) in
146 subjects with familial and sporadic GH and ACTH hypersecretion. *PAM* is a highly
147 conserved, multifunctional protein that is increasingly recognized as an important regulator
148 of peptide amidation and secretion, among many other functions, in health and disease [9].
149 We functionally evaluated 36 SNVs, demonstrating a deleterious effect on *PAM*
150 function/expression in eight variants. We also report a statistically significant association of
151 rare *PAM* SNVs with diagnoses of hyperfunction or tumors of the anterior pituitary gland.
152 These results suggest that pathogenic *PAM* variants can predispose and/or contribute to the
153 development of pathological pituitary hypersecretion.

154

155 **Results**

156 Identification of a pathogenic SNV in the *PAM* gene in a family with pituitary gigantism

157 The discovery cohort consisted of a three-member, non-consanguineous FIPA family that
158 was enrolled in the UDP of the NIHCC. The kindred consisted of monozygotic twin brothers
159 (individuals II-2 and II-3) and the eldest child of individual II-2, all of whom were affected by
160 childhood-onset pituitary gigantism (Table 1 and Figure 1A).

161 **Table 1. Summary of characteristics of seven cases with rare pathogenic *PAM* variants**

ID	Diagnosis	Gender	Pathogenic <i>PAM</i> variant(s)	Ethnicity	Age at disease onset (years)	Age at PA diagnosis (years)	Tumor size (mm)	Treatment	Other clinical diagnoses
II-2*	GH excess (gigantism)	M	c.2108G>A (p.Arg703Gln)	Caucasian	<10	35-40	4	PegV	hypogonadism, adrenal nodules, pleural masses, severe osteopenia, muscular atrophy, diverticulosis
II-3*	GH excess (gigantism)	M	c.2108G>A (p.Arg703Gln)	Caucasian	<10	35-40	5x6	TSS, SSA, PV	hypogonadism, pancreatic and colon adenocarcinoma
III-3*	GH excess (gigantism)	M	c.2108G>A (p.Arg703Gln)	Caucasian	1-5	1-5	n.a.	SSA, PegV	hypotonia
NIH26	pediatric CD	F	c.2332-2A>T (p.His778fs)	Caucasian	10-15	10-15	3	TSS	scleroderma
NIH36	pediatric CD	F	c.-133T>C	African-American	10-15	10-15	10	TSS (x3)	no
Belgium128	GH excess (gigantism)	M	c.2276T>C (p.Phe759Ser)	Latino	15-20	20's	Macro	TSS, SRL	none
Belgium197	GH excess (acromegaly)	M	c.1654G>A (p.Gly552Arg), c.1688A>G (p.Asp563Gly)	Caucasian	40's	40's	40	TSS, SRL, RTx	none

162 F, female; M, male; n.a., not applicable; PA, pituitary adenoma; PegV, pegvisomant; RTx, radiotherapy; SRL, somatostatin receptor ligand; TSS, transsphenoidal surgery.

163 *Member of the index FIPA kindred.

165 The monozygotic twins were born at 31 weeks gestation; their birth weights and lengths
166 were 1.96 kg and 48.3 cm (II-2) and 2.3 kg and 53.3 cm (II-3), respectively. They had two
167 normally sized siblings. Their father was 190 cm in height. Their mother was very tall [187
168 cm; +4.1 SDS using Centers for Disease Control (CDC) charts] but had no known history of
169 pituitary disease or established medical diagnosis of overgrowth; she died of colorectal
170 cancer in her 70's. The precise onset of the twins' excessive growth is uncertain, but it
171 began during childhood, such that by in their 10's they both measured >200 cm in height
172 (>+6.2 SDS). They were formally diagnosed with pituitary gigantism as adults (35-40 years),
173 at which time their final height was 231 cm (+7.8 SDS). At diagnosis they had clinical signs
174 of acromegaly including coarse facial features, frontal bossing, and enlarged extremities.
175 Elevated random GH (26 and 23.1 ng/mL) and IGF-1 (603 and 1130 ng/mL) levels were
176 noted, but prolactin levels were below normal levels. Only minor abnormalities were noted
177 on 3 Tesla pituitary magnetic resonance imaging (MRI), with possible small pituitary
178 microadenomas, 4 mm and 5 mm in maximum diameter (Figure 1B). Individual II-3
179 underwent transsphenoidal surgery, but no somatotropinoma tissue was identified by
180 histopathology. Both brothers required medical therapy with the GH-receptor antagonist,
181 pegvisomant, to reduce excess IGF-1. Colorectal and pancreatic adenocarcinomas were
182 diagnosed in patient II-3, and he subsequently died of the latter tumor in his 40's.

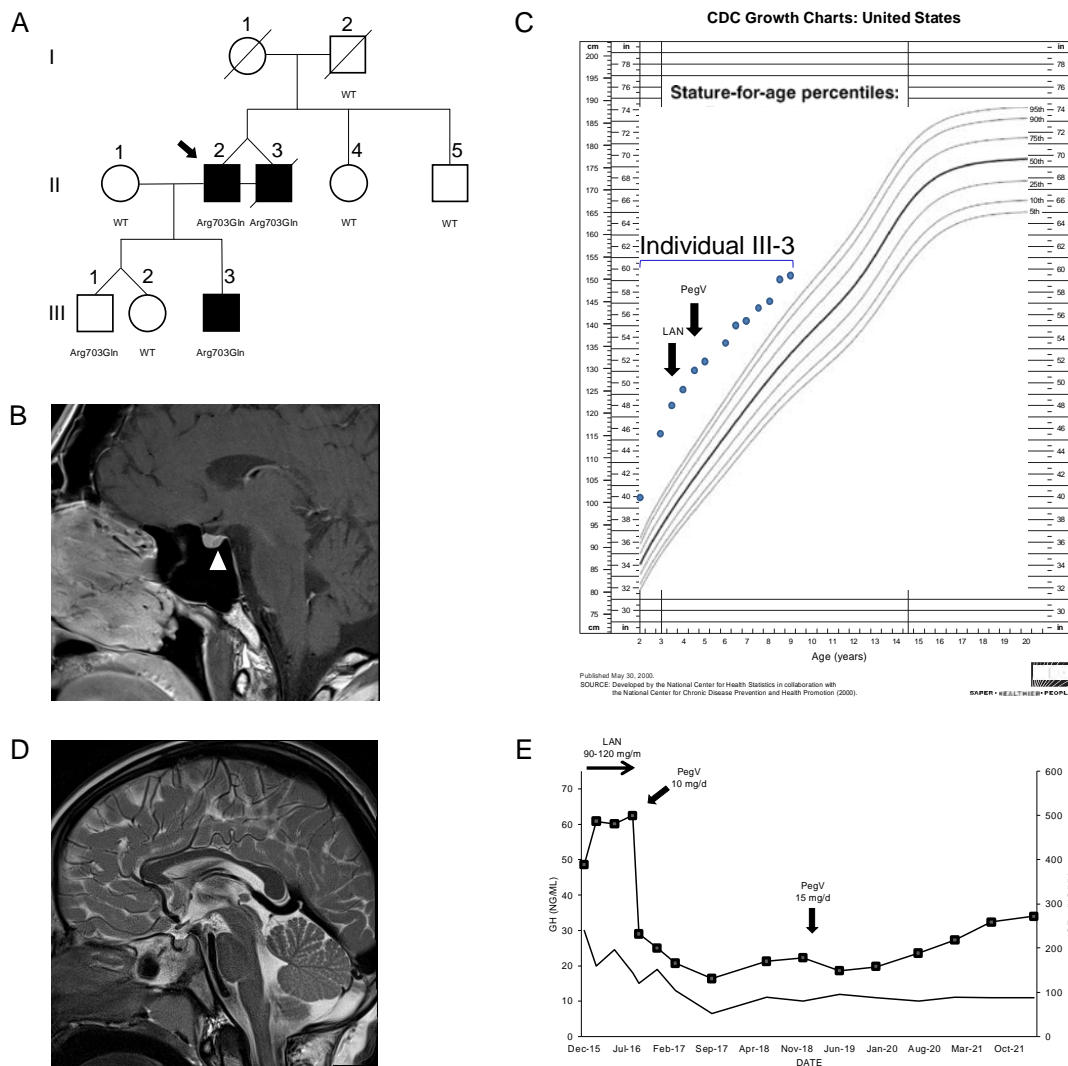
183 The third affected member was III-3, a male (son of II-2) who was born with normal length
184 and weight but developed marked overgrowth by the age of 12 months (Figure 1C). His
185 siblings are dizygotic twins with normal height. III-3 was diagnosed with GH and IGF-1
186 excess (random GH 30 ng/mL and IGF-1 389 ng/mL) before five years of age. An MRI
187 demonstrated a normal pituitary gland (Figure 1D). To control the excessive growth, the
188 patient was started on the long-acting somatostatin receptor ligand lanreotide autogel at a
189 dose of 90-120 mg/month sc. Despite this adult level dosing, the GH remained uncontrolled
190 and the IGF-1 rose to 487 ng/mL. During the lanreotide autogel treatment period, the growth
191 velocity remained accelerated, at 15.9-20 cm/year. Similar to II-2 and II-3, his condition
192 markedly improved on switching to pegvisomant (10-15 mg/day sc): IGF-1 rapidly fell from

193 500 ng/mL to 232 ng/mL and growth velocity decreased to 3.6-5.6 cm/year during
194 approximately five years of follow-up. His growth curves for height and weight remain above
195 the 99th centile but are no longer diverging (Figure 1E).

196

197 We conducted WES in peripheral blood-derived DNA isolated from the index cases (see the
198 Methods section for details). We applied a variant prioritization strategy (Figure S1 and
199 Table S3) and selected for further analysis a heterozygous missense SNV in *PAM*
200 (c.2108G>A, p.Arg703Gln), which was shared by both twins from generation II (Figure 1A).
201 DNA of their deceased mother was unavailable, so we could not confirm whether they
202 inherited the *PAM* variant from her or if it arose *de novo*; the father was WT. The
203 p.Arg703Gln variant was present in the other individual with gigantism, III-3, but also in III-1,
204 his younger brother (Figure 1A). Endocrine and growth studies showed that III-1 had no
205 evidence of growth excess or hormonal dysregulation at the time of study (age < 10 years).
206 All other family members studied were WT for *PAM* and had normal growth. Overall, these
207 results suggest incomplete penetrance. No other potentially pathogenic alterations in genes
208 were shared among the three affected members.

209



210

211 **Figure 1. Clinical findings in the index family with pituitary gigantism**

212 (A) Pedigree. Generation numbers are represented by Roman numerals, and individual numbers are
 213 in Arabic numerals. The proband is II-2, indicated by the black arrow. Open square/circle, unaffected
 214 male/female; filled square, affected male. *PAM* mutational status is shown under each screened
 215 individual. In (B) the white arrowhead points to a possible 4 mm lesion, seen in II-2. (C) Growth chart
 216 for individual III-3 before and after medical interventions. (D) A sagittal T2-weighted MRI of individual
 217 III-3 that was performed before medical therapy began did not reveal a pituitary lesion. (E) Time
 218 course of the effects of treatment modalities on GH (left axis) and IGF-1 (right axis) in individual III-3.
 219 The rapid decrease of IGF-1 after switching from lanreotide (LAN) to pegvisomant (PegV) is evident.

220

221 The *PAM* variant was notable among the 27 candidates due to its strong expression in
222 neuroendocrine tissues and gene function. As reported in *The Human Protein Atlas*, the
223 pituitary is among the tissues displaying the strongest *PAM* mRNA and protein expression
224 signal in human and other mammals. *PAM* is highly expressed in all pituitary cell types,
225 including folliculostellate cells (Figure S2A), where it plays a crucial function in post-
226 translational hormone processing and secretion [10, 11]. Staining for *PAM* and GM130, a
227 cis-Golgi marker, in normal adult human pituitary cells showed diffuse cytoplasmic
228 expression for *PAM* that partially overlapped the Golgi (Figure S2B), reflecting the
229 established trafficking of *PAM* within the secretory pathway observed in rat pituitary cells.

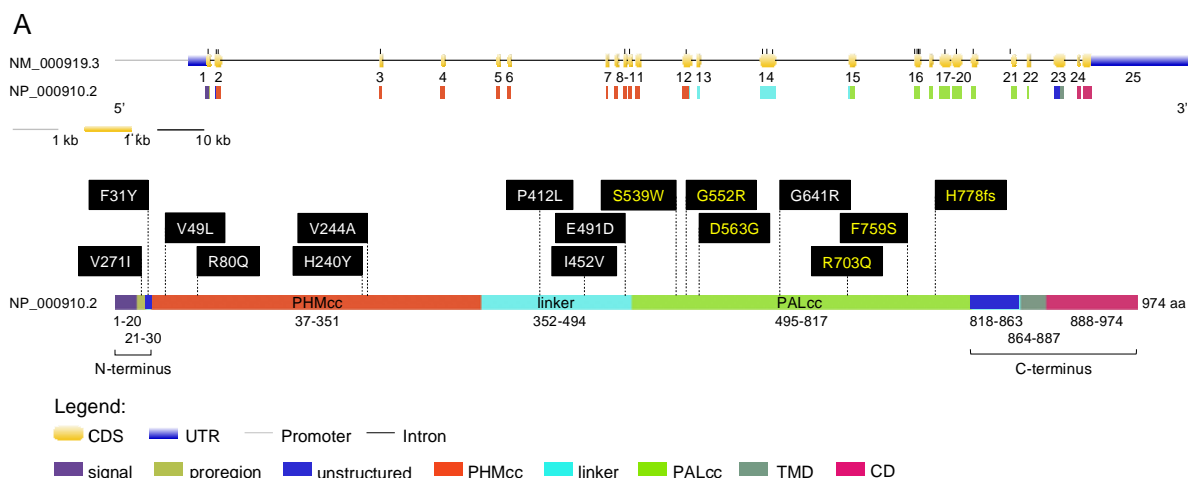
230 *PAM* is a multifunctional protein that contains two enzymatic domains, peptidylglycine α -
231 hydroxylating monooxygenase (PHM) and peptidyl- α -hydroxyglycine α -amidating lyase
232 (PAL); acting sequentially, the two domains generate C-terminally amidated peptides [10].
233 The p.Arg703Gln variant – located in the PAL domain (Figure 2A) – has an extremely low
234 minor allele frequency in controls (MAF: 0.0013%), no homozygous variant entries in
235 gnomAD, and is bioinformatically predicted to be pathogenic (Table 2).

