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# A new multi-system disorder caused by the *Gas* mutation p.F376V

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30

31 **Abbreviated title:** The Gas variant F376V causes a novel disorder

32

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35

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58

## 59 **Abstract**

60 **Context:** The alpha-subunit of the stimulatory G-protein ( $G_{\alpha s}$ ) links numerous receptors to adenylyl  
61 cyclase.  $G_{\alpha s}$ , encoded by *GNAS*, is expressed predominantly from the maternal allele in certain tissues.  
62 Thus, maternal heterozygous loss-of-function mutations cause hormonal resistance, as in  
63 pseudohypoparathyroidism type Ia, while somatic gain-of-function mutations cause hormone-  
64 independent endocrine stimulation, as in *McCune-Albright Syndrome*.

65 **Objective:** We here report two unrelated boys presenting with a new combination of clinical findings  
66 that suggest both gain and loss of  $G_{\alpha s}$  function.

67 **Design, Setting:** Clinical features were studied and sequencing of *GNAS* was performed. Signaling  
68 capacities of wild-type and mutant- $G_{\alpha s}$  were determined in the presence of different G protein-coupled  
69 receptors (GPCRs) under basal and agonist-stimulated conditions.

70 **Results:** Both unrelated patients presented with unexplained hyponatremia in infancy, followed by  
71 severe early-onset gonadotrophin-independent precocious puberty and skeletal abnormalities. An  
72 identical heterozygous *de novo* variant (c.1136T>G; p.F376V) was found on the maternal *GNAS* allele,  
73 in both patients; this resulted in a clinical phenotype that differ from known  $G_{\alpha s}$ -related diseases and  
74 suggested gain-of-function at the receptors for vasopressin (V2R) and lutropin (LHCGR), yet increased  
75 serum parathyroid hormone (PTH) concentrations indicative of impaired proximal tubular PTH1  
76 receptor (PTH1R) function. *In vitro* studies demonstrated that  $G_{\alpha s}$ -F376V enhanced ligand-independent  
77 signaling at the PTH1R, LHCGR and V2R and, at the same time, blunted ligand-dependent responses.  
78 Structural homology modeling suggested mutation-induced modifications at the C-terminal  $\alpha 5$ -helix of  
79  $G_{\alpha s}$  that are relevant for interaction with GPCRs and signal transduction.

80 **Conclusions:** The  $G_{\alpha s}$  p.F376V mutation causes a previously unrecognized multi-system disorder.

81

## 82 **Introduction**

83 Heterotrimeric G-proteins, comprised of a specific alpha ( $\alpha$ ) subunit and associated beta ( $\beta$ ) and gamma  
84 ( $\gamma$ ) subunits, activate a variety of distinct intracellular signaling pathways (1,2). These signaling events

85 are normally initiated when the G-protein heterotrimer interacts with the cytoplasmic portion of an  
86 agonist-activated G-protein-coupled receptor (GPCR) (3). Activating mutations in either a GPCR or a  
87 G-protein that increase ligand-independent signaling are the cause of several diseases (4,5).

88 The  $\alpha$ -subunit of the stimulatory G-protein ( $G_{\alpha s}$ ), encoded by *GNAS* exons 1-13, links a large number  
89 of different GPCRs to the adenylyl cyclase/cAMP pathway. Through as-yet unknown mechanisms,  $G_{\alpha s}$   
90 expression from the paternal *GNAS* allele is reduced in a tissue-specific manner, such that cells of the  
91 renal proximal tubule, thyroid, pituitary, and several other tissues express  $G_{\alpha s}$  predominantly from the  
92 maternal allele. Consequently inactivating  $G_{\alpha s}$  mutations on the maternal allele, as in  
93 pseudohypoparathyroidism type Ia (PHP1A), lead to a multi-system disorder characterized by  
94 parathyroid hormone (PTH)-resistant hypocalcemia, impaired signaling at the thyrotropin receptor  
95 leading to reduced thyroid hormone production, and several other endocrine and developmental  
96 abnormalities (6).

97 Somatic gain-of-function mutations in  $G_{\alpha s}$  that occur at either position R201 or R227 (7) are found in  
98 a variety of human cancers and benign endocrine tumors (8). In addition, mosaic expression of  $G_{\alpha s}$   
99 mutations at residue R201 give rise to the *McCune-Albright Syndrome*. Depending on the embryonic  
100 stage at which the somatic nucleotide change occurred, these patients present with a combination of  
101 different clinical and laboratory findings that can include gonadotropin-independent precocious puberty  
102 in early infancy that is resistant to treatment with GnRH agonists, hyperthyroidism, café-au-lait spots,  
103 and variable skeletal findings (9,10). R201- $G_{\alpha s}$  mutations are not transmitted through the germline,  
104 presumably because of early lethality due to excessive cAMP signaling (11).

105 Previously, two male patients with gonadotropin-independent precocious puberty and PTH-resistant  
106 hypocalcemia were shown to carry an identical  $G_{\alpha s}$  mutation, A366S, which was associated with  
107 agonist-independent activation of some GPCRs, yet hormonal resistance at others (12). Thus, these  
108 patients presented with LH-independent precocious puberty and resistance at the receptors for PTH  
109 (PTH1R) and thyrotropin (TSHR). These discrepant findings were thought to be explained by instability  
110 of the  $G_{\alpha s}$  mutant at body temperature, combined with agonist-independent activation of the lutropin  
111 receptor (LHCGR) because of accelerated release of GDP at the lower temperature of the testes.

