Landscape of familial isolated and young-onset pituitary adenomas: prospective diagnosis in *AIP* mutation carriers

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ABSTRACT

Context: Familial isolated pituitary adenoma (FIPA) due to aryl hydrocarbon receptor interacting protein (*AIP*) gene mutations is an autosomal dominant disease with incomplete penetrance. Clinical screening of apparently unaffected *AIP* mutation (*AIP*mut) carriers could identify unapparent disease.

Objective: To determine *AIP* mutational status of FIPA and young pituitary adenoma patients, analyzing their clinical characteristics, and to perform clinical screening of apparently unaffected *AIP*mut carrier family members.

Design: Observational, longitudinal study, 2007-2013.

Setting: International collaborative study, referral centers for pituitary diseases.

Participants: FIPA families (n=216) and sporadic young-onset (≤30 years) pituitary adenoma patients (n=404).

Interventions: Genetic screening of patients for *AIP*muts, clinical assessment of their family members and genetic screening for somatic *GNAS1* mutations and the germline *FGFR4* p.G388R variant.

Main Outcome Measure(s): Clinical disease in mutation carriers, comparison of characteristics of *AIP*mut positive and negative patients, results of *GNAS1* and *FGFR4* analysis.

Results: Thirty-seven FIPA families and 34 sporadic patients had *AIP*muts. Patients with truncating *AIP*muts had a younger age at disease onset and diagnosis, compared to patients with non-truncating *AIP*muts. Somatic *GNAS1* mutations were absent in tumors from *AIP*mut positive patients, and the studied *FGFR4* variant did not modify the disease behavior or penetrance in *AIP*mut positive individuals. A total of 164 *AIP*mut positive unaffected family members were identified; pituitary disease was detected on 18 of those who underwent clinical screening.

Conclusions: A quarter of the *AIP*mut carriers screened were diagnosed with pituitary disease, justifying this screening and suggesting a variable clinical course for *AIP*mut positive pituitary adenomas.

INTRODUCTION

Familial isolated pituitary adenoma (FIPA) is characterized by the presence of pituitary adenomas in two or more members of the same family in the absence of other syndromic clinical features, such as those characteristic of multiple endocrine neoplasia type 1 (MEN1) and 4 (MEN4), Carney complex or tumors related to mutations in the succinate dehydrogenase (SDH) genes. FIPA is a heterogeneous condition, encompassing cases with unknown genetic cause and patients with mutations in the aryl-hydrocarbon receptor interacting protein gene (*AIP*), with distinctive clinical characteristics. Germline *AIP* mutations (*AIP*muts) play a role not only in a subset of FIPA families (1-4), but also in sporadically diagnosed pituitary adenomas (5-9), and in the setting of somatostatin analogue (SSA)-resistant acromegaly (10). Another form of FIPA, X-linked acrogigantism (X-LAG), due to microduplications in the Xq26.3 region, has been recently identified in patients with very young-onset gigantism and pituitary adenoma/hyperplasia (11).

The phenotype of *AIP*mut-associated pituitary adenomas has been described before (2-4;12), but a systematic follow-up of cases and families is lacking, due to the relative novelty of this pathogenic association (1), the variable disease penetrance (4;12-14) and the rarity of this clinical entity. We present the clinical and genetic characteristics of a large cohort of FIPA and *simplex* (patients with germline mutation and no family history) *AIP*mut positive patients, aiming: (i) to perform a systematic follow-up of families to identify and characterize *AIP*mut positive carriers, (ii) to seek the role of disease-modifying genes on the variable phenotype and penetrance of the disease, and (iii) to confirm and extend the description of the phenotype of *AIP*mut positive patients, providing a comparison with *AIP*mut negative cases. We establish that genetic screening followed by clinical assessment identifies a large percentage of family members with pituitary abnormalities, supporting the facilitation of genetic diagnosis and follow-up of these patients and their families.

PATIENTS AND METHODS

108   Our study population (1725 subjects, Table 1) was recruited via the collaborative research network of

109   the International FIPA Consortium (15). Pituitary adenoma patients were grouped into 11 clinical

110   diagnostic categories (Supplemental Table 1). The diagnoses of acromegaly,

111   acromegaly/prolactinoma, gigantism, gigantism/ prolactinoma, and mild acromegaly (16) were

112   grouped together under the category of 'GH excess' for some analyses.