236 **Table 2. Prioritized heterozygous missense, truncating, and regulatory PAM variants that were functionally analyzed**

DNA change	Protein change	SNP ID	Location in gene	Location in protein	MAF in our cohort (%)	Global Control MAF (%)	Highest Control Population MAF (%)	# of homozygotes in gnomAD	P-value	<i>In silico</i> prediction	Individuals harboring the variant
c.-607A>G †	n.a.	rs76396377	promoter	n.a.	0.4	1.479	2.943 (African)	24	n.s.	1 TF replaced	1 from 1 FIPA (PRL)
c.-550G>T †	n.a.	rs1007572715	promoter	n.a.	0.4	0.0072	0.0415 (South Asian)	0	<0.05	2 TFs lost	1 acro
c.-361G>A	n.a.	rs143617515	5'UTR (exon 1)	n.a.	1.21	0.6275	3.647 (Finnish)	12	n.s.	2 TFs lost, 4 TFs created	2 gigantism, 1 NFPA
c.-133T>C *	n.a.	rs1413721196	5'UTR (exon 1)	n.a.	0.25	0.0007	0.0015 (European)	0	<0.05	1 TF lost	1 pediatric CD
c.-109G>C	n.a.	Novel	5'UTR (exon 1)	n.a.	0.25	n.a.	n.a.	n.a.	n.a.	1 TF replaced	1 acro
c.-35C>G	n.a.	rs201016377	5'UTR (exon 1)	n.a.	0.15	0.0749	0.9040 (Finnish)	1 (Finnish)	n.s.	n.a.	1 gigantism
c.79G>A **	p.Val27Ile	rs199856250	exon 1	proregion	0.16	0.0004	0.0008 (European)	0	<0.05	VUS (1, 6, 12)	1 acro
c.92T>A	p.Phe31Tyr	rs114014768	exon 2	linker region	0.63	0.8654	2.814 (African)	19	n.s.	likely benign (0, 1, 16)	2 acro, 1 gigantism, 1 pediatric CD
c.145G>C	p.Val49Leu	rs2230458	exon 2	PHMcc	0.94	0.9278	1.803 (Finnish)	5 (European)	n.s.	likely benign (0, 2, 17)	2 gigantism, 3 pediatric CD, 1 PRL
c.239G>A	p.Arg80Gln	rs753307443	exon 3	PHMcc	0.16	0.0026	0.0066 (Latino)	0	<0.05	likely benign (1, 5, 13)	1 from 1 FIPA (GH excess)
c.718C>T ††	p.His240Tyr	rs761898981	exon 9	PHMcc	0.15	0.0039	0.0478 (Other)	0	<0.05	VUS (0, 11, 7)	1 gigantism
c.731T>C **	p.Val244Ala	rs201009674	exon 10	PHMcc	0.16	0.0533	0.6632 (Ashkenazi Jewish)	0	n.s.	likely pathogenic (6, 12, 0)	1 pediatric CD
c.1235C>T **	p.Pro412Leu	rs761619241	exon 14	linker region	0.16	0.0016	0.0029 (Latino)	0	<0.05	likely pathogenic (6, 9, 4)	2 from 1 FIPA (heterogeneous)
c.1354A>G	p.Ile452Val	rs145710876	exon 14	linker region	0.16	0.0151	0.0555 (African)	0	n.s.	likely benign (0, 0, 18)	1 gigantism
c.1473A>C	p.Glu491Asp	rs61736661	exon 14	linker region	0.16	0.6662	1.241 (European)	8 (European)	n.s.	likely benign (0, 1, 16)	1 from 1 FIPA (PRL)
c.1616C>G	p.Ser539Trp	rs78408340	exon 16	PALcc	0.15	0.4169	0.7250 (European)	2 (European)	n.s.	likely pathogenic (10, 6, 2)	1 acro
c.1654G>A ††	p.Gly552Arg	rs201249509	exon 16	PALcc	0.15	0.0053	0.0103 (European)	0	<0.05	likely pathogenic (10, 6, 2)	1 acro
c.1688A>G	p.Asp563Gly	rs35658696	exon 16	PALcc	4.00	3.109	5.657 (European)	108	n.s.	likely pathogenic (4, 11, 1)	13 acro, 5 gigantism, 4 PRL, 4 CD (3 pediatric, 1 silent)
c.1921G>C	p.Gly641Arg	rs775364358	exon 18	PALcc	0.15	0.0033	0.0197 (Latino)	0	<0.05	likely benign (0, 3, 16)	1 PRL
c.2108G>A	p.Arg703Gln	rs761130902	exon 19	PALcc	0.15	0.0013	0.0193 (East Asian)	0	<0.05	likely pathogenic (10, 9, 0)	4 (3 affected) from 1 FIPA (gigantism)
c.2276T>C ††	p.Phe759Ser	rs375364507	exon 20	PALcc	0.15	0.0092	0.0206 (European)	0	n.s.	likely pathogenic (7, 11, 0)	1 gigantism
c.2332-2A>T §	p.His778fs	rs1006675725	intron 20	PALcc	0.15	0.0007	0.0015 (European)	0	<0.05	pathogenic (disrupts canonical SA)	2 (1 pediatric CD and unaffected parent)
c.*1455C>T ¶¶	n.a.	rs146343559	3'UTR (exon 25)	n.a.	0.67	0.0362	0.1047 (Latino)	0	n.s.	creates new miRNA binding sites	1 pediatric CD

237 Variants were annotated using the NC_000005.9(NM_000919.3) reference sequence. All variants were observed in heterozygosis. MAFs were retrieved from
238 the gnomAD database ver. 3.1.2. “European” excludes the Finnish population; “African” includes African-American; “Latino” includes Admixed American. For
239 *in silico* predictions, the computational verdict was based on the combined outputs of 19 softwares available in Varsome (within parentheses, the first number
240 represents the pathogenic verdicts, the second number the uncertain verdicts, and the third number the benign verdicts) for all missense variants; for the
241 truncating and 3’UTR variants we used Alamut predictions; for the regulatory variants we used Genomatix MatInspector. Individual variant allele frequencies
242 in the study population were compared with the global frequencies reported in gnomAD using the Fisher’s exact test or the chi-square test, as appropriate.
243 acro, acromegaly; CD, Cushing disease; FIPA, familial isolated pituitary adenoma; MAF, minor allele frequency; n.a., not available/applicable; NFPA, non-
244 functioning pituitary adenoma; n.s., not significant; PALcc, catalytic core of peptidyl- α -hydroxyglycine α -amidating lyase; PHMcc, catalytic core of
245 peptidylglycine α -hydroxylating monooxygenase; PRL, prolactinoma; SA, splice acceptor site; TF, transcription factor; VUS, variant of uncertain significance.
246 †This individual harbors the PAM p.Glu491Asp variant in exon 14.
247 ‡This individual harbors the PAM p.Val49Leu variant in exon 2.
248 ¥This individual harbors the PAM p.Phe31Tyr variant in exon 2 and a somatic heterozygous p.Ser718del variant in the *USP8* gene [12].
249 ††This individual is compound heterozygote for p.[His240Tyr];p.[Phe759Ser].
250 ††This individual is compound heterozygote for p.[Gly552Arg];p.[AspD563Gly].
251 ¥¥This individual harbors a somatic heterozygous p.Ser718Pro variant in the *USP8* gene [12].
252 ¥¥¥This individual harbors a somatic heterozygous p.Ser718del variant in the *USP8* gene [12].
253 §Somatic hotspot variants in the *USP8*, *BRAF*, and *USP48* genes were ruled out in this case.
254 **Allele frequency obtained from gnomAD ver. 2.1.1.

255 Arg703 is conserved throughout evolution (Figure 2B). Along with Tyr651, crystallographic
256 studies identified Arg703 as part of the PALcc catalytic dyad; the catalytic activity of soluble
257 recombinant rat PALcc, in which Arg703 was replaced by Ala or Gln, was greatly reduced
258 [13], a result consistent with its location at the active site (Figure 2C). Altogether, these data
259 suggested that a LOF *PAM* variant could be associated with pituitary gigantism, prompting
260 us to pursue this lead.
261



B

PAL: Ser539Trp (Ex15/16); **inactive** PAL: Gly552Arg (Ex16); **6% active** PAL: Asp563Gly (Ex16); **14% active**

	536		539		542		
hum	D	G	N	S	F	D	S
chi	D	G	N	S	F	D	S
rat	D	G	N	S	F	D	S
apl	N	A	A	S	F	D	I
dro	D	V	N	T	F	N	E
chl	K	S	D	T	F	D	R

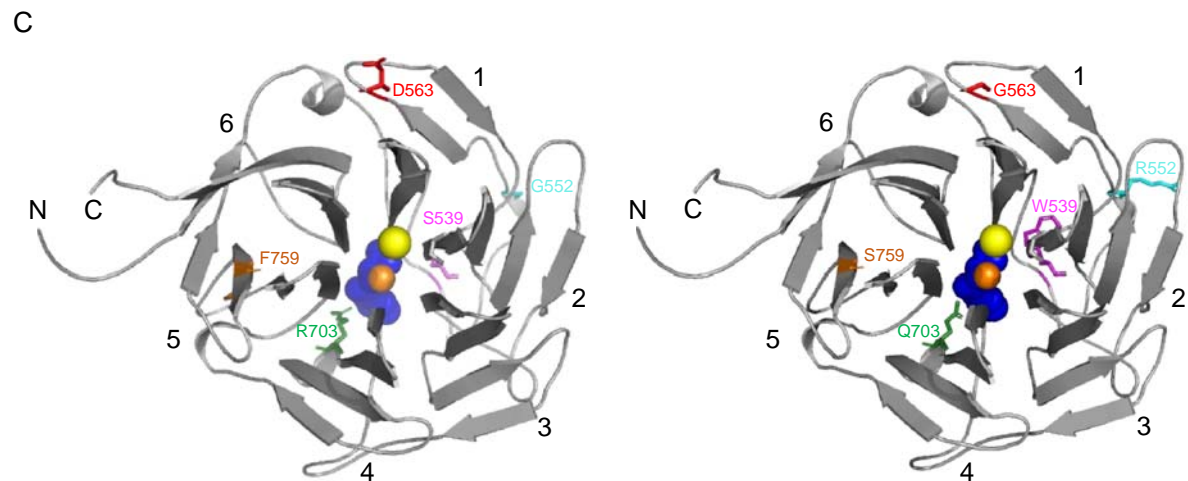
	549		552		555		
hum	I	G	L	G	P	I	E
chi	I	G	L	G	P	I	E
rat	R	G	L	G	P	I	E
apl	F	Q	D	S	P	I	T
dro	I	E	Y	G	P	I	K
chl	T	R	K	E	P	V	P

	560		563		566		
hum	L	V	I	D	P	N	N
chi	L	V	I	D	P	N	N
rat	L	V	I	D	P	N	N
apl	L	V	T	D	S	T	G
dro	Y	V	L	D	A	K	T
chl	L	N	M	N	P	D	T

PAL: Arg703Gln (Ex19); **inactive** PAL: Phe759Ser (Ex20); **12% active**

	700		703		706		
hum	V	A	D	R	E	N	G
chi	V	A	D	R	E	N	G
rat	V	A	D	R	E	N	G
apl	V	A	D	R	E	N	G
dro	I	A	D	R	E	N	M
chl	V	A	S	R	E	G	R

	756		759		762		
hum	V	Q	G	F	V	M	N
chi	V	Q	G	F	V	M	N
rat	V	Q	G	F	V	M	N
apl	V	Q	G	F	T	V	D
dro	V	R	G	F	T	I	D
chl	P	T	Q	F	W	T	L



262

263

264 **Figure 2. Location and evolutionary conservation of the missense and frameshift *PAM* SNVs**
265 **that were functionally tested**

266 (A) Schematic representation of the *PAM* gene (GenBank: NM_000919.3, *PAM*-1, 25 exons) and
267 encoded protein (NP_000910.2, 974 amino acids), including functional domains, with 15 missense
268 and a frameshift SNVs. Gene and protein structures were drawn with the Gene Structure Display
269 Server (GSDS ver. 2.0) [14]. Variants found to have deleterious effects on *PAM* function/expression
270 ($p < 0.01$) are shown in yellow lettering, while those without major effects are shown in white
271 lettering. Brackets identify the non-catalytic regions that precede PHMcc (N-Terminus) and follow
272 PALcc (C-Terminus); CD, cytosolic domain; CDS, coding sequence; PALcc, catalytic core of peptidyl-
273 α -hydroxyglycine α -amidating lyase; PHMcc, catalytic core of peptidylglycine α -hydroxylating
274 monooxygenase; TMD, transmembrane domain; UTR, untranslated region. (B) Protein sequence
275 alignments for five of the variants with deleterious effects. Conserved affected residues are shown in
276 yellow. PAL activities, indicated in red, refer to functional experiments in PEAkrapid cells. (C) The
277 crystal structure of rat PALcc (PDB entry 3FW0) was used to contextualize the missense variants
278 categorized as likely pathogenic based on *in silico* analyses; the WT residue is shown on the left and
279 the mutant residue on the right. PAL folds as a β -propeller, with six blades (numbered 1 to 6)
280 positioned around a central cavity. The calcium and mercury ions are depicted as yellow and orange
281 spheres, respectively. The mercury ion was used instead of zinc to capture the binding of a
282 nonpeptide substrate, α -hydroxyhippuric acid, depicted in blue. The affected residues are highlighted
283 in purple (Ser or Trp 539), cyan (Gly or Arg 552), red (Asp or Gly 563), green (Arg or Gln 703), and
284 orange (Phe or Ser 759), along the ribbon visualization of WT rat PALcc in grey. Arg703 is positioned
285 at the active site and participates in substrate binding. Interestingly, p.Gly552Arg and p.Asp563Gly
286 are located on the same face of the β -propeller. C, C-terminus; N, N-terminus.

287

288 *PAM* screening in a validation cohort revealed multiple likely pathogenic SNVs by *in silico*
289 prediction

290 We next determined whether predicted pathogenic *PAM* variants are associated with other
291 types of PAs, both hereditary and sporadic. To do so, we screened a diverse group of 326
292 germline and 60 tumor DNAs from PA patients for *PAM* SNVs (see the Methods section and
293 Table S1 for details). Sequencing covered the *PAM* CDS and exon–intron junctions.

294 Germline CNVs at the *PAM* locus were analyzed by ddPCR in a cohort of 137 individuals
295 with PAs (16 with gigantism and 121 with different types of PAs). Germline DNA sequencing
296 detected 51 SNVs (Table 2 and Table S4). There were no germline CNVs, or
297 rare/pathogenic somatic DNA SNVs (data not shown). A subset of the germline SNVs (15 of
298 51) was prioritized for further screening together with the p.Arg703Gln variant (Table 2). This
299 selection was based on the following non-mutually exclusive criteria: MAF < 1%, *in silico*
300 analysis with multiple algorithms predicting a deleterious effect on PAM function or splicing,
301 and published functional data indicating pathogenicity [15]. To complement these data, we
302 used Clustal and structure-based analyses to identify variants that could impact protein
303 structure/function (Figure 2B and 2C, Figure S3). The remaining, non-characterized germline
304 SNVs are listed in Table S4.