112 We now report two unrelated male patients, who were referred to us with an unexplained combination

113 of severe asymptomatic infantile hyponatremia, skeletal and growth plate abnormalities, early-onset  
114 pubertal development and apparent PTH-resistance in the proximal, but not in the distal renal tubules.  
115 The same novel *Gas* mutation (p.F376V) was identified in both boys and *in vitro* studies revealed  
116 GPCR-specific signaling abnormalities, which explained the patients unusual phenotypes, i.e. symptoms  
117 that suggested both gain- and loss-of function.

118

## 119 **Patients and Methods**

120 The parents of both patients have consented to the reported investigations. Two unrelated boys presented  
121 first with unexplained hyponatremia in infancy and subsequently with early-onset gonadotrophin-  
122 independent precocious puberty, elevated serum PTH concentrations and unique skeletal abnormalities.  
123 This previously unreported combination of symptoms and laboratory findings was indicative of both  
124 gain- and loss-of-function at different GPCRs that had not been encountered in other *Gas*-related  
125 diseases (clinical data are summarized in [Table 1](#), comparative phenotypic information is provided in  
126 [Table 2](#), and detailed patient information are available in the supplementary material).

127

### 128 ***DNA sequence analyses***

129 *GNAS* exons 1-13 underwent direct nucleotide sequence analysis for patients 1 and 2; whole exome  
130 sequencing was furthermore performed for patient 1. To determine the parental origin of the mutation,  
131 a PCR fragment was amplified from both patients and their parents that extends from intron 6 to the 3'-  
132 non-coding region telomeric of exon 13, i.e. the exon that comprises the patient's mutation. The PCR  
133 products from each patient were cloned into a TA cloning vector (Life technologies, Darmstadt,  
134 Germany) and 10 independent colonies underwent nucleotide sequence analysis.

135

### 136 ***Construction of G-alpha variants***

137 Wild-type (wt) human *Gas* (NM\_000516) was cloned into the pcDps expression vector, as described  
138 previously (13); investigated *Gas* variants were introduced by standard mutagenesis technique. To  
139 determine if G-protein expression is modified in the presence of tested GPCRs, a luciferase (NanoLuc)  
140 was introduced between amino acids 324 and 325 of the wild-type and mutant *Gas*.

141

142 ***Determination of WT-Gas and Gas-variant expression via luciferase activity***

143 HEK293 cells lacking Gas (GSG-5 cells) were seeded in white 96-well plates ( $1.5 \times 10^4$ /well). The  
144 following day, cells were co-transfected with the NanoLuc-modified wild-type and mutant Gas, in  
145 combination with the TSHR, LHCGR, MC4R, PTH1R, V2R, or empty vector using Metafectene®  
146 (Biontex, Munich, Germany) according to manufacturer's protocol using Opti-MEM without phenol  
147 red. 24h after transfection, 25  $\mu$ l/well NanoLuc substrate Nano-Glo™ (50  $\mu$ M) was injected using  
148 Berthold Mithras LB 940. The luciferase activity was measured at 460 nm  $\pm$  25nm in triplicates.

149

150 ***Determination of signaling properties of Gas-variants***

151 Functional characterization of wt and mutant Gas was performed using mouse fibroblast 2B2 cells that  
152 lack *Gnas* exon 2 on both alleles (14) and GSG-5 cells, derived from the human embryonic kidney cell  
153 line (HEK293), which had been engineered by CRISPR-Cas9 to lack Gas; the latter cells were stably  
154 transfected to express the luciferase-based GloSensor™ cAMP reporter. Cells were seeded in 96-well  
155 plates ( $1 \times 10^4$ /well). Next day the different GPCRs (TSHR, MC4R, PTH1R, LHCGR or V2R) were co-  
156 transfected with wt or mutant Gas at a ratio 1:1 using Metafektene (Biontex, Munich, Germany) for 2B2  
157 cells or Lipofectamine-2000 (Invitrogen Promega) for GSG-5 cells according to the manufacturers'  
158 protocols. Two days later cAMP accumulation assays were performed at 37°C as previously described  
159 (15). For GSG-5 cells, intracellular cAMP was monitored by treating the cells with luciferin (5 mM)  
160 and measuring changes in luminescence over time at room temperature using a PerkinElmer Envision  
161 plate reader. Basal cAMP was measured for 14 minutes immediately following the addition of luciferin,  
162 and ligand-stimulated cAMP responses were subsequently measured for 88 minutes following the  
163 addition of PTH(1-34) (100 nM).

164

165 ***Data analysis***

166 All data are given as raw data (mean  $\pm$  SEM) obtained from four independent experiments performed  
167 in triplicates. Bar graphs and dose-response curves as well as statistical analyses were generated using  
168 GraphPad Prism Version 6.0 (GraphPad Software, San Diego, CA). Data obtained with GSG-5 cells are

169 reported as means  $\pm$  SEM of six independent assays; for each assay, data from replicate wells (twelve  
170 for basal and two for each PTH concentration) were averaged before combining the data to obtain the  
171 mean values for the six independent experiments.