113

114   Between January 2007 and January 2014, we recruited patients from 35 countries from two different

115   groups: either members of FIPA families, defined by the presence of pituitary adenomas in two or

116   more members of a family without other associated clinical features (1-3;17) ('familial' cohort), or

117   sporadically-diagnosed pituitary adenoma patients with disease onset at ≤30 years of age ('sporadic'

118   cohort). As an exception to these inclusion criteria, one *AIP*mut positive >30 years sporadic patient

119   was found thanks to *AIP* screening in the setting of a research study, and the screening of his relatives

120   detected a second *AIP*mut positive pituitary adenoma case; this family was included in the familial

121   cohort. The first patient reported in each FIPA family and all the sporadic patients were considered

122   'probands'. All the patients received treatment and were followed up in accordance with the

123   guidelines and clinical criteria of their respective centers. Relevant clinical and family structure data

124   were received from clinicians and/or patients, and genetic screening was performed in the families of

125   all the *AIP*mut positive probands, selecting individuals according to their risk of inheriting the

126   mutation, based on their position in the family tree, and extending the screening to as many

127   generations as possible. In both familial and sporadic cases, other causes of familial pituitary

128   adenomas, such as MEN1 and 4, Carney complex, pheochromocytoma/paraganglioma and pituitary

129   adenoma syndrome and X-LAG were ruled out by clinical, biochemical and, in some cases, genetic

130   tests, as appropriate. The study population included a great majority of new cases, but also previously

131   diagnosed patients being followed-up by the participating centers and a few historical cases,

132   corresponding to deceased members of FIPA families (further details in Supplemental Results). Four

133   *AIP*mut positive patients (two with diagnosis of acromegaly and two with gigantism) died in the post-

134   recruitment period. Three of the deaths were due to cardiovascular causes (stroke, chronic heart

135 failure and acute coronary syndrome), while the exact cause of death is unknown in the fourth, a

136 patient with long-standing untreated familial acromegaly.

137

138 All the patients and family members included agreed to take part by providing signed informed

139 consent forms approved by the local Ethics Committee. Further details on the study population and

140 the procedures for genetic/clinical screening and search for disease-modifying genes are described in

141 the Supplemental Material.

142

143 **Statistical analysis**

144 The qualitative, categorical variables were expressed as percentages and compared using the chi-

145 squared test or the Fisher's exact test, as appropriate. The normal distribution of the quantitative

146 variables was verified using the Shapiro-Wilk and the Kolmogorov-Smirnov tests for normality.

147 Means and standard deviations were used to report parametric data, and non-parametric data were

148 expressed as median and interquartile ranges. Parametric data were analyzed with the unpaired t-test,

149 with a 95% confidence interval, while the Mann-Whitney U test was used for the non-parametric data.

150 Statistical significance was considered when the *P* value was <0.05. All the statistical analyses were

151 carried out using the GraphPad Prism 6 (GraphPad Software Inc.) and Stata 12 (StataCorp LP)

152 statistical software.

153

154 **RESULTS**

155

156 **Study population**

157 The familial cohort was composed of 216 FIPA families, including 156 new families (989 subjects:

158 337 patients and 652 unaffected family members) and 60 previously described families (3;12), where

159 46 new subjects (15 patients and 31 unaffected family members) were added to the previously

160 reported 196 individuals (150 patients and 46 unaffected family members). The sporadic cohort

161 originally included 409 pituitary adenoma patients ≤30 years old at disease onset, with no known

162 familial history of pituitary adenoma, but we excluded five patients from further analysis due to

163   harboring an Xq26.3 microduplication. Of the remaining 404 sporadic patients, six were reported

164   previously (3). In addition to the *AIP*mut screening, a subset of *AIP*mut negative FIPA (n=55) and

165   sporadic (n=45) patients underwent genetic screening for other endocrine neoplasia-associated genes

166   (Supplemental Table 2). All of these tests were negative for pathogenic variants. After the genetic

167   screening and follow-up of the patients and carriers, 60 individuals in the familial cohort and seven in

168   the sporadic cohort were classified as 'not at risk' of inheriting an *AIP*mut, and were excluded from

169   further analysis. Twenty three individuals initially thought to be unaffected were identified with

170   pituitary abnormalities (see details in the 'Prospective diagnosis' section).

171

172   **Genetic screening results**

173   Thirty-seven (17.1%) out of 216 FIPA families screened and 34 out of 404 sporadic patients (8.4%)

174   were positive for pathogenic or likely pathogenic *AIP*muts, accounting for a total of 71 *AIP*mut

175   positive kindreds and 144 *AIP*mut positive patients (76.4% familial and 23.6% *simplex*, Table 2). We

176   also identified 164 *AIP*mut positive apparently unaffected family members (see 'Follow-up and

177   prospective diagnosis'). Samples were not available from family members of 25 *AIP*mut positive

178   *simplex* cases to establish the presence or lack of *de novo* mutations. We identified three pituitary

179   adenoma patients (two with clinically non-functioning pituitary adenoma [NFPA] and one with a

180   microprolactinoma) belonging to *AIP*mut positive FIPA families and being 'at risk' of inheriting, but

181   not carrying an *AIP*mut; therefore they were considered as phenocopies.