305

306 Four of the 16 heterozygous nonsynonymous and splice site-affecting SNVs prioritized for
307 functional studies were in PHMcc and seven were in PALcc; the remainder were in the non-
308 catalytic regions of PAM (Figure 2A). Based on *in silico* predictions, one of the four SNVs in
309 PHMcc and six of the seven in PALcc were predicted to be pathogenic (Table 2). The
310 prioritized variants were not significantly spatially clustered when we considered the
311 geometric mean distance between all pairs of variants normalized to cDNA length ($p = 0.35$).
312 However, when we classified the variants based on their location within the N-terminus,
313 PHMcc, linker, PALcc or C-terminus (Figure 2A), the N-terminus harbored more variants
314 than expected by random chance ($p = 0.02$, Table S5).

315

316 Expression of prioritized *PAM* SNVs revealed deficits in protein expression, enzymatic
317 activity, and glycosylation

318 To understand the impact of the prioritized *PAM* variants on the function of this membrane
319 enzyme, we transiently expressed each PAM-1 variant in PEAkRapid (HEK-293 derivative)
320 cells. To facilitate accurate assessment of the PHM and PAL activity of each *PAM* variant, a
321 non-ionic detergent (TX-100) was used to solubilize membrane proteins from a crude

322 particulate fraction (Figure 3A). PAM expression levels and protein integrity were evaluated
323 by Western blot analysis (Figure 3B) and PAM trafficking was assessed by analysis of N-
324 and O-linked oligosaccharide maturation (Figure 3C and 3D).

325 To establish the efficacy of our expression system, we compared the properties of WT PAM
326 to those of missense PAM variants designed to inactivate PHMcc (p.Thr189Ile) or PALcc
327 (p.His529Arg and p.Gly796Glu, Figure S4). PHM and PAL activity measurements for WT
328 PAM and for each engineered control were normalized to the expression of that PAM protein
329 [(PHM or PAL activity)/PAM protein], providing a measure of its specific activity; the PHM
330 and PAL specific activities of each engineered control were then compared to the PHM and
331 PAL specific activities of WT PAM. As expected, the PHM activity of the p.Thr189Ile mutant
332 and the PAL activity of the p.His529Arg and p.Gly796Glu mutants were reduced more than
333 ten-fold compared to WT PAM (Figure S4 and Table 3).

334

335

336 **Table 3. Summary of effects on enzymatic activity and glycosylation for all PAM variants that**
 337 **were functionally tested**

PAM variant	PHM activity (% WT)	PAL activity (% WT)	PNGaseF (N-sugars)	Neuraminidase (sialic acid)
WT	100 ± 2	100 ± 3	yes	yes
Val27Ile	79 ± 6	103 ± 15	(yes)	(yes)
Phe31Tyr	76 ± 16	84 ± 1	(yes)	(yes)
Val49Leu	80 ± 26	93 ± 1	(yes)	(yes)
Arg80Gln	75 ± 6	87 ± 9	(yes)	(yes)
Thr189Ile [†]	6 ± 3**	23 ± 5**	yes	no
His240Tyr	111 ± 24	98 ± 22	yes	no
Val244Ala	113 ± 32	80 ± 10	(yes)	(yes)
Pro412Leu	81 ± 32	85 ± 28	(yes)	(yes)
Ile452Val	112 ± 45	113 ± 30	(yes)	(yes)
Glu491Asp	133 ± 75	138 ± 55	(yes)	(yes)
His529Arg [†]	41 ± 13	7 ± 1**	n.a.	n.a.
Ser539Trp	49 ± 7**	0 ± 1**	yes	no
Gly552Arg	41 ± 6**	6 ± 2**	yes	no
Asp563Gly	44 ± 8**	14 ± 3**	yes	no
Gly641Arg	104 ± 0	116 ± 10	(yes)	(yes)
Arg703Gln	92 ± 17	0 ± 1**	yes	yes
Phe759Ser	110 ± 25	12 ± 3**	yes	no
His778fs	23 ± 3**	0 ± 1**	n.a.	n.a.
Gly796Glu [†]	55 ± 8	4 ± 1**	(yes)	(yes)

338 Values are presented as mean ± SEM.
 339 n.a., not assessed; WT, wild-type; (yes), based on SDS-PAGE profile.
 340 [†]Engineered inactive mutants.
 341 ** p < 0.0001.
 342

343

344 The structure of bifunctional PAM has not yet been experimentally determined, but for all
345 three engineered variants, inactivating mutations placed into one domain resulted in a
346 modest decrease in the activity of the other domain.

347 Activity and Western blot data for all studied SNVs are shown in Figure 3A and 3B,
348 respectively. No significant decrease in PHM activity was seen for SNVs p.Val27Ile,
349 p.Phe31Tyr, p.Val49Leu, p.Arg80Gln, p.His240Tyr or p.Val244Ala. The three non-catalytic
350 linker domain SNVs tested exhibited no significant change in PHM or PAL activity and were
351 not extensively assayed. Six of the seven SNVs located in PALcc exhibited a dramatic
352 decrease in PAL activity. While some PALcc variants reduced PHM activity, others did not.
353 Although the PAL activity of p.Arg703Gln was undetectable and the PAL activity of
354 p.Phe759Ser was 12% of WT, their PHM activities were equal to that of WT. In contrast, the
355 dramatic reductions in PAL activity observed for p.Ser539Trp, p.Gly552Arg, p.Asp563Gly,
356 and p.His778fs were accompanied by at least a two-fold decrease in PHM activity. Normal
357 levels of both PHM and PAL activity were detected for p.Gly641Arg.

358

359 Maturation of N-linked oligosaccharides is generally completed only when the newly
360 synthesized protein exits the Golgi complex. Sialylation of both the N- and O-linked
361 oligosaccharides attached to rat PAM-1 occurs just before it exits the Golgi complex. The
362 stepwise manner in which the maturation of N- and O-linked glycans occurs often results in
363 glycoprotein heterogeneity, contributing to the diffuse bands observed for WT PAM and
364 many of the prioritized SNVs (Figure 3B, FLAG Ab and PHM Ab). In contrast, we
365 consistently observed a slightly smaller, more compact band for p.Thr189Ile (engineered
366 variant), p.His240Tyr, p.Ser539Trp, p.Gly552Arg, p.Asp563Gly, and p.Phe759Ser.

367

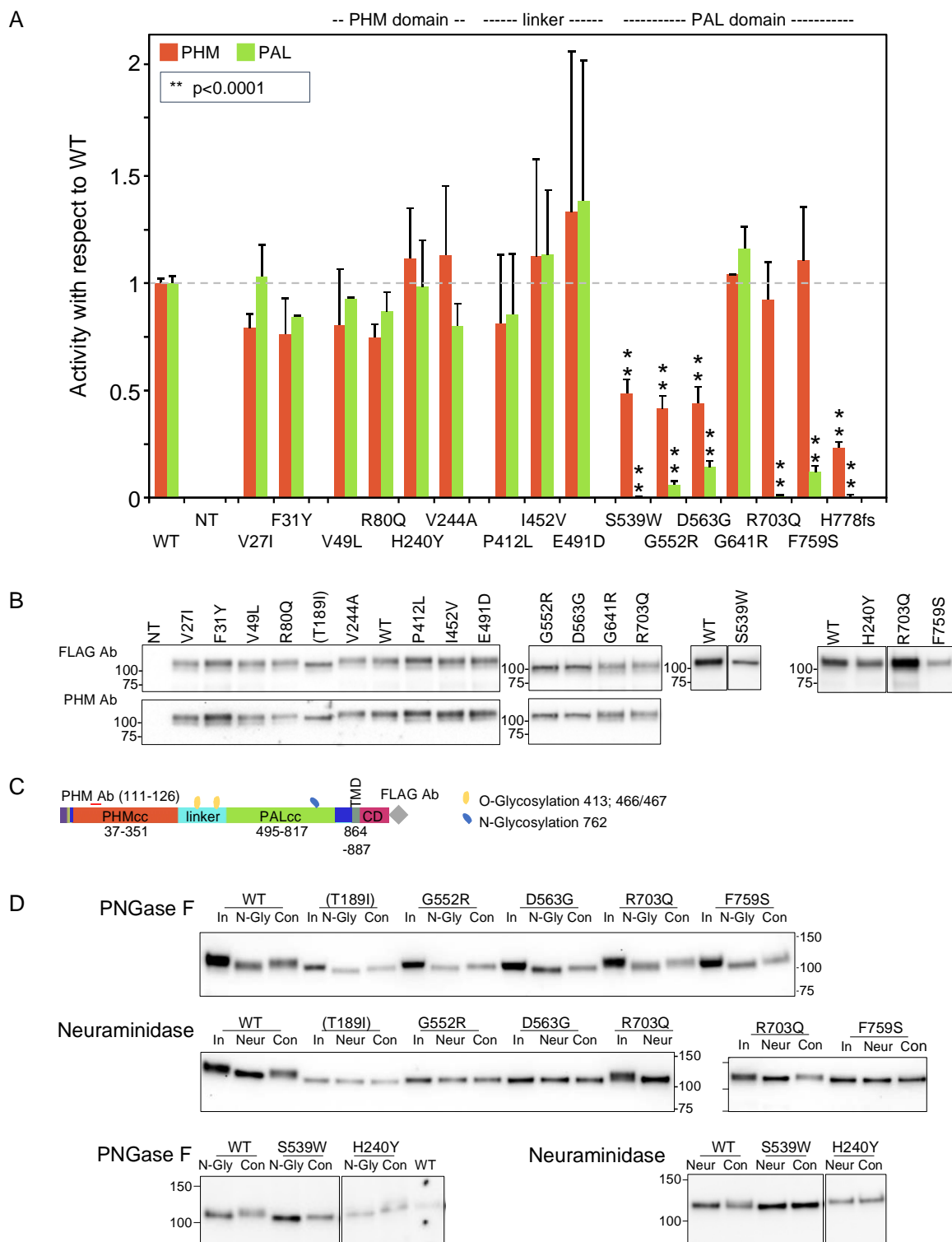
368 Since altered access to the enzymes responsible for the maturation of N- and O-linked
369 glycans can reflect altered protein trafficking, we utilized PNGase F to remove N-linked
370 oligosaccharides and neuraminidase to remove sialic acid (Figure 3D). The 2 to 3 kDa
371 reduction in apparent molecular mass caused by PNGase F treatment demonstrated that the

372 engineered control lacking PHM activity (Thr189Ile) and that each of these six variants had
373 been N-glycosylated. As expected, neuraminidase treatment brought about a slight
374 decrease in the apparent molecular mass of WT PAM. Strikingly, neuraminidase treatment
375 failed to reduce the mass of any of these six SNVs, indicating that their glycans had not
376 undergone normal sialylation. Despite this, p.His240Tyr, one of the variants exhibiting
377 altered sialylation, had normal levels of both PHM and PAL activity. Altered trafficking in the
378 secretory and/or endocytic pathway could limit sialylation.

379

380 A summary of the effect of each prioritized and engineered SNV on PAM expression,
381 enzymatic activity and oligosaccharide maturation is presented in Table 3.

382



383

384 **Figure 3. Enzymatic activity, protein expression, and glycosylation pattern of PAM variants**

385 (A) PHM and PAL activity. As described in Methods, TMT solubilized particulate fractions prepared
 386 from transiently transfected PEAKrapid cells were assayed for PHM activity and for PAL activity. Data

387 for the level of expression of WT PAM and each full-length variant were determined by quantifying the
388 FLAG-tag signal. Levels of p.His778fs were assessed as described in Methods. NT, not transfected;
389 WT, wild-type; **p < 0.0001. (B) PAM protein expression. SNV expression was assessed using a
390 FLAG tag antibody and an antibody to a peptide contained in PHMcc (JH246). The lines separating
391 WT and p.His240Tyr from p.Arg703Gln and p.Phe759Ser indicate that data for two intervening
392 samples were removed. Molecular weight standards are indicated. (C) PAM protein diagram,
393 indicating the location of the JH246 epitope (red horizontal line), the FLAG tag (grey diamond), and
394 the expected location of N- and O-glycans (blue and yellow freeform shapes, respectively). (D)
395 Glycosylation is altered in a subset of PAM variants. Cell lysates were treated with PNGase F or
396 neuraminidase as described in Methods. Proteins were visualized using the FLAG antibody. The
397 samples treated with Neuraminidase were analyzed on two separate gels, with the p.Arg703Gln
398 samples appearing in part on both gels. Con, control; In, Input; N-Gly, PNGase; Neur, neuraminidase;
399 NT, not transfected.

400

401 Three SNVs with diminished PAL activity were found in subjects with GH excess leading to
402 sporadic acromegaly (p.Ser539Trp, p.Gly552Arg) or gigantism (p.Phe759Ser). The relatively
403 common p.Asp563Gly variant (3.11% global MAF) was observed in persons with different
404 types of PAs (Table 2). Interestingly, two males with GH excess – one with acromegaly, and
405 one with gigantism – were compound heterozygotes for p.[Gly552Arg];[Asp563Gly] and
406 p.[His240Tyr];[Phe759Ser], respectively (Figure S5, Table 1). Although neither the PHM nor
407 PAL activity of p.His240Tyr (located in PHMcc) differed from WT, its heterogeneous band
408 pattern and lack of sensitivity to neuraminidase suggest structural alterations sufficient to
409 alter its trafficking (Figures 3A, 3B, and 3D); its significantly higher prevalence in the cohort
410 of subjects with PAs vs. controls, and the absence of homozygous individuals in gnomAD,
411 argue for its classification as a variant of uncertain significance (VUS). Further studies are
412 necessary to establish or disprove pathogenicity.

413

414 Three of the 15 SNVs were identified in individuals from FIPA families (one variant in each
415 family, Table 2). SNVs p.Arg80Gln and p.Pro412Leu, which are very rare (MAF: 0.002%),

416 are significantly more prevalent in our cohort vs. controls, and are without homozygous
417 variant entries in gnomAD. The p.Arg80Gln SNV is located within PHMcc, while the
418 p.Pro412Leu and p.Glu491Asp SNVs are within the linker region. The p.Arg80Gln did not
419 segregate with the phenotype in the FIPA kindred and functional studies showed no
420 significant effect of p.Arg80Gln on PHM enzymatic activity. In contrast, the p.Pro412Leu
421 variant segregated with the FIPA phenotype and was seen in both the proband (acromegaly)
422 and the daughter (prolactinoma). Neither of the p.Pro412Leu or p.Glu491Asp variants in the
423 non-enzymatic linker region had a functional effect on PHM or PAL activity (Figure 3A, Table
424 3). Altogether, combining allele frequencies in our cohort vs. controls, *in silico* predictions,
425 functional data, and segregation analysis, our results indicate that the p.Arg80Gln and
426 p.Glu491Asp variants should be classified as likely benign, while p.Pro412Leu linker region
427 change is a VUS.