172

### 173 ***Structural homology modeling***

174 A structural homology model of Gas in its inactive conformation was generated to study the interactions  
175 of F376 in the context of the wild-type protein. We compared the inactive model with the already known  
176 active and receptor-bound Gas conformation (16). In brief, an inactive state structure of Gas without  
177 the N-terminal helix is already available (PDB entry 1AZT (17)) as well as for the trimeric Gi protein  
178 (PDB entry 1GP2 (18)). To build a completed inactive Gas model, the available Gas crystal structure  
179 was superimposed onto the  $\alpha$ -subunit of Gi and the N-terminal  $\alpha$ 1-helix (which has not yet been resolved  
180 for the Gas structure) and was inserted by substituting the N-terminal helix of G $\alpha$ i into the incomplete  
181 Gas structure. The mutant amino acids were then introduced *in silico* into the chimeric template of wild-  
182 type Gas. The resulting completed heterotrimeric Gas model was refined by geometry optimization and  
183 energy minimization of the side chains until converging at a termination gradient of 0.2 kcal/mol\*Å  
184 with constraint backbone atoms, which were finally released in a second minimization step until  
185 converging at a termination gradient of 0.1 kcal/mol\*Å. The Gas-variants (p.F376V, p.F376Y, and  
186 p.F376M) were introduced into the inactive wild-type Gas model to unravel the potential local impact  
187 of these side chain variations. All structural modifications were performed using the software SYBYL-  
188 X 2.0 (Certara, NJ, US). The AMBER F99 force field was used for energy minimization. Structure  
189 images were produced using the PyMOL Molecular Graphics System, Version 1.5, Schrödinger, LLC.

190

## 191 **Results**

### 192 **A previously unrecognized phenotype in two unrelated patients**

193 Both male patients, 7 and 3.5 years of age at last follow-up, respectively, were born at term after  
194 uneventful pregnancies to unrelated parents with no family history of note. A more detailed patient  
195 description and discussion is provided in the supplemental material.

196



197 ***Nephrogenic syndrome of inappropriate anti-diuresis (NSIAD)***

198 Both patients presented in the first few days of life with clinically asymptomatic hyponatremia in the  
199 absence of hyperkalemia, which was detected incidentally when analyzing blood glucose and gases.  
200 Investigations revealed no evidence for defects in mineralocorticoid production (hypoaldosteronism) or  
201 action (i.e. pseudohypoaldosteronism) and both patients were deemed euvolemic. A mutation in  
202 vasopressin receptor 2 (AVPR2; protein name V2R) causing NSIAD was suspected in patient 1 but  
203 excluded by direct nucleotide sequence analysis; treatment with sodium supplementation was started.  
204 In patient 2, hypertension induced by sodium supplements and mineralocorticoids (fludrocortisone) was  
205 treated with amlodipine. Copeptin, a marker of arginine vasopressin (AVP) secretion, also known as  
206 CT-proAVP, was low (<3.6 pmol/l) on four occasions when serum sodium was normal or low, yet urine  
207 osmolality remained persistently elevated (>800 mosm/kg). A tolvaptan (V2R antagonist) challenge  
208 failed to increase urine output and to decrease urine osmolality consistent with NSIAD. Treatment with  
209 sodium alone or with sodium and fludrocortisone were discontinued in both patients at 3 years of age.

210

211 ***PTH-resistance in the proximal, but not the distal, renal tubules***

212 Both patients had persistently elevated PTH serum concentrations in association with total calcium  
213 concentrations that were within the normal range, and serum phosphate levels that were at the upper end  
214 of the normal range or slightly elevated. 25(OH)D and 1,25(OH)<sub>2</sub>D concentrations were within the  
215 normal range. Both had a low urinary calcium/creatinine ratio (patient 1: 0.05 mol/mol, patient 2: 0.09  
216 mol/mol) and there was no evidence of nephrocalcinosis. Treatment of patient 1 with calcitriol and of  
217 patient 2 with 1-alpha calcidol at relatively low doses reduced PTH serum concentrations.

218

219 ***Evidence for increased PTH1R activity in bone and growth plates***

220 Both patients had enlarged and persistently open anterior fontanelles beyond the age of one year, rather  
221 short distal phalanges, and subtle widening of the growth plates, but neither had, until the age of 3 yrs,  
222 clinical or radiographic features of AHO such as brachymetacarpy, and there was no evidence for fibrous  
223 dysplasia. Patient 1 developed severe deformities of the lower limbs and suffered a spontaneous right  
224 tibial fracture at 1.5 years of age, which required surgery [Fig. 1]. At the age of 1.25 years, a skeletal

225 survey of patient 2 showed subperiosteal erosions, particularly at the proximal radius, metacarpals and  
226 middle phalanges, and evidence of acro-osteolysis at the distal phalanges of hands and feet.

227

### 228 *Gonadotrophin-independent precocious puberty*

229 Both patients were noted to have symmetrical testicular enlargement and signs of precocious puberty at  
230 about 2 years of age that were associated with accelerated growth and significantly advanced bone age.