182

183   Thirty-one different *AIP*muts (ten not previously reported) were identified in our study population: 12

184   exclusively in familial cases, 12 in *simplex* cases only and seven in both settings (Table 3 and

185   Supplemental Figure 1). Of the total mutations, 70.8% (22/31) predict a truncated or missing protein,

186   and were termed as 'truncating *AIP*muts' (Supplemental Figure 2). We also identified 11 apparently

187   non-pathogenic *AIP* variants (three of them novel) in our population (Supplemental Table 3).

188

189   A multiple regression analysis was performed to determine which clinical features could more

190   accurately predict the likelihood of a patient to carry an *AIP*mut. An age at diagnosis ≥ 10 and < 20

191   years conferred an odds ratio (OR) of 5.8 (*P*=0.000, 95% CI 3.1-10.8) of having an *AIP*mut, while the

192   OR was 2.8 if the age at diagnosis was ≥20 and <30 years (*P*=0.000, 95% CI 1.3- 5.7); thus, an age at

193   diagnosis between 10 and 30 years is the best predictor of *AIP*muts. Inversely, a diagnosis of

194   prolactinoma resulted in an OR of 0.2 (*P*=0.000, 95% CI 0.1-0.5).

195

196   **Genotype-phenotype correlation within the *AIP*mut positive cohort**

197   Truncating mutations accounted for 78.9% (15/19) of the *AIP*muts found in the familial cohort, and

198   for 57.9% (11/19) of those detected in the sporadic cohort. To study a possible difference in disease

199   penetrance between truncating and non-truncating mutations, we compared the number of affected

200   individuals with truncating *AIP*muts in the familial (85/110 [77.3%]) and *simplex* cohorts (21/34

201   [61.8%]), finding no significant difference, although a trend was observed (*P*=0.0729, analysis

202   included prospectively diagnosed patients). No significant differences were found regarding the

203   proportion of GH excess cases, number of patients per family, maximum tumoral diameter, frequency

204   of macroadenomas, extrasellar invasion or number of treatments received between the patients with

205   truncating and non-truncating mutations. However, patients with truncating mutations were

206   significantly younger at disease onset (median 16 [IQR 15-25] vs. 22 [IQR 17.3-27.8] years,

207   *P*=0.0046, Figure 1a) and at diagnosis (median 21 [IQR 16-30] vs. 27 [IQR 20.8-37] years,

208   *P*=0.0028, Figure 1b), and the occurrence of pediatric cases was more common in this group (60%

209   [57/95], Figure 1c), compared to the patients with non-truncating *AIP*muts (33.3% [12/36],

210   *P*=0.0064). In concordance with these differences, gigantism accounted for a significantly higher

211   proportion of the GH excess cases in the patients with truncating *AIP*muts (54.7% [47/86]), compared

212   to those with non-truncating *AIP*muts (30% [9/30], *P*=0.0200). As p.R304* was the most common

213   *AIP*mut in our study population (20 kindreds), we analyzed if these patients behaved differently to

214   other patients with truncating mutations, finding more affected individuals per family (median 4 [IQR

215   2.5-5]), compared to families with other *AIP*muts (median 2 [IQR 2-3], *P*=0.0133). When considering

216   all the *AIP*mut positive patients together (familial and sporadic), we found a higher proportion of

217   pediatric patients among those with the *AIP* p.R304* mutation (65.8% [25/38] vs. 46.5% [40/86],

218   *P*=0.0475).

219

Clinical and histopathological features

Findings regarding gender distribution, age at onset/diagnosis, distribution of clinical diagnoses,

tumor size/extension, pituitary apoplexy, histopathological features, extrapituitary tumors and specific

analyses of patients with GH excess and with gigantism are detailed in the Supplemental Material.

224

Disease penetrance

To calculate the penetrance of pituitary adenomas among *AIP*mut positive families, complete data is

needed both for phenotype and genotype. Therefore, we have selected three families (two with

p.R304*, and one with p.A34_K39del mutations) where complete data was available in three or more

generations for consenting 'at risk' individuals. The *AIP* genotype was known in 76.6% (range 68.4-

94.7%) of the individuals at risk; of them, 16.8% were patients and 83.2% were unaffected carriers.

The gender distribution was similar between patients and unaffected carriers. The mean penetrance in

these three families was 28.6% (19-38.1), and it decreased to 22.7% (18.2-26.7) when 50% of the

individuals at risk with unknown genotype were considered as unaffected carriers. When the

prospectively diagnosed patients were omitted from this calculation, the total penetrance of pituitary

adenomas was 12.5%, highlighting the importance of the follow-up of apparently unaffected carriers

for the correct calculation of the disease penetrance.