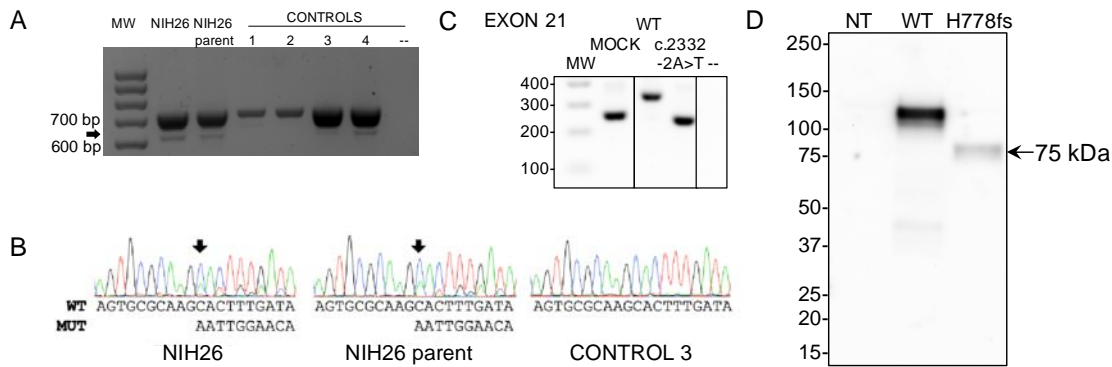
428

429 Functional evaluation of a truncating variant associated with Cushing disease

430 A variant affecting a canonical splice acceptor site (c.2332-2A>T) was present in a pediatric
431 female (NIH26) with ACTH excess leading to Cushing disease (Table 1). Her parent also
432 carried the variant and had evidence of disrupted circadian cortisol rhythm, based on
433 elevated midnight serum cortisol, but did not present clinically overt Cushing disease. The
434 variant was predicted to cause skipping of exon 21, generating a frameshift that introduces a
435 premature stop codon, eliminating the C-terminal region of PALcc (p.His778fs, Table 2 and
436 Figure S6). Skipping of exon 21 was confirmed by testing both the affected subject and
437 parent blood-extracted RNA (Figure 4A and 4B) and by using a minigene splicing assay
438 (Figure 4C). Transient expression of p.His778fs in PEAKrapid cells demonstrated, as
439 expected, that the truncated protein lacked detectable PAL activity; in addition, significantly
440 reduced levels of PHM activity were also observed (Figure 3A, Table 3). Western blotting
441 indicated that the mutant protein is produced but at lower levels than those observed for WT.
442 Moreover, the observed band was smaller than predicted (75 vs. 90 kDa, respectively),

443 suggesting that the mutant might lack some post-translational modifications and/or is
 444 cleaved at a pair of basic residues introduced by the frameshift (Figure 4D).

445



446

447 **Figure 4. Functional studies of the c.2332-2A>T (p.His778fs) truncating variant**

448 (A) RT-PCR analysis of *PAM* exon 21 splicing was performed using blood-extracted RNA from two
 449 family members carrying the c.2332-2A>T variant (NIH26 and parent) and four WT control cDNAs.
 450 Primers were designed on exons 17 and 22. Both carriers and controls showed a normally spliced
 451 transcript (713 bp, upper band), while only the carriers showed an additional alternatively spliced
 452 transcript lacking exon 21 (the 613 bp band, identified by the arrow). MW, molecular weight marker.

453 (B) The identity of the PCR products (panel A) was confirmed by Sanger sequencing. The arrows
 454 point to the variant-specific peaks present only in the carriers. MUT, mutated; WT, wild-type. (C)

455 Minigene assay. After transfection into HEK-293 AD cells, mRNA synthesis from the plasmids using
 456 the cells' own transcription and splicing machinery led to mRNA products containing (WT) or lacking
 457 (variant) exon 21 of *PAM* flanked by two exons from the pSPL3 vector. The RT-PCR analysis of the
 458 minigene transcripts was conducted using vector-specific primers. MW, molecular weight marker;
 459 MOCK, cDNA from empty vector-transfected cells consisting of a 260 bp band made up of fragments
 460 of pSPL3 exons; --, negative control (RT-PCR without cDNA). The lines separating MOCK and
 461 negative control from WT and c.2332-2A>T indicate that data for intervening samples were removed.

462 (D) An expression vector lacking exon 21 of human PAM-1 (H778fs) was transiently expressed in
 463 PEAKrapid cells. Proteins were visualized using an antibody to PHMcc (JH246 PHM Ab). Expression
 464 of WT PAM produces a major band at 114 ± 1 kDa and a minor one at 105 kDa. The only band visible
 465 in the cells expressing p.His778fs migrated at 75 kDa; after signal peptide removal, the mass

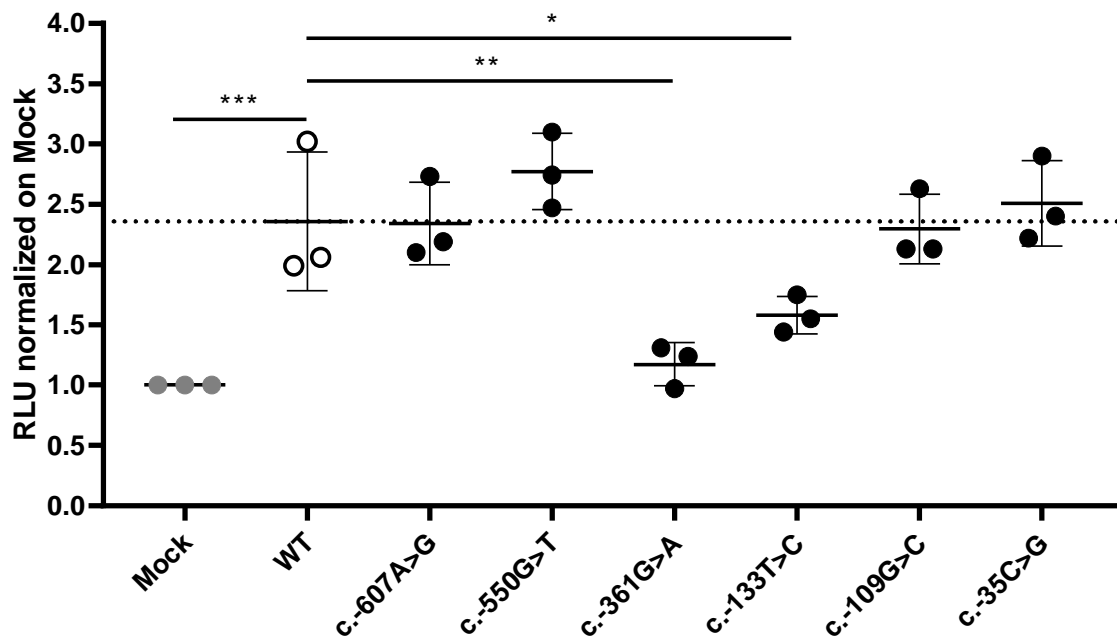
466 predicted for this protein – which includes only the first 777 residues of WT PAM-1 but extends 45
467 residues beyond residue 777 before reaching a stop codon – is 90.31 kDa. NT, not transfected.

468

469 Screening for PAM SNVs located in regulatory regions identified two variants that reduce
470 transcription

471 Next, we extended our screening to regulatory regions of *PAM*, namely the promoter and
472 untranslated regions (UTRs), to investigate whether there are SNVs with the potential to
473 impact *PAM* expression. We identified multiple variants and prioritized seven – two in the
474 promoter, four in the 5'UTR, and one in the 3'UTR – for functional studies (Table 2 and
475 Figure S7). All SNVs in regulatory regions were observed in the heterozygous state. The
476 selection criteria we applied for prioritization were analogous to those used for
477 nonsynonymous variants. *In vitro* evaluation in HEK-293 AD cells using reporter assays, in
478 which we cloned either the promoter-5'UTR upstream or the 3'UTR downstream of the
479 luciferase coding sequence, identified two 5'UTR variants, c.-361G>A and c.-133T>C, that
480 significantly reduced luciferase activity when compared to the WT sequence (Figure 5A). In
481 contrast, the 3'UTR variant c.*1455C>T, which was predicted to create new miRNA binding
482 sites, did not produce any effect on luciferase activity when expressed alongside the
483 identified miRNAs (Figure S8), indicating that it does not impair *PAM* mRNA stability and
484 expression. The c.-361G>A variant was identified in two patients with gigantism and in one
485 individual with a non-functioning PA, while the c.-133T>C variant was present in a pediatric
486 subject who also harbored the p.Phe31Tyr variant and was affected by CD (NIH36, Table 1);
487 this genotype will be identified as c.-133T>C(;;)p.Phe31Tyr. Based on *in vitro* enzyme
488 assays, the p.Phe31Tyr missense SNV diminished neither PHM nor PAL activity to a
489 significant extent (Figure 3A and Table 3).

490



491

492 **Figure 5. WT and mutant PAM promoter activity**

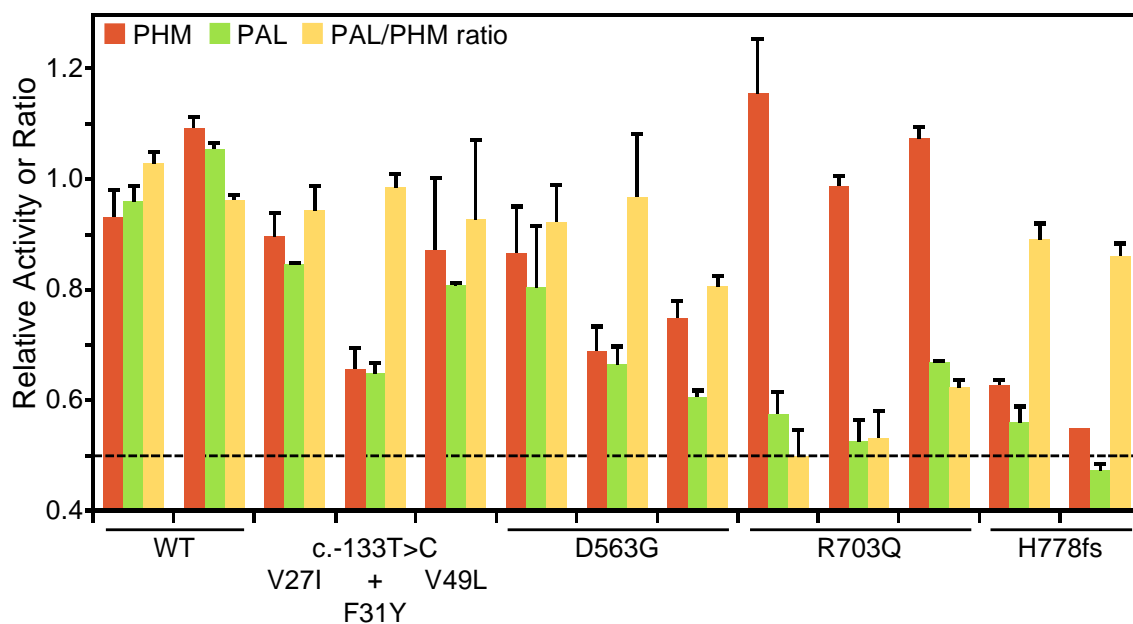
493 A 5 kb PAM promoter-5'UTR WT sequence was cloned upstream of a luciferase reporter. Six mutant
494 constructs were created by site-directed mutagenesis. The constructs were transiently transfected
495 into HEK-293 AD cells together with a *Renilla* luciferase reporter for normalization. *Firefly* and *Renilla*
496 luciferase activities were measured 24 h post-transfection. Two SNVs, c.-361G>A and c.-133T>C,
497 have significantly lower transcriptional activity than the WT hybrid transcript. Mock, empty pRMT-Luc
498 vector. Differences between experimental groups were analyzed by 1-way ANOVA with Dunnett's
499 post hoc test, using WT as the control group. RLU, relative luciferase activity. *, p = 0.0443; **, p =
500 0.0019; ***, p = 0.0005.

501

502 Quantification of PHM and PAL catalytic activities in serum

503 Endoproteolytic cleavage of PAM-1 can occur within the regulated secretory pathway and
504 after the endocytic retrieval of PAM-1 from the cell surface [16-18]. In addition, several PAM
505 splice variants encode soluble bifunctional PAM proteins that are secreted along with the
506 peptide hormones stored in secretory granules [10]. As a result, both PHM and PAL activity
507 are readily detected in human serum [19, 20]. The anti-coagulants added during the
508 collection of plasma make the accurate assessment of PHM activity in plasma very difficult.

509 As a result, we could only assess PHM and PAL activity in the small number of serum
510 samples collected from our cohort. As shown in Figure 6, we measured PHM and PAL
511 activity and calculated the ratio of PAL to PHM activity for 13 subjects (11 affected
512 individuals/carriers and two WT controls). To control for the fact that the serum samples
513 assayed had been stored for varying periods of time, we evaluated the statistical
514 significance of changes in activity by normalizing PAL activity to PHM activity.
515



516

517 **Figure 6. PHM and PAL activity in human sera**

518 Assays for PHM and PAL activity were carried out on sera from individuals harboring variants
519 showing deleterious effects in *in vitro* assays (p.Asp563Gly, p.Arg703Gln, and p.His778fs), subjects
520 with more benign variants (p.Val27Ile and p.Val49Leu) and controls (WT). In NIH36, the p.Phe31Tyr
521 variant occurs along with the 5'UTR c.-133T>C variant. The dashed line at 0.5 indicates the activity
522 level expected with one completely inactive allele. WT, wild-type.

523

524 Several serum samples were available from patients with two SNVs that resulted in the
525 almost total loss of the PAL activity of PAM-1, p.Arg703Gln (three samples) and p.His778fs
526 (two samples); consistent with our *in vitro* activity assays of transiently expressed PAM-1
527 and the total elimination of one allele, serum PAL activity fell by a factor of two for both

528 variants. Also consistent with the data shown in Table 3, serum PHM activity was unaffected
529 in the p.Arg703Gln variant and was reduced substantially in the p.His778fs variant. Three of
530 the serum samples available came from subjects carrying SNVs located in PHMcc, but none
531 significantly reduced the PHM or PAL activity of transiently expressed PAM-1. Strikingly,
532 both PHM and PAL activities were reduced in the serum sample from NIH36, who harbors
533 an expression-inhibiting 5'UTR SNV (c.133T>C) in addition to the p.Phe31Tyr SNV. Taken
534 together, our data indicate that the observed reduction in serum PHM and PAL activity can
535 be attributed to the 5'UTR SNV, thus confirming the *in vitro* finding. Sera from three different
536 subjects harboring the p.Asp563Gly variant showed substantial, but variable, reductions in
537 both PHM and PAL activity. A better interpretation of serum PHM and PAL activity requires
538 further insight into its multiple potential sources. Altogether, analysis of the limited set of
539 serum samples available emphasizes the utility of using a wide variety of *in vitro* systems to
540 evaluate the functional effects of PAM SNVs.