231 **Elevated serum testosterone concentrations were noted; yet there was no increase in luteinizing hormone**  
232 **(LH) and follitropin (FSH) secretion after challenge with gonadotropin-releasing hormone (GnRH), i.e.**  
233 **findings** consistent with gonadotrophin-independent precocious puberty [Tab 1]. Treatment with an  
234 aromatase inhibitor (Anastrozole) and initially cyproterone acetate (anti-androgen) was therefore  
235 commenced in both patients; the latter medication was subsequently substituted by Bicalutamide. **Based**  
236 **on the radiographic findings at follow-up after 5 years of treatment in patient 1 and after 1.5 years in**  
237 **patient 2**, these medications have had no significant effect on bone age advancement. The  
238 gonadotrophin-independent precocious puberty resembles that seen in boys with MAS or testotoxicosis  
239 due to activating LHCGR mutations (19).

240

### 241 *Other findings*

242 In Patient 1, hypothyroidism was suspected because of a single low fT4 concentration (0.75 ng/ml,  
243 reference range: 0.89 – 2.22) with a serum TSH serum concentration in the upper normal range (4.08  
244 mU/l, age related reference range: 0.4 -5.97). Thyroid hormone replacement (25 µg/day) was therefore  
245 started at the age of 1 year. In patient 2, TSH concentrations were normal (1.79 mU/l) at 1.2 yrs (age  
246 related reference range 0.3-4.0 mU/l).

247 Patient 2 has two small café-au-lait patches, but neither patient has additional cutaneous features  
248 characteristic of MAS and neither had palpable cutaneous ossifications as can be observed in PHP1A.  
249 Both patients have delayed motor development and patient 2 also has significantly delayed expressive  
250 language. **There is no evidence for abnormalities in the GHRH/GH/IGF1 axis, as determined by IGF1**  
251 **measurements. However, an effect of excessive GH production on growth may have been masked by**  
252 **accelerated bone maturation due to severe precocious puberty [Suppl. Fig. 1].**

253

254 ***GNAS nucleotide sequence analyses***

255 Direct nucleotide sequence analysis of *GNAS* exons 1-13 revealed in both patients an identical  
256 heterozygous missense *de novo* mutation (c.1126T>G; p.F376V) in exon 13, which was not identified  
257 in the parents. Furthermore, whole exome sequencing of patient 1 provided no obvious additional  
258 candidate variants explaining the clinical and metabolic phenotype. To determine whether the identified  
259 mutation resides on the maternal or the paternal *GNAS* allele, **genomic DNA from both patients was**  
260 **amplified across an informative SNP in *GNAS* intron 6 (rs919196) and the c.1126T>G variant; note both**  
261 **patients are C/T, while both mothers are homozygous for C, and both fathers are homozygous for T.**  
262 **Allele separation was performed by cloning the PCR products.** Nucleotide sequence analysis of ten  
263 independent clones revealed that the *GNAS* exon 13 mutation always segregated together with the  
264 maternal cytosine for SNP rs919196, thus documenting that the *Gas* mutation resides on the maternal  
265 allele.

266

267 ***Functional characterization of *Gas* variants***

268 The complex phenotype exhibited by both patients suggested agonist-independent activation of some  
269 GPCRs, yet hormonal resistance at others. The *Gas*-F376V mutant was therefore functionally assessed  
270 in cultured cells by co-expressing wild-type and mutant *Gas* with the GPCRs implicated in the different  
271 abnormalities, namely V2R (water reabsorption), LHCGR (gonadotrophic action/puberty), PTH1R  
272 (mineral ion homeostasis, bone metabolism, and growth plate development), TSHR (thyroid function)  
273 or melanocortin-4 receptor (MC4R, weight regulation) [Tab. 2].

274 To ensure that expression of the G proteins is not influenced by co-transfected receptors, protein levels  
275 of wt-*Gas* and *Gas*-F376V were determined by measurement of luciferase activity in the presence of all  
276 tested receptors. Cellular levels of wild-type and mutant *Gas* were indistinguishable in the presence of  
277 TSHR, V2R, LHCGR and PTH1R; however, in the presence of MC4R, expression of *Gas*-F376V was  
278 slightly increased [Suppl. Fig. 2]. To gain further insights into how specific side chain properties at  
279 position 376 might affect signaling function two additional *Gas* variants, p.F376M or p.F376Y were  
280 introduced and functionally characterized.

281 In 2B2 cells, basal cAMP levels were indistinguishable when co-expressing either Gas-F376V or wt-  
282 Gas with the TSHR or MC4R [Fig. 2A,B]. However, when co-expressed with V2R, LHCGR, or PTH1R,  
283 the Gas-F376V variant revealed increased basal cAMP accumulation that was 1.6-, 1.5- and 2.3-fold,  
284 respectively, higher than that observed with cells co-transfected with wt-Gas [Fig. 2]. In contrast,  
285 agonist-dependent cAMP accumulation was 40-50% lower for all receptors when co-transfected with  
286 the F376V mutant, as compared with wt-Gas [Fig. 2-3]. To validate results obtained in the 2B2 cell  
287 system, a second cell system, namely HEK293 cells deficient in Gas (GSG-5), were used to test the  
288 PTH1R in the presence or absence of PTH(1-34) (20). Co-transfection of GSG-5 cells with Gas-F376V  
289 and the PTH1R resulted in approximately 2-fold higher rates of basal cAMP accumulation, as compared  
290 to cells co-transfected with wt-Gas and the PTH1R. However, in comparison to cells expressing wt-Gas  
291 and the PTH1R, the response to PTH(1-34) was blunted in cells co-transfected with Gas-F376V and the  
292 PTH1R [Suppl. Fig. 2].

293

294 *Additional F376 variants:* Substitution of phenylalanine at position 376 with tyrosine (p.F376Y)  
295 resulted in strongly diminished signaling after agonist stimulation when co-expressed with the different  
296 GPCRs [Fig. 2-3], which was similar to the findings with the Gas-F376V mutant. In the absence of  
297 agonist, Gas-F376V revealed similarly enhanced cAMP signaling when coexpressed with V2R,  
298 LHCGR and PTH1R [Fig. 2-3]. In contrast, Gas-F376Y mutant displayed functional properties similar  
299 to those of the patient's mutation leading to the conclusion that the additional hydrophilic hydroxyl-  
300 group of the tyrosine modifies the essential hydrophobic core interactions observed with phenylalanine  
301 present in wild-type Gas at this position [Fig. 4].

302 Surprisingly, the Gas-F376M substitution revealed more complex signaling properties. For V2R and  
303 LHCGR, ligand-induced signaling was comparable to that observed with wild-type Gas [Fig. 2-3], but  
304 was reduced for TSHR [Fig. 2-3]. In contrast, maximal signaling response was enhanced for the PTH1R  
305 and the MC4R (1.5- to 2-fold) compared to signaling with wild-type Gas [Fig. 2-3]. Importantly,  
306 methionine at this position does not lead to a significant increase in basal signaling.

307

308 *Insights from structural homology models*

309 Amino acid F376, located in the C-terminal  $\alpha 5$ -helix, is highly conserved in the alpha-units of different  
310 G proteins [Suppl. Fig. 3]. In the inactive *G $\alpha$ s* conformation, the F376 side chain is embedded into a  
311 hydrophobic/aromatic cage formed by L43, M60, H64, F212, F219 and M221, located in the  $\beta 1$ -,  $\beta 2$ -,  
312  $\beta 3$ -strands and the N-terminal helix 1 [Fig. 4A1]. These residues rearrange considerably during the  
313 activation process to thereby modulate the GDP/GTP-binding pocket. However, unlike disease caused  
314 by mutations at residue R201 (9,10), R227 or A366 (12), F376 is not immediately proximal to the  
315 nucleotide binding site. Nevertheless, substitution with valine at position 376 (p.F376V) is predicted to  
316 disturb the critical network of hydrophobic interactions and to thus indirectly perturb the catalytic site  
317 environment [Fig. 4A2]. In contrast, substitution with methionine (p.F376M) largely maintains the  
318 essential side chain interactions, which is consistent with the near-normal signaling properties observed  
319 for the *G $\alpha$ s*-F376M mutant [Fig. 2-3]. The mutation in our patients, *G $\alpha$ s*-F376V, with a shorter and more  
320 branched side chain compared to phenylalanine, most likely leads to a loss of tight hydrophobic  
321 interactions, which then can cause, in comparison to the wild-type protein, modifications in the relative  
322 spatial  $\alpha 5$ -helix orientation. In addition, the p.F376Y mutant with functional properties similar to that  
323 of the mutation identified in our patients, strongly supports the conclusion that the additional hydrophilic  
324 hydroxyl-group also interrupts the essential hydrophobic core interactions.

325

## 326 **Discussion**

327 We here describe a previously unrecognized congenital human disorder caused by a novel, maternal  
328 *G $\alpha$ s* mutation, namely p.F376V, that is characterized by a unique combination of clinical and  
329 laboratory findings, including NSIAD and GnRH-independent precocious puberty. In addition, PTH  
330 was elevated because of resistance in the proximal tubules where *G $\alpha$ s* is derived predominantly from  
331 the maternal *GNAS* allele, thus leading to secondary hyperparathyroidism. However, due to biallelic  
332 *G $\alpha$ s* expression in most other tissues, including osteoblasts, bone turnover was found to be increased  
333 leading to skeletal changes reminiscent of hyperparathyroidism, i.e. changes that can be encountered  
334 in PHP1B (21). Furthermore, it appears likely that elevated PTH concentration, combined with  
335 normal paternal *G $\alpha$ s* expression in chondrocytes, resulted in growth plate changes reminiscent of  
336 rickets similar to those encountered in a few young patients with PHP1B (22). Irrespective of the

337 underlying mechanisms, the co-incidental identification of two unrelated patients, who presented with  
338 indistinguishable, unusual combinations of clinical and metabolic features, strongly suggest that the  
339 identified *de novo* p.F376V mutation on the maternal *GNAS* allele accounts for this thus far not  
340 described phenotypic profile of the disorder.