541

542 Loss of heterozygosity (LOH) studies

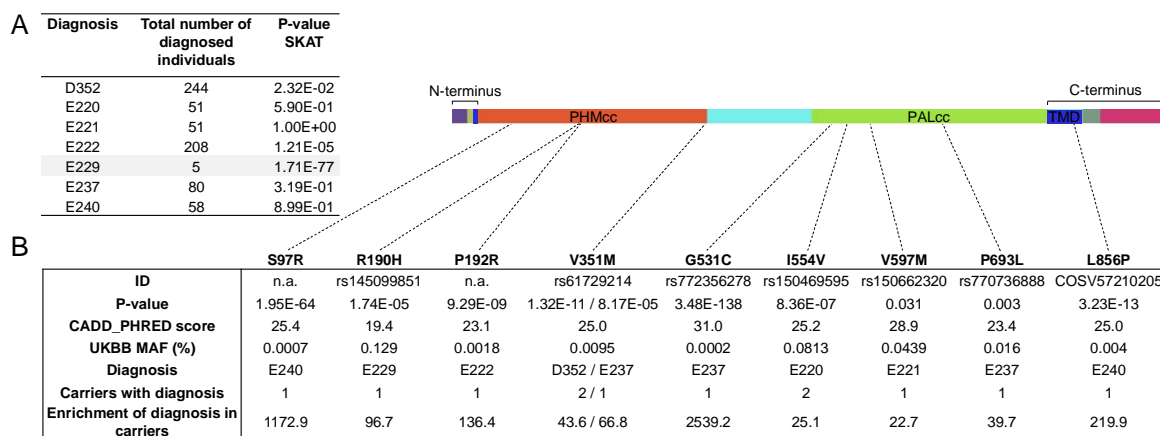
543 Next, we explored whether PAM acts as a tumor suppressor gene and requires a second
544 somatic hit affecting the WT allele, as would be expected by the Knudson two-hit hypothesis
545 [21]. Therefore, we extracted genomic DNA from the PAs surgically removed from
546 individuals harboring variants with demonstrated deleterious effects and studied LOH by
547 Sanger sequencing. The available tumors that we analyzed harbored the following variants:
548 c.-133T>C(;);p.Phe31Tyr, p.Ser539Trp, p.[Gly552Arg];[Asp563Gly], and p.Asp563Gly (n = 1
549 tested for each SNV). We did not identify loss of the WT allele, suggesting that *PAM* does
550 not behave like a typical tumor suppressor but may rather be a haploinsufficient gene.
551 Unexpectedly, in the individual compound heterozygous for p.[Gly552Arg];[Asp563Gly], we
552 observed loss of the mutant allele causing the change at codon 552 (Figure S9). This likely
553 represents an instance of *in vivo* reversion to normal of an inherited variant (revertant
554 mosaicism) [22].

555

556 *PAM* SNVs are significantly associated with a hyperfunctioning pituitary gland

557 Having detected multiple SNVs with deleterious effects on *PAM* protein function in our
558 cohort, we decided to test whether SNVs within the *PAM* locus are statistically associated
559 with a pituitary disease phenotype in a larger and more heterogeneous cohort. To this end,
560 we interrogated the UKBB, a powerful resource for genetic association analyses that
561 contains exome sequencing data of 200,643 adult individuals with different and well-
562 annotated pathologies. First, we performed a gene-based association study using the
563 sequence kernel association test (SKAT) [23, 24]. For this analysis, we considered ICD-10
564 codes associated with sellar lesions (D352, E220, E221, E222, E229, E237, E240, covering
565 the following conditions: benign neoplasm of pituitary gland; acromegaly and pituitary
566 gigantism; hyperprolactinemia; syndrome of inappropriate secretion of antidiuretic hormone;
567 hyperfunction of pituitary gland, unspecified; disorder of pituitary gland, unspecified; and
568 pituitary-dependent Cushing's disease, respectively) and SNVs at the *PAM* locus for which
569 we assigned a weight based on their detrimental effect on protein (see Methods for details).
570 We set a MAF cutoff of 0.1% for rare variants. *PAM* was identified as significantly associated
571 (exome-wide) with a diagnosis of hyperfunction of the pituitary gland (E229, $p = 1.71 \times 10^{-77}$,
572 Figure 7A). Next, we performed single-variant analyses restricted to rare (MAF < 1%) *PAM*
573 variants with a predicted deleterious effect (CADD_PHRED score ≥ 15). We identified nine
574 missense heterozygous variants (Figure 7B and Figure S10, Table S6), four in PHMcc
575 (p.Ser97Arg, p.Arg190His, p.Pro192Arg, p.Val351Met), four in PALcc (p.Gly531Cys,
576 p.Ile554Val, p.Val597Met, p.Pro693Leu) and one in the C-terminal non-catalytic region
577 (p.Leu856Pro). The p.Ser97Arg variant is located in the loop connecting the β -strand
578 containing the vicinal His residues that bind Cu_H to the preceding β -strand and the
579 p.Arg190His and p.Pro192Arg variants are both in the short linker that connects the two
580 homologous domains of PHMcc. PHMcc terminates with Val351; although the final three C-
581 terminal amino acids are not identified in the crystal structure, removal of four C-terminal
582 residues produces a protein lacking PHM activity. Two of the PAL variants (p.Gly531Cys

583 and p.Ile554Val) are situated in or near the long loop that forms its hydrophobic substrate
 584 binding pocket and connects β -strands 2 and 3 of Blade 1. The other two PAL variants
 585 (p.Val597Met and p.Pro693Leu) are contained within the structural repeats that form Blades
 586 2 and 4. A study aimed at understanding the effects of luminal pH on the trafficking of rat
 587 PAM identified the juxtamembrane region that encompasses p.Leu856Pro as a key
 588 determinant [25].



589

590 **Figure 7. Gene- and variant-based association analyses for PAM in the UKBB**

591 (A) Results of SKAT analysis of PAM variants for diagnoses of hyperfunction of the pituitary gland
 592 (identified from UKBB fields 41270 and 41204 - primary and secondary ICD-10 diagnoses from
 593 hospitalization records - and 20002 - self-reported diagnosis) in 200,000 UKBB participants. The
 594 SKAT CommonRare algorithm was used for analysis. (B) Significant enrichment for PAM pathological
 595 missense PAM variants in subjects diagnosed with hyperfunction of the pituitary gland.

596

597 To complement the analyses conducted on the UKBB, we examined the outputs of a gene-
 598 specific metric, the gene damage index (GDI) [26]. The GDI score predicts the liability of a
 599 gene to contain disease-causing mutations by considering the influences of selection and
 600 genetic drift [27]. For this analysis, we considered 20 genes that harbor germline or somatic
 601 variants known to predispose to PAs in order to estimate a GDI cutoff above which a gene is
 602 unlikely to cause a pituitary disease, i.e., it is considered a false positive. PAM was reported
 603 to have a Phred-scaled GDI score of 3.98, below the calculated cutoff of 4.84 (Table S7).

604 Therefore, our analysis indicates an intolerance to mutational changes for *PAM* in large
605 population samples, supporting the notion that the observed LOF variants are more likely to
606 contribute to disease.

607

608 Functional analysis of splicing in other rare variants

609 Among the rare SNVs identified in the UKBB, an intronic SNV (c.2746+3A>T) was predicted
610 to disrupt the canonical splice donor site of intron 24 (Figure S11A and Table S6); this SNV
611 was associated with a diagnosis of acromegaly and pituitary gigantism (E220). Our splicing
612 analysis ascertained that this SNV caused skipping of exon 24 in most transcript molecules;
613 the expected outcome is the in-frame deletion of 19 residues located within the cytoplasmic
614 domain (p.(Ala897_Arg915del)). While the absence of exon 24 does not alter the PHM or
615 PAL activity of PAM, previous studies indicate that tissue-specific splicing at this site occurs
616 in the rat and could affect the trafficking of membrane PAM [28-30]. PAM mRNA expression
617 levels for the mutant allele were about half of WT levels in *in vitro* studies (Figure S11B).
618 Therefore, an individual harboring this variant is predicted to make 25% less biologically
619 active PAM protein than healthy controls; if trafficked correctly, a reduced level of this variant
620 might support normal PAM functions.

621 The finding of two non-coding SNVs affecting *PAM* splicing (albeit with different outcomes)
622 and recent studies underscoring the importance of evaluating splicing defects as a disease
623 mechanism for both conditions of hyper and hypopituitarism [31, 32], prompted us to extend
624 the corresponding functional studies to selected synonymous and intronic variants found in
625 our cohort (Figure S12A and Table S8). The selection criteria were analogous to those
626 applied for other prioritized variants. This analysis did not reveal any splicing impairment.
627 We observed only a possible reduction in mRNA expression by the c.195G>T allele (Figure
628 S12B), but this was not subsequently confirmed by qPCR studies (Figure S12C).

629 Taken together, these findings indicate that impairment of PAM function or expression via
630 abnormal splicing is not frequently associated with PAs.

631

632 **Discussion**

633 Studies over the last 30 years have identified a small number of germline and somatic
634 genetic defects associated with the development of different types of PAs. However, the
635 majority of tumors still do not have a known genetic cause [8]. Here, we report that several
636 rare variants in the *PAM* gene are associated with pituitary gland hypersecretion and that
637 most have a deleterious effect on protein function.

638

639 We initially identified a deleterious missense *PAM* variant (p.Arg703Gln) in a FIPA family
640 with a severe form of childhood-onset pituitary gigantism in which other established causes,
641 such as X-linked acrogigantism (X-LAG) due to duplications involving *GPR101*, germline *AIP*
642 mutations/deletions or others, had been ruled out [33, 34]. Among the genes filtered from the
643 WES screening, the high level of *PAM* expression in the pituitary gland and its known
644 functions in the pituitary and other endocrine cells made *PAM* the prioritized candidate. A
645 bifunctional enzyme that is essential for the biosynthesis of multiple pituitary and
646 hypothalamic peptide hormones, *PAM* affects regulated secretion and is involved in the
647 biogenesis of secretory granules [10, 11, 35, 36]. Based on site-directed mutagenesis and
648 crystallographic studies of rat PALcc, this Arg residue (Arg 706 in rat *PAM*) is located at the
649 active site and participates in substrate binding; site-directed mutagenesis of this residue to
650 Gln reduced the activity of PALcc by a factor of four, and mutagenesis to Ala decreased
651 activity by 97% versus WT [13]. Or functional studies on bifunctional human *PAM* with the
652 Arg703 to Gln mutation revealed an even greater decrease in its PAL activity (1% relative to
653 WT human *PAM*), while retaining fully normal PHM activity.

654

655 This intriguing finding prompted us to explore whether LOF *PAM* variants (SNVs and CNVs)
656 were associated with other types of anterior pituitary adenomas, occurring both in familial
657 and sporadic settings. No other likely deleterious *PAM* SNVs were observed in the familial
658 cases examined, making the estimated frequency of *PAM* LOF variants in our FIPA kindreds

659 5.56% (1/18). A very rare VUS, p.Pro412Leu, located within the linker region connecting the
660 two enzymatic domains was identified in a heterogeneous FIPA kindred.

661

662 We detected six germline SNVs with deleterious effects on protein function/expression in
663 sporadic PA cases. Two variants (p.Ser539Trp, p.Asp563Gly) are relatively common in the
664 general population (global MAF range: 0.42-3.11%). The remaining four variants are very
665 rare (MAF < 0.01%), were found in one subject each, and were significantly enriched in our
666 cohort compared to the general population. Two of these variants (p.Gly552Arg and
667 p.Phe759Ser) were identified in subjects with GH excess (2/173, frequency: 1.16%) and two
668 (c.-133T>C and p.His778fs) in cases with pediatric CD (2/81, frequency: 2.47%).

669

670 The p.Ser539Trp and p.Asp563Gly mutants were previously implicated as major risk factors
671 for type 2 diabetes [37, 38], and an important question is whether these proteins are
672 enzymatically active. When introduced into human PAM-1 and assayed in cell lysates, the
673 p.Ser539Trp variant had 50% of normal PHM activity, lacked detectable PAL activity, and
674 was not normally sialylated, suggesting its abnormal intracellular trafficking. When
675 expressed as a soluble protein containing both PHMcc and PALcc, secretion of the
676 p.Ser539Trp protein was not detected, precluding assessment of its catalytic activity [15].
677 We prepared two independent p.Ser539Trp plasmid constructs, sequenced both and verified
678 that both constructs were correct. The p.Ser539Trp mutation disrupted the normal trafficking
679 of membrane PAM; when present in the soluble protein, the same variant may inhibit its
680 secretion [15]. Using an assay to quantify PAM activity (PHM followed by PAL) by measuring
681 glyoxylate production from hippuric acid, *Thomsen et al.* reported that the p.Asp563Gly
682 mutation was about 50% active [15]. Our assays, which assess PHM and PAL activity
683 separately, showed that the PHM activity of p.Asp563Gly was slightly less than half that of
684 WT PAM, while its PAL activity was only one seventh that of WT PAM.

685

686 Our *in vitro* functional tests indicated an impairment in enzymatic activity for five missense
687 SNVs and the splicing variant (in PAL). *PAM* expression was decreased for two 5'UTR
688 variants and the COOH-terminal region splicing variant. In addition, glycosylation was
689 abnormal for four of the five missense SNVs. Experimental truncations used to define the C-
690 terminus of rat PALcc are consistent with the lack of PAL activity observed for p.His778fs,
691 with the same inability of the truncated rat protein to fold correctly, thereby preventing its
692 secretion [39]. Our functional data indicate a similar behavior for the human variant, along
693 with a significant reduction in PHM activity.

694

695 We also showed that measurement of PHM and PAL activity in the serum of subjects
696 harboring *PAM* variants correlates well with the corresponding values for each single variant
697 determined using the PEAKrapid assay system. The CD case that harbored a regulatory
698 variant reducing *PAM* expression was instructive in this regard. In that case, both PHM and
699 PAL activities were lowered to the same extent (about 40% compared to WT), with the PAL
700 to PHM ratio showing a normal value, suggesting that enzymatic activity was not affected.
701 These observations indicate that it is valuable to explore whether assays of PHM and PAL
702 activity in sera could be used as a biomarker of pituitary disease. The recent development of
703 an efficient high-throughput amidation assay [20] could aid in this endeavor.

704

705 The association of loss-of-function *PAM* SNVs with PAs was further explored by screening
706 200,000 exomes from the UKBB. Supporting our findings, *PAM* was significantly associated
707 with a diagnosis of pituitary hyperfunction (ICD-10 code E229) and nine rare (MAF < 1%)
708 missense variants, all predicted to be pathogenic, were associated with diagnoses of sellar
709 lesions. Although the UKBB cohort has an inherent selection bias towards healthy
710 volunteers, and disease annotation may be self-reported in some cases or only after hospital
711 admission in others (thereby potentially excluding mild or undiagnosed pituitary
712 hypersecretion diagnoses), the validation of our findings related to *PAM* variants in an
713 independent cohort more representative of the general population strengthens the observed

714 associations. Based on previous studies, each of the nine *PAM* variants identified in the
715 UKBB (four in PHMcc, four in PALcc, and one in the juxtamembrane region of the luminal
716 domain) is expected to disrupt enzymatic activity or trafficking of membrane *PAM* bearing
717 these mutations.

718

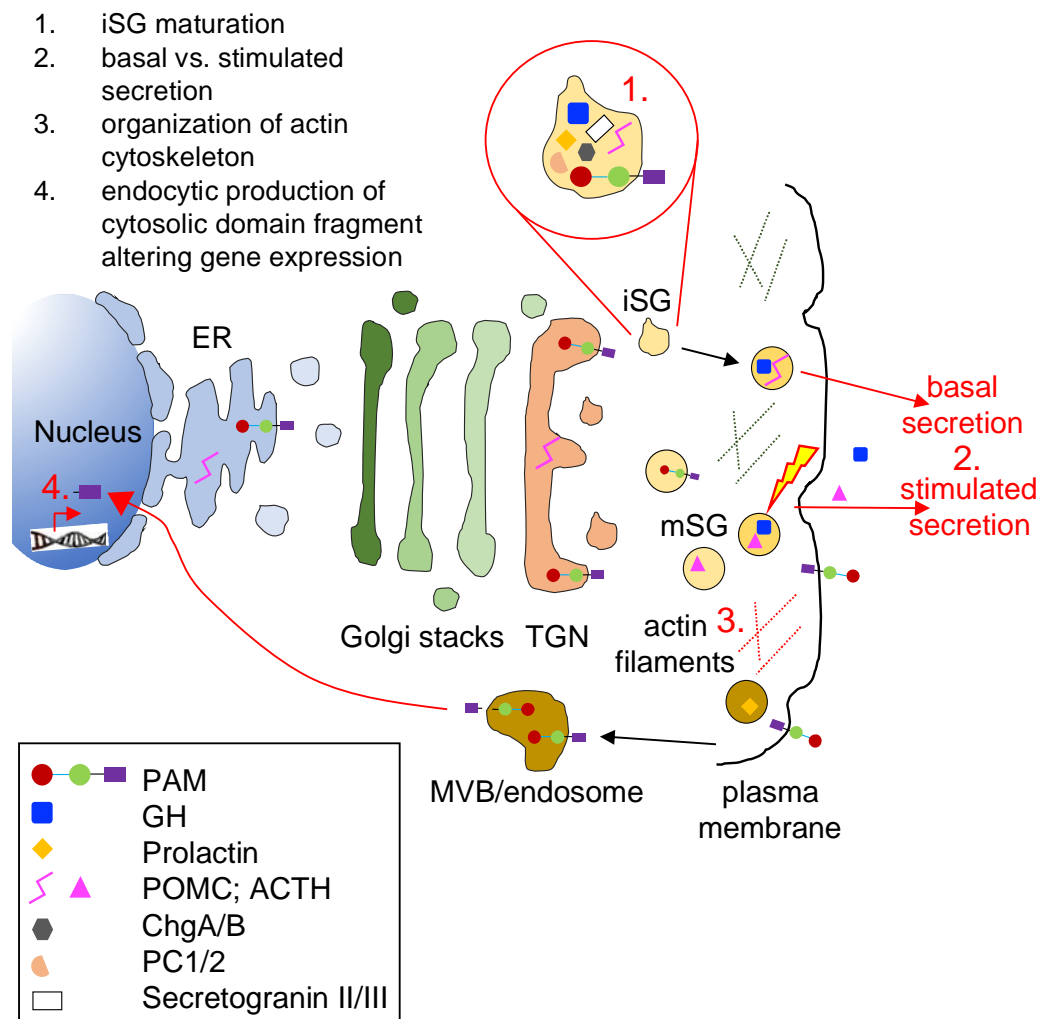
719 We did not observe LOH for *PAM* variants. This suggests that *PAM* does not behave as a
720 classical tumor suppressor gene, leading us to propose that pathogenic LOF *PAM* variants
721 act as haploinsufficient alleles. This hypothesis is supported by *in silico* predictions (HI and
722 HIPred scores for *PAM* are 0.588 and 0.7057, respectively) [40, 41], and by previous
723 observations that *Pam* haploinsufficiency impairs several interdependent organ systems in
724 *PAM*^{+/-} mice. Although a complete lack of *Pam* is not compatible with life [42], mice with a
725 single functional copy of *Pam* developed increased adiposity and mild glucose intolerance
726 with age [42], were deficient in their ability to maintain body temperature in the cold [43], and
727 showed an increase in anxiety-like behavior [44]; these findings are consistent with defects
728 in β -cell function, the vascular system, and neuronal/neuroendocrine function, respectively.
729 Production of the few amidated peptides tested was not greatly altered by *PAM*
730 heterozygosity [42, 45, 46], but the production of amidated TRH in the hypothalamus was
731 compromised and the mice were deficient in their pituitary response to hypothyroidism [43].
732 Moreover, pituitary levels of POMC-derived amidated peptides (diAc- α -MSH, α -MSH, and
733 JP) were significantly increased in *PAM*^{+/-} mice [46]. These data, together with our
734 observation that rare and functionally deleterious *PAM* SNVs are associated with anterior
735 pituitary hyperfunction, suggest that the pituitary gland is particularly sensitive to *PAM*
736 haploinsufficiency. Moreover, they indicate that changes in gene expression associated with
737 *PAM* heterozygosity are tissue-specific and that both amidation-dependent and amidation-
738 independent mechanisms (for instance, the secretagogue-mediated translocation of the
739 soluble cytoplasmic *PAM* fragment to the nucleus affecting gene expression in a cell-type
740 specific manner) [11, 47, 48] likely underlie the described deficits. It will be interesting to

741 study in further detail whether any of the phenotypes observed in PAM^{+/-} mice are also
742 present in individuals with PAs that harbor LOF *PAM* variants.

743

744 Limitations to our current study include the fact that only a few subjects with sporadic non-
745 functioning tumors and families with multiple endocrine neoplasia were screened. To better
746 estimate the prevalence of *PAM* variants and their possible contribution to pituitary
747 tumorigenesis, the search for *PAM* variants needs to be extended to those subtypes as well
748 as cohorts with hyperprolactinemia (tumoral or non-tumoral), the most common form of
749 pituitary hypersecretion. In particular, it would be interesting to better assess the risk that the
750 relatively common p.Asp563Gly variant confers to the development of pituitary
751 hyperfunction, particularly in the homozygous state. The variable PAL activity measured in
752 PA subjects that were heterozygous for this variant suggests that other factors, such as
753 genetic, biochemical or dietary (e.g., copper or vitamin C availability, hypoxia) [19],
754 contribute to the regulation of *PAM* expression and/or activity and, hence, predisposition to
755 pituitary dysfunction and potentially tumorigenesis. Second, our LOH and serum activity
756 analyses were limited to the small number of available PAs and serum samples,
757 respectively. It will be important to screen more tumors harboring deleterious *PAM* variants
758 to definitively exclude the absence of LOH. Likewise, assessing circulating PHM and PAL
759 activity in more *PAM* variant carriers will better define their risk profile. Third, the question of
760 how disordered *PAM* function leads to increased hormonal secretion and/or pituitary tumor
761 development remains to be determined. *PAM* is a complex, multifunctional protein involved
762 in several pathways related to the regulation of secretion; disruption of one or more of these
763 pathways could play a role in hormonal hypersecretion (Figure 8).

764



765

766 **Figure 8. Biological processes and cellular components that might be affected by PAM**
 767 **haploinsufficiency in pituitary hormone-secreting cells**

768 The trafficking of integral membrane PAM and pituitary hormones through the regulated secretory
 769 pathway is depicted. Immature secretory granules (iSG) budding from the *trans*-Golgi network (TGN)
 770 contain prohormones and processing enzymes like the prohormone convertases (PCs), granins
 771 [chromogranins A and B (ChgA/B) and secretogranins II and III], and PAM. Mature secretory granules
 772 (mSG) release their soluble content during regulated exocytosis (the yellow lightning bolt represents
 773 the external stimulus triggering secretion). Basal rates of hormone secretion from pituitary cells are
 774 less than 1% of the cell content/hour. Although PAM appears on the cell surface during exocytosis, its
 775 rapid endocytosis means that very little PAM remains on the plasma membrane at steady-state.
 776 Cleavage by secretase-like enzymes can generate a soluble, cytosolic fragment of PAM that can
 777 enter the nucleus, where it relays information about the status of the secretory granule pool. Several

778 *in vitro* studies have shown that PAM misexpression can affect a variety of steps in the regulated
779 secretory pathway; these are highlighted in red in the cartoon and include SG formation, basal and
780 stimulated secretion, actin cytoskeleton organization, and gene expression. ER, endoplasmic
781 reticulum; GH, growth hormone; MVB, multivesicular body.

782

783 In our study pathological PAM variants were associated with hypersecretion of both
784 amidated and non-amidated pituitary hormones, which suggests that the altered PAM
785 function may affect a different target than the hormones themselves. Granin proteins,
786 including chromogranin A and B are crucial factors in hormonal secretion. Chromogranin A-
787 derived peptides such as pancreastatin are themselves targets for amidation by PAM [49],
788 such that changes in PHM and PAL function could alter the packaging and release of
789 hormones contained in secretory granules. Separately, the linker region connecting the PHM
790 and PAL enzymes is O-glycosylated and pH sensitive and is known to influence PAM
791 function and its effects on vesicular trafficking [50].

792 In AtT-20 cells, expression of the PAM-1 isoform without soluble PHM and PAL leads to
793 accumulation of uncleaved POMC in the trans-Golgi network; however, while PAM-1
794 expression decreased secretagogue-stimulated secretion by mature granules, it increased
795 basal secretion of larger POMC peptides [35]. More recently, it has been shown that in atrial
796 myocytes proANP storage is a highly PAM-dependent process even though neither proANP
797 nor ANP itself are amidated proteins. Also, in murine atrial cells devoid of PAM that have
798 depleted proANP storage, the reintroduction of PAM facilitated proANP storage. Crucially,
799 this effect occurred irrespective of whether normal or enzymatically inactivated PAM was
800 used [51, 52]. Interestingly, the depletion of proANP in the absence of PAM was
801 unaccompanied by decreased proANP mRNA or protein biosynthesis, but rather occurred
802 due to 3-fold increased basal ANP secretion. Bäck, Mains & Eipper recently suggested that
803 PAM may also have a role as a cargo receptor that directly binds to and protects proANP
804 soon after its synthesis in the rough endoplasmic reticulum through to newly formed
805 granules [9, 51]. A potential role for PAM as a cargo receptor in pituitary cells remains to be

806 explored. Taken together, these data underline the multiple roles that PAM can play in
807 secretion of hormones. Studies of emerging PAM actions will be needed to address the
808 influence of genetic variants in this study on PAM-mediated pituitary hormone release.

809

810 In conclusion, *PAM* was identified as a candidate gene associated with pituitary
811 hypersecretion. Our comprehensive analysis of the impact of multiple *PAM* variants on
812 protein function can also improve our understanding of other endocrine disorders recently
813 linked to PAM defects, such as type 2 diabetes. Together, these new results raise the
814 prospect of therapeutically targeting PAM function to influence disease in the pituitary and
815 elsewhere across the endocrine system.

816

817 **Materials and Methods**

818

819 The study population consisted of index cases and a validation cohort.

820 The index cases were part of a three member FIPA acro-gigantism family referred to the
821 National Institutes of Health Clinical Center (NIHCC) as part of the Undiagnosed Diseases
822 Program (UDP) [53-55]. The presence of germline SNVs/CNVs in genes known to
823 predispose to pituitary tumors (*AIP*, *GPR101*, *CDKN1B*, *MEN1*, *PRKAR1A*, *GNAS*, and
824 *DICER1*) had been previously ruled out in all affected individuals.

825

826 The validation cohort consisted of 359 individuals with PAs (Table S1). Germline DNA was
827 studied in 299 unrelated individuals with sporadic PAs, in 17 FIPA families (including 19
828 individuals), and in three families with syndromic presentations of PAs and other endocrine
829 tumors (MEN-like syndromes) [7, 56]. Among the sporadic cases, 173 had somatotroph
830 adenomas (40 had pituitary gigantism), 84 had Cushing disease associated with corticotroph
831 adenomas (CD, 81 were pediatric - age < 18 years at diagnosis - and one had Nelson
832 syndrome), four had silent corticotroph adenomas, 25 had lactotroph adenomas (six were
833 pediatric, one was a carcinoma), and 13 had clinically non-functioning or silent adenomas
834 (one was pediatric). Somatic PA DNA was available from additional 38 subjects with
835 sporadic acromegaly; five of them had matched germline DNA [7, 57]. Twenty-two of the
836 pediatric CD cases (one familial and 21 sporadic) had matched tumor DNA available [56].
837 Subjects for the study were recruited from the Centre Hospitalier Universitaire (CHU),
838 University of Liège (231), the NIH (90), and the Bicêtre Hospital and Foch Hospital Paris
839 (38).

840

841 UDP patients provided written informed consent under clinical protocol 76-HG-0238,
842 “Diagnosis and Treatment of Patients with Inborn Errors of Metabolism and Other Genetic
843 Disorders”, approved by the NHGRI Institutional Review Board. Other individuals from the
844 NIH were recruited between 1997-2017 under research protocol 97-CH-0076

845 (ClinicalTrials.gov: NCT00001595). The study was approved by the *Eunice Kennedy Shriver*
846 National Institute of Child Health and Human Development (NICHD) Institutional Review
847 Board and by the Ethics Committee of the University of Liège, Belgium. Affected subjects or
848 their relatives signed informed consent or assent forms approved by the local ethics
849 committee. Parents and siblings of the affected individuals were also recruited, when
850 appropriate and available.