341 A novel feature of both patients was agonist-independent activation of the V2R-regulated pathway  
342 leading to NSIAD with the p.F376V mutation in *Gαs*. Activation of the V2R which is caused by  
343 constitutively increased signaling has not been described in MAS nor in any other *Gαs*-related disease  
344 reported to date. The resolution of hyponatremia after early infancy was likely due to adaptive  
345 mechanisms, such as changes in nutrition and self-regulation of thirst-driven fluid intake, comparable  
346 to that encountered in patients with NSIAD due to gain-of-function mutations in *AVPR2* (23). However,  
347 excessive free water intake may in the future again lead to hyponatremia.

348 Gonadotrophin-independent precocious puberty can be caused by mosaic of *Gαs* mutated at residue  
349 R201, as observed in MAS (10) , or by activating somatic or germline LHCGR mutations (19). In the  
350 latter case, the disorder presents with rapid symmetrical growth of testes associated with premature  
351 testosterone production. This is clinically similar to the findings in our two patients, but distinct from  
352 the frequently, but not always, observed asymmetrical testicular volume encountered with somatic  
353 expression of the abnormal *Gαs*-R201 alleles (24).

354 Serum PTH concentrations were elevated, yet serum phosphate concentrations were at the upper end of  
355 normal or slightly elevated, but not reduced. This is consistent with reduced PTH-responsiveness of the  
356 PTH1R/*Gαs*-F376V complex in the proximal tubule cells where *Gαs* is expressed predominantly from  
357 the maternal *GNAS* allele. As a consequence of increased serum PTH concentrations, augmented PTH1R  
358 signaling is predicted to occur when this GPCR is coupled to wt-*Gαs* derived from the paternal allele,  
359 i.e. in tissues with biallelic *Gαs* expression. The urinary calcium-to-creatinine ratio was low in both  
360 patients indicating that calcium reabsorption is fully functional in the distal tubules, where *Gαs* is  
361 expressed from both parental alleles. Thus, either PTH can stimulate efficiently the complex comprising  
362 the PTH1R and paternal wt-*Gαs*, as is observed in PHP1B and PHP1A, and/or ligand-independent  
363 calcium reabsorption occurs due to the complex between the PTH1R and the maternal *Gαs*-F376V  
364 mutant.

365 The skeletal changes leading to bowing of weight-bearing bones as well as the subperiosteal erosions  
366 and acro-osteolysis, particularly of the distal phalanges [Fig. 1], were in keeping with enhanced  
367 activation of the PTH1R expressed in osteoblasts. These changes might be caused by agonist-  
368 independent signaling mediated by complexes formed between the unoccupied PTH1R and Gas-F376V  
369 in osteoblasts which could lead to increased rates of bone turnover and resorption. However, the agonist-  
370 independent effects on osteoblasts, and chondrocytes as outlined above, could be augmented also by  
371 elevated serum PTH concentrations and thus sustained activation of the PTH1R coupled to wt-Gas,  
372 which is reminiscent of secondary hyperparathyroidism in children with PHP1B or chronic kidney  
373 disease (21,22).

374

375 To better understand the mechanisms responsible for this previously unreported human disorder, we co-  
376 expressed Gas variants with a sub-set of GPCRs that are expressed in tissues, which are known to be  
377 associated with the symptoms observed in our patients [Tab. 2]. In fact, ligand-independent signaling  
378 was induced when the Gas-F376V mutant was co-expressed with the V2R, PTH1R and LHCGR in  
379 concordance with the clinical symptoms of the two patients, but not with the TSHR and MC4R [Fig. 2-  
380 3]. Also consistent with *in vivo* evidence for PTH-resistance in proximal tubules our *in vitro* experiments  
381 revealed a blunted response to PTH(1-34) when the PTH1R was co-expressed with the Gas-F376V  
382 mutant [Fig. 3 and Suppl. Fig. 3]. The p.F376V mutation resides in both patients on the maternal allele,  
383 which contributes in some tissues, such as the proximal renal tubules, most or all Gas protein. *In vitro*,  
384 the response to PTH was blunted at the PTH1R in the presence of the Gas mutant, which is likely  
385 responsible for the PTH elevation caused by PTH1R resistance in this portion of the kidney. It thus  
386 appears plausible that the presence of the p.F376V mutation on the paternal *GNAS* allele could lead only  
387 to NSIAD and gonadotrophin-independent precocious puberty without PTH resistance. The fact that the  
388 maternal p.F376V mutation facilitates ligand-independent cAMP formation in a receptor-specific  
389 manner is consistent with the clinical findings observed in both boys. Three different mechanisms are  
390 likely to explain the unusual combination of findings in our patients, which include features of both  
391 loss- and gain-of-function of Gas: (*i*) loss-of-function effects that are caused by impaired activity of the  
392 Gas mutant and thus hormonal resistance in those target tissues that rely predominantly or exclusively

393 on maternal *Gas* expression, (ii) GPCR-dependent gain-of-function effects that are agonist-independent,  
394 and (iii) effects that are caused by the elevated PTH concentrations and activation of the PTH1R when  
395 coupled to the wt-*Gas* transcribed from the paternal *GNAS* allele.