851

852 DNA sequencing

853 Index cases: DNA was extracted from peripheral whole blood samples of the twin probands
854 affected with gigantism from generation II, their father, two unaffected siblings, and the three
855 individuals from generation III (sons of II-2). Whole exome sequencing (WES) was
856 performed by a commercial vendor (Axeq Technologies, Rockville, Maryland) using the
857 TruSeq Human Exome 62 Mb capture kit for library preparation for individuals from
858 generation I and II. Exome sequencing was completed using the Illumina HiSeq2000
859 platform resulting in raw sequence FASTQ files. WES in individuals from generation III (and
860 in II-2 as control for kit-specific artifacts) was performed using the NimbleGen SeqCap EZ
861 Exome Library v3.0 kit (Roche) for library preparation. Exome sequencing was completed
862 using the Illumina NovaSeq 6000 platform. Alignments were made to human reference
863 assembly GRCh38 using BWA-MEM [58]. Variant calling and joint genotyping were
864 performed using GATK Best Practices (GATK ver. 4.2.3.0, HTSJDK ver. 2.24.1, Picard ver.
865 2.25.4) [59]. The VCF files were annotated with population data from the gnomAD database
866 (ver. 3.1.2). Functional and predictive annotations were attached using Ensembl Variant
867 Effect Predictor (ver. 102) [60] and the dbNSFP (ver. 4.3a) [61] database. Pathogenicity
868 predictions used in filtering came from SIFT, ClinPred, PolyPhen-2, FATHMM, and
869 Combined Annotation Dependent Depletion (CADD) score. No known disease-causing or
870 damaging biallelic/hemizygous variants segregating to the affected status were identified.
871 Slivar [62] was used to filter the variants and apply pedigree analysis. We prioritized rare
872 heterozygous damaging variants with nonzero pituitary median expression from GTEx that

873 appear in affected individuals for downstream validation and analysis, excluding variants
874 with a population minor allele frequency (MAF) > 1%. Variants reported in the ClinVar and/or
875 the UniProt databases as benign or likely benign were also excluded. PCR (GoTaq Green
876 Master Mix, M7123, Promega) and Sanger sequencing (BigDye Terminator 3.1 Cycle
877 Sequencing Kit, 4337456, ThermoFisher Scientific) were used to confirm the *PAM* variant
878 identified by WES. Sequences were aligned and analyzed using the SeqMan Pro 11.1.0
879 (DNASTAR Lasergene) software. Sequence chromatograms were visualized using
880 SnapGene Viewer ver. 5.0.4.

881

882 Validation cohort: WES data on germline and tumor DNA samples from subjects with CD
883 were generated as described previously [56]. These WES datasets were manually assessed
884 using Integrative Genomics Viewer ver. 2.12.3 platform (Broad Institute) [63]. For all other
885 subjects in the validation cohort, genomic DNA was extracted from peripheral blood and
886 screened for *PAM* variants by Sanger sequencing. Whenever a *PAM* pathogenic/likely
887 pathogenic variant was identified and family members were available for analysis, cascade
888 screening was initiated. This led to two instances of genotype-positive family members. In 38
889 individuals with sporadic acromegaly, tumor DNA was available for analysis. However, due
890 to the limited amount of DNA available, Sanger sequencing was targeted to specific regions
891 of *PAM* where the majority of likely pathogenic variants were previously observed (Figure 2),
892 i.e., the proximal promoter and exons 9, 16, 18, 19, 20, and 21. The primers included in
893 Table S2 were used to amplify the promoter and 5'UTR regions, the coding regions and
894 exon–intron junctions by PCR, and for Sanger sequencing. In the available adenomas, DNA
895 was extracted from unstained sections using the Pinpoint Slide DNA Isolation System
896 (D3001, Zymo Research) and loss of heterozygosity (LOH) was investigated by Sanger
897 sequencing. The NC_000005.9(NM_000919.3) reference sequence was used to annotate
898 *PAM* variants.

899

900 Single cell RNA sequencing (scRNA-seq) analysis: Figure S2A was derived from data
901 published in the NCBI Gene Expression Omnibus (GEO) Series (accession number
902 GSE132224) using methods described in detail [64]. Briefly, scRNA-seq data were derived
903 from freshly dispersed rat anterior pituitary cells from postpubertal males (3,562 cells) and
904 diestrus females (3,334 cells). Cell type clusters were identified using known genetic
905 markers for secretory cell types, folliculostellate cells, erythrocytes, leukocytes, and
906 endothelial cells. Here, we examined the expression levels of the *Pam* gene and the
907 percentage of cells of each type expressing this gene, plotted as tSNE map [65] and
908 percent-expressing heatmaps using Matlab (R2018b).

909

910 CNV analysis

911 Sixteen pituitary gigantism patients and 121 other individuals with different subtypes of PAs
912 were tested for germline *PAM* deletions/duplications by droplet digital PCR (ddPCR) using
913 five FAM-labeled TaqMan CNV assays spanning the entire *PAM* gene (Hs03560663_cn in
914 intron 2, Hs03041534_cn in intron 5, Hs06720527_cn in intron 13, Hs06071620_cn in intron
915 16, Hs06028580_cn in exon 25, all ThermoFisher Scientific). The VIC-labeled *RPP30*
916 (*Rnase P*) assay (4403326, ThermoFisher Scientific) was used as an internal control. All
917 PCR reactions were prepared using the ddPCR SuperMix for Probes (no dUTP, 1863024,
918 Bio-Rad) and the HindIII restriction enzyme in a QX200 Droplet Digital PCR System (Bio-
919 Rad). Results were analyzed with Quanta Soft software ver. 1.7.4.0917 (Bio-Rad).

920

921 Allele phasing

922 The PacBio long seq run was employed to determine the phasing of *PAM* variants c.718C>T
923 (p.His240Tyr) and c.2276T>C (p.Phe759Ser), located 60 kb apart (exons 9 and 20,
924 respectively), in a person with sporadic gigantism. His parents were not available for genetic
925 studies. Briefly, we employed Single Molecule, Real Time (SMRT) Sequencing technology
926 and a Sequel sequencer (Pacific Biosciences, Menlo Park, CA). The general strategy was to
927 generate a whole-genome library, then perform a hybridization-based pulldown to enrich for

928 the genomic region including the two variants. The probes were targeted against
929 chr5:102,939,611-103,019,811 (hg38 coordinates). This encompasses the positions of the
930 two variants plus an extra 10 kb upstream and downstream for a total of about 83 kb. The
931 biotinylated xGen Lockdown Probes were designed and synthesized by IDT (Coralville, IA)
932 with one probe approximately every 1 kb, for a total of 86 probes. The long reads enable
933 heterozygous variant walking to determine phasing of the variants. The library was
934 constructed according to the Pacific Biosciences protocol given at [https://www.pacb.com/wp-](https://www.pacb.com/wp-content/uploads/Procedure-Checklist-%E2%80%93-Multiplex-Genomic-DNA-Target-Capture-Using-IDT-xGen-Lockdown-Probes.pdf)
935 [content/uploads/Procedure-Checklist-%E2%80%93-Multiplex-Genomic-DNA-Target-](https://www.pacb.com/wp-content/uploads/Procedure-Checklist-%E2%80%93-Multiplex-Genomic-DNA-Target-Capture-Using-IDT-xGen-Lockdown-Probes.pdf)
936 [Capture-Using-IDT-xGen-Lockdown-Probes.pdf](https://www.pacb.com/wp-content/uploads/Procedure-Checklist-%E2%80%93-Multiplex-Genomic-DNA-Target-Capture-Using-IDT-xGen-Lockdown-Probes.pdf), except that SeqCap EZ reagents and the
937 SeqCap Library SR User's Guide (Roche, Indianapolis, IN) were used for the hybridization
938 pulldown. Briefly, the genomic DNA was fragmented using a g-TUBE (Covaris, Woburn, MA)
939 and Pacific Biosciences linear adapters were ligated to the ends to generate a library with an
940 average insert length of about 10 kb. The region of interest was pulled down using a pool of
941 400 attomoles of each of the 86 probes described above. The resulting material was
942 amplified and SMRTbell adapters were ligated to the ends. This library was sequenced on a
943 Sequel (Pacific Biosciences) on a SMRT Cell 1M v2 cell with Sequencing kit 2.1 and a 10 hr
944 acquisition time. The resulting reads were analyzed using the program "Targeted Phasing
945 Consensus" from Pacific Biosciences ([https://github.com/PacificBiosciences/targeted-](https://github.com/PacificBiosciences/targeted-phasing-consensus)
946 [phasing-consensus](https://github.com/PacificBiosciences/targeted-phasing-consensus)) to perform the read phasing and alignment using CCS reads (circular
947 consensus sequences from 3+ passes), basic subreads, and the hg38 genome for the target
948 region.

949

950 A male with sporadic acromegaly harbored *PAM* variants c.1654G>A (p.Gly552Arg) and
951 c.1688A>G (p.Asp563Gly), both located in exon 16. To determine the phasing of these
952 closely located SNVs, exon 16 was PCR-amplified from his germline DNA and the PCR
953 product cloned into the TOPO-TA vector. Transformed bacterial colonies were screened for
954 the presence of the variants by PCR and Sanger sequencing.

955

956 Bioinformatic analyses

957 Variant pathogenicity and gene damage predictions: Besides the bioinformatic tools used in
958 the variant prioritization process, Alamut Visual ver. 2.9 software (Interactive Biosoftware)
959 was used for annotation, *in silico* prediction, and for determining the frequency in public
960 databases of all the *PAM* variants identified. For nonsynonymous variants, unless specified,
961 the computational verdict was based on the combined outputs of 19 software packages
962 available in Varsome (accessed on 11/24/2022. Five algorithms (Splice Site Finder, MaxEnt,
963 NNSplice, GeneSplicer and Human Site Finder) integrated in Alamut were used for splicing
964 variants. Variants were considered probably damaging or affecting splicing when most of the
965 algorithms agreed; otherwise, they were considered variants of uncertain significance (VUS).
966 The gene damage index (GDI) [26] score for *PAM* and other genes known to predispose to
967 pituitary tumors was retrieved from The Gene Damage Index Server.

968

969 Spatial clustering analysis: The spatial clustering of a set of variants is obtained by
970 calculating the geometric mean distance between all pairs of variants and normalizing them
971 to the gene's cDNA length (2,922 bp, isoform P19021-5). An empirical p-value is calculated
972 by randomly-generating 100,000 permutations of the variants and comparing their clustering
973 distance against the clustering of the actual variants. As input, we used the 16 prioritized
974 missense and truncating variants found in the index cases and validation cohort and
975 reported in Table 2. We then classified the number of variants located in particular domains
976 of the protein (converted to cDNA coordinates) and counted the number of variants
977 appearing in each domain across all the permutations to calculate an empirical p-value
978 comparing the actual number of variants found in each domain to the number of variants in
979 each domain of the 100,000 permutation analysis.

980

981 Promoter analysis: The computational search for transcription factor binding sites within the
982 promoter region of *PAM* was executed using Genomatix. The MatInspector ver. 3.1 tool of
983 Genomatix (Matrix Library 11.0) was used with a core similarity threshold of 0.75 and an

984 optimized matrix similarity threshold to search for the presence of transcription factor binding
985 sites. We limited our search to vertebrate general core promoter elements and the + strand.

986

987 Protein sequence alignment and structural analysis: Protein sequence alignments were
988 performed using the following sequences from UniProt and Clustal Omega: human PAM-1
989 (P19021-5), chimpanzee PAM-1 (A0A2I3SM67-1), rat PAM-1 (P14925-1), *Aplysia* PAM-1
990 (Q9NJ14-1), *Drosophila* PHM (O01404-1) and PAL2 (Q9W1L5-1), and *Chlamydomonas*
991 PAM (A0A0S2C767-1). The enzymatically active cores for PHM and PAL were aligned.
992 Enzyme activity has been demonstrated for each of these proteins except *Aplysia* and
993 chimpanzee [39, 66-71]. We included chimpanzee as this species is evolutionarily close to
994 human, rat because both its PHM and PAL domains have been crystalized and extensively
995 analyzed [13, 72], *Aplysia* and *Drosophila* to cover other major families, and
996 *Chlamydomonas* as the most evolutionarily distant species at the boundary of the
997 animal/plant kingdoms. Full-length alignments are available in the Supplemental Methods.
998 Ribbon diagrams of the crystal structures of the catalytic cores of rat PHM (PHMcc) (PDB:
999 1OPM) and PAL (PALcc) (PDB: 3FW0) were used to contextualize the identified variants.
1000 3D models were generated using the PyMOL molecular graphics system (Schrödinger,
1001 LLC). Rat PHMcc and PALcc are, respectively, 93% and 92% identical to human PHMcc
1002 and PALcc.

1003

1004 Analysis of PAM SNV enrichment in pituitary diagnoses from the UK Biobank (UKBB) cohort
1005 UKBB dataset and disease annotation: The UKBB is a prospective study that recruited
1006 502,611 participants aged 38 to 73 years from 22 sites across the UK with baseline
1007 measures collected between 2006 and 2010 [73]. This research was conducted using the
1008 UKBB Resource (Application Number 48008). The UKBB obtained ethics approval from the
1009 North West Multi-centre Research Ethics Committee (approval number: 11/NW/0382) and
1010 obtained informed consent from all participants. Diagnoses of hyperfunctioning pituitary
1011 diseases were retrieved from hospital inpatient diagnosis fields 41270, 41202, 41204, with

1012 International classification of diseases (ICD-10) codes E220 (acromegaly and pituitary
1013 gigantism), E221 (hyperprolactinemia), E222 (syndrome of inappropriate secretion of
1014 antidiuretic hormone), E229 (unspecified hyperfunction of the pituitary gland), E240-E248-
1015 E249 combined as E240 (pituitary-dependent Cushing disease), D352-D359 combined as
1016 D352 (benign neoplasm of pituitary gland), E237 (disorder of pituitary gland), or as self-
1017 reported medical condition with codes 1237 (disorder of pituitary gland combined with ICD-
1018 10 code E237), 1238 (pituitary adenoma/tumor combined with ICD-10 code D352), 1239
1019 (Cushing's syndrome combined with ICD-10 code E240), 1431 (hyperprolactinemia
1020 combined with ICD-10 code E221), 1429 (acromegaly combined with ICD-10 code E220).

1021

1022 SKAT analysis: Gene-based diagnosis-association analysis was performed on 200,643
1023 individuals for whom exome sequencing data were available using the Sequence Kernel
1024 Association Test (SKAT). The R package SKAT was employed [24]. We used the
1025 CommonRare algorithm for binary traits with the adaptive sum method to account for
1026 common and rare variants within the *PAM* gene, using a MAF cutoff of 0.1% and considering
1027 the weight of the SNVs based on their detrimental effect on protein. SNV weights were
1028 assigned as previously described [74] and adapted as follows:

- 1029 • weight of 5 for UTR variants, synonymous variants, splice region variants;
- 1030 • weight of 10 for protein altering variants;
- 1031 • weight of 20 for start lost, stop lost, in-frame deletions, in-frame insertions;
- 1032 • for missense variants, weight of 20 + score from combined PolyPhen-2 and SIFT
1033 predictions: PolyPhen-2 +10 if possibly damaging or +20 if probably damaging or +5
1034 if unknown; SIFT +20 if deleterious, +0 if tolerated;
- 1035 • weight of 75 for frameshift variants, nonsense, splice acceptor variants, splice donor
1036 variants;
- 1037 • weight of 100 for transcript ablation.