396

397 At the protein structural level phenylalanine 376 is located near the C-terminus of the *Gas*  $\alpha$ 5-helix and  
398 a phenylalanine at this position is highly conserved among all G-protein subtypes [Suppl. Fig. 4]. In the  
399 inactive conformation, its aromatic side chain is embedded into a hydrophobic core that is formed by  
400 side chains projecting from several different portions of the *Gas* scaffold [Fig. 4A1]. This localization  
401 enables F376 to act as a key transducer of the conformational forces that propagate from the C-terminal  
402 portion of the  $\alpha$ 5-helix as it engages an activated receptor (25,26) [Fig. 4B] to the interior components  
403 of the G-protein that directly participate in the GDP/GTP exchange mechanism (27). A valine mutation  
404 at F376 likely causes a marked perturbation of the hydrophobic core configuration and consequently  
405  $\alpha$ 5-helix changes that differ from those of the wild-type protein [Fig. 4B1]. This hypothesis for *Gas* is  
406 supported by *in vitro* studies at *Gi*, where mutations at the corresponding phenylalanine lead to rotation  
407 of the  $\alpha$ 5-helix (28). Moreover, *in vitro* studies at the corresponding phenylalanines of *Gat* (29) or *Gai*  
408 (30) revealed constitutive G-protein activation with respect to an increased GDP/GTP exchange, but  
409 they are (partially) resistant to stimulation by agonist-occupied GPCRs. Amino acid F376 in *Gas*  
410 corresponds to F341 in *G $\alpha$ 11* where the p.F341L mutation causes autosomal dominant hypocalcemia  
411 (31).

412 Our experimental data indicate that the p.F376V mutation, combined with the proposed spatial  
413 displacement of the  $\alpha$ 5-helix, decreases ligand-induced down-stream signaling at the investigated  
414 receptor/ligand complexes [Fig. 2,3]. As already known from a crystallized GPCR/Gs complex (16)  
415 [Fig. 4B], *Gas* undergoes major global and local structural movements during activation (25,27,28,32).  
416 This general finding has recently been confirmed at the structural level also for GPCRs complexed with  
417 *Gi* or *Go*, as elucidated by cryo-electron microscopy (33-35). In the active state, F376 within the  
418 GPCR/*Gas* complex (16) is located spatially close to the intracellular loop 2 (IL2) of the receptors, but  
419 a direct contact was not observed in these GPCR/G-protein complexes without bound nucleotides.  
420 Therefore it can only be speculated that F376 may play a crucial role for a potential pre-coupled state,



421 the transition between the inactive and fully-active state, or for dissociation of the G-protein from the  
422 GPCRs. In addition, our findings based on side chain variations at position 376 support two hypotheses:  
423 (1) The receptors are different in their fine-tuned interaction process with G $\alpha$ s, which would be in  
424 accordance with the differences in basal activity observed for the tested GPCRs in complex with the  
425 G $\alpha$ s-F376V mutant. (2) Maintenance of the hydrophobic side-chain at this position is essential to  
426 facilitate signaling and a methionine can maintain these properties in contrast to a valine.

427

428 In summary, the *in vivo* and *in vitro* findings suggest that NSIAD is caused by a recurrent maternal G $\alpha$ s  
429 mutation (p.F376V), which also leads to precocious puberty and skeletal dysplasia [Tab. 2]. It can  
430 therefore be expected that other more common diseases, such as precocious puberty or skeletal dysplasia  
431 or NSIAD of unknown origin, can be caused by G $\alpha$ s mutations similar to p.F376V, if these reside on  
432 the paternal *GNAS* allele thus enhancing GPCR-independent signaling. The knowledge gained from  
433 unique ligand-independent and ligand-dependent consequences caused by this G $\alpha$ s variant will be  
434 helpful in the future development of targeted therapies, and for the clinical management of these patients,  
435 for example, restricted fluid intake in view of the predisposition to hyponatremia.

436

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446

447 **Author contributions:** H.B. - designed, conducted and evaluated all functional studies, interpretation of  
448 functional data, wrote manuscript, figure preparations; G.K. - design of protein models, wrote the

449 manuscript, interpretation of functional and structural data, contribution to study design, figure  
450 preparations; D.S. - initiated clinical studies, clinical data generation; D.B. - initiated clinical studies  
451 and manuscript preparation; L.C.W. - clinical phenotype discussions, contributions to manuscript  
452 preparation; I.T. - clinical phenotyping, genetic studies; S.K.- clinical phenotyping, contributed to  
453 manuscript preparation; P.S. - design of protein models, wrote the manuscript, interpretation of  
454 functional and structural data, figure preparations; M.R. - conducted data generation and analysis and  
455 interpretation; S.P. - performed functional studies; J.W.G. - initiated clinical studies and clinical data  
456 generation and commented on manuscript drafts; J.A. - clinical phenotyping; H.K. - clinical phenotype  
457 discussions; M.M. - contributed to the data interpretation and provided insights into experimental  
458 models; T.G. - contributed to the data interpretation and provided insights into experimental models;  
459 M.D. - designed the clinical and experimental studies, supervised data generation, wrote the manuscript;  
460 H.J. - designed the clinical and experimental studies, supervised data generation, interpreted the results  
461 and wrote the manuscript; A.G. - designed the clinical and experimental studies, supervised data  
462 generation, figure preparations, interpreted the results and wrote the manuscript

463

#### 464 **Abbreviations**

465 AVP, arginine vasopressin; cAMP, cyclic adenosine monophosphate; CG, chorio-gonadotropin; GNAS,  
466 G protein alpha subunit; HEK, human embryonic kidney cells; FSH, follitropin; LH, lutropin; LHCGR,  
467 lutropin/choriogonadotropin receptor; MAS, McCune-Albright Syndrome; MC4R, Melanocortin-4  
468 receptor; PCR, polymerase chain reaction; PTH, parathyroid hormone; PTH1R, parathyroid hormone  
469 receptor; PHP, pseudohypoparathyroidism; V2R, vasopressin-2 receptor; NSIAD, Nephrogenic  
470 syndrome of inappropriate anti-diuresis; TSHR, thyroid stimulating hormone receptor; GPCR, G-  
471 protein-coupled receptor; TM, transmembrane helix; EL1/2/3, extracellular loops 1/2/3; ILs 1/2/3,  
472 intracellular loops 1/2/3; PDB, Protein Data Bank; wt, Wild-type

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477 **References**

478

479 supplementary material is deposited under: Biebermann H. et al., Figshare repository, 30. September  
480 2018. <https://figshare.com/s/5954228387e060979a42>)

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577

578 **Table and Figure legends**

579

580 **Table 1: *Clinical and metabolic findings in the two patients.*** Clinical and biochemical findings in the  
581 patients with p.F376V compared to patients with pseudohypoparathyroidism and precocious puberty  
582 (p.A366S) caused by protein instability (12) or found in McCune Albright Syndrome (p.R201H) (10).  
583 Characteristic findings of the three different diseases are highlighted. “+” present, “-“ not present, “+/-  
584 “ unilateral testes enlargement

585

586 **Table 2: *Review of inherited human diseases caused by impaired GPCR function.*** Human diseases  
587 caused by variants of GPCR or Gas of either loss- or gain-of-function consequences including the novel  
588 phenotypes caused by variant p.F376V studied in this report. Receptor +/Gas + = gain-of-function  
589 variants, Receptor -/Receptor - = Loss-of-function variants

590

591 **Figure 1: *Radiographic studies of skeletal features in the patients.*** Radiographs of patient 1 showing  
592 the skeletal abnormalities such as antecurvatura of the left distal tibia, bowing of both upper legs,  
593 enlargement of the metaphyses. **A) Radiographic study of patient 1, at 1 year 8 months and 7 years. B)**  
594 **Radiographic study of patient 2 at 1 year and 3 months.**

595

596 **Figure 2: *Functional characterization of wt Gas and variants co-expressed with several different***  
597 ***GPCRs.*** 2B2 cells were co-transfected with GPCRs, and either wild-type or mutant Gas, as indicated.  
598 After two days, cAMP accumulation was determined in the absence or presence of maximal  
599 concentration of indicated ligands by alpha screen technology. Results of four independent experiments  
600 performed in triplicated were shown as mean ± SEM. Statistical analysis was performed with one-way  
601 ANOVA with Kruskal-Wallis test, indicated receptors + Gas were tested against receptors in the  
602 presence of different Gas variants; \* p ≤ 0.05, \*\* p ≤ 0.01, \*\*\*p ≤ 0.001, \*\*\*\*p ≤ 0.0001.

603

604 **Figure 3: *Functional characterization of wt Gas and variants co-expressed with different GPCRs.***  
605 2B2 cells were co-transfected with GPCRs, and wild-type or mutant Gas, as indicated. After two days,

606 cAMP accumulation was determined in the presence of decadic increasing concentration of indicated  
607 ligands by alpha screen technology. Results of four independent experiments performed in triplicates  
608 were shown as mean  $\pm$  SEM.

609

610 **Figure 4: Phenylalanine 376 in different G $\alpha$ s conformations and activity states. A-A1)** In the inactive  
611 state conformation of the heterotrimeric G $\alpha$ s amino acid F376 is cage-like embedded by several  
612 hydrophobic and/or aromatic amino acids (shown as green sticks). F376 is not directly located at the  
613 GDP/GTP (shown as orange spheres) binding pocket unlike known pathogenic G $\alpha$ s mutations, *e.g.*  
614 residues A366, R201 or Q227 (7) (magenta sticks). **A2).** Mutational variants such as p.F376V, p.F376Y  
615 and p.F376M lead to specific changes in the tight hydrophobic/aromatic environment at position 376.  
616 Methionine is aliphatic in contrast to phenylalanine, but is hydrophobic and interacts with aromatic ring  
617 systems. Valine is characterized by a shorter and more branched side chain compared to phenylalanine.  
618 The patient mutant F376V therefore most likely leads to a loss of the tight interactions, which finally  
619 can cause structural modifications in the relative spatial  $\alpha$ 5-helix orientation compared to wild-type. **B)**  
620 From the crystal structure G $\alpha$ s/ $\beta$ -2AR complex (PDB entry 3SN6 (16)) it is known that the G $\alpha$ s-protein  
621 undergoes structural re-organization during interaction with the receptor, *e.g.* a global movement of the  
622 helical domain (arrow), enabled by ligand-induced conformational changes in the receptor. **B1)** The  
623 superimposition between the active and the inactive G $\alpha$ s crystal structure shows that F376 changes the  
624 spatial localization during G-protein activation related to local movements of the C-terminal  $\alpha$ 5-helix.