1038 Statistical significance for gene-based tests was set at a Bonferroni-corrected threshold of p
1039 $< 2 \times 10^{-6}$ (threshold for 25,000 genes).

1040

1041 Analysis of SNVs in UKBB: Analysis of SNVs in UKBB individuals with pituitary diagnoses
1042 was performed with PLINK and a custom code in Python ver. 3.7. The CADD v1.660 VEP
1043 plugin was used to provide prediction scores for their detrimental effect. A CADD_PHRED
1044 score of ≥ 15 and a MAF $< 1\%$ were considered as pathogenicity criteria.

1045

1046 Functional characterization of SNVs

1047 Cell lines: The PEAKrapid (ATCC CRL-2828) and HEK-293 AD (ATCC CRL-1573) cells are
1048 lines derived from the Human Embryonic Kidney (HEK)-293 cell line. The PEAKrapid cells
1049 were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12, with 10% fetal
1050 bovine serum (FBS, Hyclone), pen-strep, and HEPES to net 25 mM, while the HEK-293 AD
1051 cells were maintained in DMEM containing low glucose, pyruvate, 2 mM glutamine (Gibco),
1052 with 10% FBS (Gemini Bio Products), and 1% antibiotic-antimycotic (Gibco). Both cell lines
1053 were kept in a humidified atmosphere at 37°C with 5% CO₂.

1054

1055 Vectors and mutagenesis: All cloning reactions were performed using the In-Fusion cloning
1056 system (Clontech). Cloning primers are reported in Table S2. Tissue-specific alternative
1057 splicing generates multiple PAM isoforms [75]. The longest human isoform is a 974 amino
1058 acid integral membrane protein identified as P19021-5 in UniProt. Since a GenBank search
1059 revealed that this isoform is more prevalent than the one chosen as the canonical sequence
1060 (UniProt P19021-1/NM_001177306.2, 973 amino acid-long), we regarded it as human PAM-
1061 1, corresponding to what was previously extensively characterized as rat PAM-1, a 976
1062 amino acid integral membrane protein. The ORF expression clone for wild-type (WT) human
1063 PAM-1 (NM_000919.3) cloned into pReceiver-M02 (EX-A3104-M02-GS, Genecopoeia) was
1064 subcloned into the pCMV-FLAG-C vector (635688, Clontech) using the EcoRI and XhoI
1065 restriction sites. The resulting PAM-1 protein has an in-frame C-terminal FLAG tag

1066 (DYKDDDDK) separated from its normal C-terminus by a five amino acid spacer (LEVPA). A
1067 5,373 bp sequence including the human WT *PAM* promoter (5,000 bp) and 5'UTR (373 bp)
1068 was generated by *de novo* gene synthesis (Blue Heron Biotech) and cloned into the pRMT-
1069 Luc vector (PR100001, Origene) using the EcoRI and MluI restriction sites located upstream
1070 of *Firefly* luciferase. The WT and mutant (harboring the c.*1455C>T variant) 3'UTRs of
1071 human *PAM* (2,040 bp) were PCR-amplified from human genomic DNA and cloned into
1072 psiCHECK-2 (Promega) using the XhoI and NotI restriction sites located downstream of
1073 *Renilla* luciferase. WT and mutant *PAM* sequences consisting of seven different human
1074 *PAM* exons (exon 2, 6, 16, 18, 19, 20, 21) together with their flanking intronic sequences
1075 (100/300-bp-long) were PCR-amplified from human genomic DNA and cloned into the
1076 EcoRI-digested pSPL3 vector (Invitrogen). See Supplemental Methods for more details.

1077

1078 Mutagenic primers (reported in Table S2) were used to introduce *PAM* SNVs using the
1079 QuikChange II XL site-directed mutagenesis kit (Agilent Technologies). Mutagenesis
1080 products were verified by Sanger sequencing. Since our findings (see the Results section)
1081 showed a discrepancy with what was previously reported by *Thomsen et al.* [15] for the
1082 p.Ser539Trp variant, we prepped the construct harboring this variant twice and sequence-
1083 verified it again. To introduce the deletion caused by the splice-altering c.2332-2A>T variant
1084 into the PAM-1_pCMV-FLAG-C vector, the PCR splicing technique was used. A pair of
1085 primers flanking the region where the deletion occurs (entire exon 21) and a pair of
1086 complementary primers comprising a region of -15 bp to +15 bp related to the junction point
1087 are reported in Table S2.

1088

1089 Antibodies: Antibodies used for the various experiments included the following: an affinity-
1090 purified rabbit polyclonal antibody (Ab JH629) raised to purified bacterially expressed Exon
1091 A (exon 16 in rat *Pam*) [76]; a rabbit polyclonal antibody (Ab JH246) raised to a synthetic
1092 peptide corresponding to human PAM(111-126), which is identical to rat PAM(116-131) [77];

1093 a mouse anti-FLAG monoclonal antibody (F1804, Sigma-Aldrich); and an anti-GM130
1094 antibody (#610822, BD Biosciences).

1095

1096 Biochemical analysis of nonsynonymous and splice site-affecting SNVs: Transient
1097 transfection of PAM-1 WT and mutant vectors was performed 24 h after plating using
1098 PEAKrapid cells and TransIT-2020 (MIR 5400, Mirus Bio) [78]. Cells were harvested 24-36 h
1099 after transfection. Protein extraction and western blotting were performed as described
1100 previously [76]. After rinsing with serum-free medium, cells were scraped into serum-free
1101 medium and pelleted. Cell pellets were extracted into ice cold 20 mM Na TES, 10 mM
1102 mannitol, 1% TX-100, pH 7.4 (TMT) containing protease inhibitors and subjected to three
1103 freeze/thaw cycles. Particulate material was removed by centrifugation and protein
1104 concentrations were determined using the bicinchoninic acid assay with bovine serum
1105 albumin (BSA) as the standard. For enzyme assays, lysates were diluted using TMT
1106 containing 1 mg/ml BSA. For Western blot analysis, lysates were denatured using 2X or 4X
1107 Laemmli Sample Buffer (Bio-Rad).

1108

1109 PHM and PAL assays were performed using [¹²⁵I]-Ac-Tyr-Val-Gly and [¹²⁵I]-Ac-Tyr-Val- α -
1110 hydroxy-Gly with 0.5 μ M unlabeled substrate, as described previously [79]. Samples were
1111 assayed in triplicate, within the linear range of the assay. Data for mutants were normalized
1112 to data for human PAM-1 analyzed in parallel. Normalized data from multiple different
1113 transfections were averaged to ensure that each mutant was assessed in at least two
1114 independent transfections. Human serum samples were diluted 10-fold into 20 mM Na TES,
1115 10 mM mannitol, 1 mg/ml bovine serum albumin (PHM diluent). Assays for PHM and PAL
1116 activity were carried out in duplicate or triplicate at pH 5.5; PHM assays included 4 μ M
1117 CuSO₄ and PAL assays contained 1 mM CoCl₂.

1118

1119 Analysis of the glycosylation patterns for PAM WT and mutants was performed as described
1120 previously [39]. Cells were harvested 24 h after transfection. Cell pellets were frozen and
1121 thawed in 20 mM Na TES, 10 mM mannitol, pH7.4 containing a protease inhibitor mix.
1122 Following centrifugation at 17,000 g for 20 min at 4°C, the pellets were extracted in TMT with
1123 protease inhibitors and the supernatants were denatured using SDS and digested with
1124 PNGase F or neuraminidase as described by the manufacturer (NEB). For PAM and for
1125 each PAM variant, one aliquot remained on ice (In, Input) and two aliquots were prepared for
1126 enzymatic digestion; both were incubated at 37°C for 60 min, but one received enzyme (N-
1127 Gly or Neur) and one did not (Con). After SDS-PAGE and transfer to PVDF membranes,
1128 epitope-tagged PAM and its variants were visualized using a FLAG antibody. Neuraminidase
1129 removes sialic acid from both N-linked and O-linked oligosaccharides. Samples treated with
1130 neuraminidase were analyzed on two separate gels, with the p.Arg703Gln samples
1131 appearing in part on both gels.

1132

1133 Analysis of splicing by minigene assay: Transient transfection of WT and mutant pSPL3
1134 vectors was performed using HEK-293 AD cells and TurboFect (ThermoFisher Scientific).
1135 Cells were transfected 24 h after plating; 24 h after transfection, total RNA was extracted
1136 using the RNeasy Plus Mini Kit (Qiagen). RNA (500 ng) was reverse transcribed to cDNA
1137 using the Superscript III Kit (ThermoFisher Scientific). mRNA synthesis from the plasmids
1138 using the cells' own transcription and splicing machinery led to mRNA products containing
1139 the tested *PAM* exon flanked by two exons from the pSPL3 vector. Splicing products were
1140 analyzed by RT-PCR using the vector-specific SD6 and SA2 primers (Table S2). The empty
1141 pSPL3 vector was used as negative control.

1142

1143 Analysis of SNVs in regulatory regions by luciferase-based reporters: SNVs located
1144 upstream (promoter and 5'UTR) and downstream (3'UTR) of *PAM* CDS, were investigated
1145 using luciferase-based reporter assays transfected into HEK-293 AD cells. The empty
1146 pRMT-Luc and psiCHECK-2 vectors were used as negative controls. For the 3'UTR

1147 experiments, four microRNA (miRNA) mimics (hsa-miR-138-2-3p, MC12814; hsa-miR-192-
1148 3p, MC12893; hsa-miR-3143, MC17293; hsa-miR-556-3p, MC12806; all ThermoFisher
1149 Scientific) were transfected alongside the WT and mutant reporter vectors. Cells were lysed
1150 24 h after transfection and *Firefly* and/or *Renilla* luciferase activities were measured using
1151 the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's
1152 protocol. When appropriate, ratios of *Firefly/Renilla* luminescence signals, serving as a
1153 measure for reporter activity normalized for transfection efficiency, were determined using a
1154 FLUOstar Omega microplate reader (BMG Labtech).

1155

1156 Immunohistochemistry

1157 Immunofluorescence analysis was performed on histological sections from non-pathological
1158 pituitary cells (control). The full protocol has been detailed elsewhere [80]. Briefly, all slides
1159 were blocked with 10% normal donkey serum in 1X PBS for 1 h at room temperature and
1160 then incubated overnight at 4°C with affinity-purified 1:500 anti-PAM JH629 and anti-GM130.
1161 All slides were incubated for 1 h with 1:500 donkey anti-rabbit 488 and donkey anti-mouse
1162 594 (respectively, A-11055 and A-21207, ThermoFisher Scientific). Slides were imaged at
1163 40x on a Keyence BZ- X710 microscope.

1164

1165 Statistical analysis

1166 All graphs were plotted as mean \pm standard error of the mean (SEM). Data distributions
1167 were assessed for approximate normality. Differences between experimental groups were
1168 analyzed by two-tailed Student's t-test or 1-way ANOVA with Dunnett's *post hoc* test, or
1169 corresponding non-parametric tests, as appropriate. *PAM* SNV allele frequencies in the
1170 study population were compared with frequencies in the general population reported in the
1171 gnomAD public database using Fisher's exact test or the chi-square test, as appropriate.
1172 Data were analyzed using GraphPad Prism (GraphPad, San Diego, CA, USA). p-values <
1173 0.05 were considered statistically significant.

1174

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1184

1185 **Data and Code Availability**

1186 The code used to conduct the spatial clustering analysis can be found at
1187 https://github.com/NICHD-BSPC/spatial_clustering.

1188

1189 **Web Resources**

1190 BWA, <https://github.com/lh3/bwa>

1191 CADD, <https://cadd.gs.washington.edu/>

1192 ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>

1193 Clustal Omega, <https://www.ebi.ac.uk/Tools/msa/clustalo/>

1194 dbNSFP (ver. 4.3a), <http://database.liulab.science/dbNSFP>

1195 dbSNP, <https://www.ncbi.nlm.nih.gov/snp/>

1196 Ensembl VEP (ver. 102), <https://grch37.ensembl.org/info/docs/tools/vep/index.html>

1197 GATK (ver. 4.2.3.0), <https://gatk.broadinstitute.org/hc/en-us/articles/4409678362139-GATK-4-2-3-0-release>

1199 Genomatix, <https://www.genomatix.de/>

1200 gnomAD (ver. 3.1.2), <https://gnomad.broadinstitute.org/>

1201 Gene Structure Display Server (GSDS 2.0), <http://gsds.cbi.pku.edu.cn/>

1202 GTEx Project, <https://www.gtexportal.org/home/>

- 1203 HIPred score, <https://github.com/HAShahab/HIPred>
- 1204 HTSJDK (ver. 2.24.1), <https://samtools.github.io/htsjdk/>
- 1205 OMIM, <https://www.ncbi.nlm.nih.gov/omim>
- 1206 PCR splicing technique, http://www.methods.info/Methods/Mutagenesis/PCR_splicing.html
- 1207 Picard (ver. 2.25.4), <http://broadinstitute.github.io/picard/>
- 1208 PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>
- 1209 Primer3, <https://www.ncbi.nlm.nih.gov/omim>
- 1210 Protein Molecular Weight, https://www.bioinformatics.org/sms/prot_mw.html
- 1211 Slivar, <https://github.com/brentp/slivar>
- 1212 The Gene Damage Index (GDI) Server, <http://pec630.rockefeller.edu:8080/GDI/>
- 1213 The Human Protein Atlas, <https://www.proteinatlas.org/>
- 1214 UniProt, <https://www.uniprot.org/>
- 1215 Varsome, <https://varsome.com/>

1216

1217 **Declaration of Interests**

1218 Dr. Beckers, Dr. Daly, Dr. Faucz, Dr. Stratakis and Dr. Trivellin hold a patent on the *GPR101*

1219 gene and its function (US Patent No. 10,350,273, Treatment of Hormonal Disorders of

1220 Growth). Dr. Stratakis holds patents on technologies involving *PRKAR1A* and related genes

1221 causing adrenal, pituitary, and other tumors. In addition, his laboratory has received

1222 research funding support by Pfizer Inc. for investigations on growth-hormone producing

1223 pituitary adenomas. Dr. Stratakis also has consulted within the last 12 months with

1224 Lundbeck Pharmaceuticals and Sync, LLC, and is currently employed by ELPEN

1225 Pharmaceuticals. Dr. Beckers and Dr. Daly have received research funding from Pfizer Inc.

1226 and Novo-Nordisk. Dr. Jaffrain-Rea is part of the advisory board of Recordati Rare diseases

1227 since 2022. The authors declare that they have no conflicts of interest with the contents of

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1229

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