237

As penetrance cannot be appropriately calculated for *AIP*mut negative families, we assessed the

number of affected family members. The *AIP*mut positive families had more affected individuals per

family than the *AIP*mut negative families (*P*<0.0001, Supplemental Figure 7e). While 84.9%

(152/179) of the *AIP*mut negative families had only two affected members, 48.6% (18/37) of the

*AIP*mut positive families had three or more pituitary adenoma patients per family. The maximum

number of affected individuals within the same family was eight (six of them prospectively

diagnosed) in a family carrying the p.R304* *AIP*mut, and the maximum number of cases of gigantism

in the same family was five, in a FIPA family with the p.E24* *AIP*mut.

246

Follow-up and prospective diagnosis

Out of the 164 originally identified *AIP*mut carriers, 160 were available and advised to undergo biochemical and clinical screening. Prospective diagnosis of a pituitary adenoma was established in 11.3% (18 subjects, 11 males) of the individuals originally considered as unaffected *AIP*mut carriers.

Six of the prospectively diagnosed patients had acromegaly (one of them with PRL co-secretion), one patient had gigantism, two patients were diagnosed with mild acromegaly (16) and nine patients harbored NFPAs. Out of the 142 individuals remaining as apparently unaffected *AIP*mut carriers, 79 (55.6%) underwent clinical assessment and one or more biochemical or imaging tests, while 63 subjects (44.4%) had only clinical evaluation.

The prospective cases were diagnosed at an older age than the rest of the patients (median 30 [IQR 22.8-39.5] vs. 23 [IQR 16-33] years, *P*=0.025). At diagnosis, seven of the prospectively diagnosed patients were symptomatic (headaches, arthralgias, acral growth, facial changes, weight gain or hyperhidrosis). Five of the 18 prospectively diagnosed tumors were macroadenomas, in contrast with a predominance of macroadenomas (89.9%, 71/79) in the rest of the *AIP*mut positive FIPA patients (*P*<0.0001). The maximum diameter was significantly smaller for prospective cases (median 5.8 [IQR 4.7-14.4] vs. 16.5 [IQR 10-29], *P*=0.0002). Four of the patients with macroadenomas had surgery and the histopathological study confirmed GH or GH/PRL positive adenomas. The fifth macroadenoma was identified in a 68-year-old male patient with well controlled hypertension and diabetes mellitus and no other comorbidities or symptoms, who did not wish to receive any treatment. In addition, one *AIP*mut negative pituitary adenoma patient, harboring a 25mm NFPA, was prospectively diagnosed as part of an *AIP*mut positive family (brother of the *AIP*mut positive proband).

Further seven subjects had abnormalities in their screening tests, but a pituitary disease was not confirmed: five individuals had slightly elevated IGF-1 levels for their age/gender, one patient displayed acromegaloid features but normal pituitary MRI and biochemistry, and a 17-year-old female had repeatedly borderline high IGF-1 and incompletely suppressed GH on OGTT, but her bulky

275   pituitary gland (11mm in height), normal at this age group, is not changing during follow-up and her

276   biochemical results are now within the normal range, after three years of follow-up.

277

278   The global penetrance of pituitary adenomas among the individuals initially considered as unaffected

279   *AIP*mut carriers was 11.3% (18/160). However, the penetrance was higher in the group of carriers

280   who underwent biochemical and imaging investigations, varying between 18.6 and 28.1% depending

281   on the depth of screening (Figure 2). Overall, these data suggest that approximately 20-25% of the

282   apparently unaffected *AIP*mut carriers screened with biochemical or imaging tests will be identified

283   with a pituitary adenoma at some point in their lives.

284

285   Clinical screening was not systematically performed in the *AIP*mut negative FIPA unaffected family

286   members. Nevertheless, due to the increased disease awareness given by the existence of previous

287   pituitary adenoma cases within their families, four individuals (three females and one male) from

288   three different *AIP*mut negative FIPA families were prospectively diagnosed. Three of them harbored

289   NFPAs, but we lack complete information about the fourth patient. The mean age at diagnosis in the

290   three NFPA cases was 37 years, and only one patient referred symptoms at diagnosis (galactorrhea,

291   not clearly associated to stalk compression, and lethargy). All of them had microadenomas, with a

292   mean diameter of 6.5mm, and did not require any therapeutic intervention other than hormonal

293   replacement in one case. The characteristics of these cases resemble those of incidentalomas;

294   however, the occurrence of two prospective cases in the same family supports an apparent inherited

295   component.

296

297   **Disease-modifying genes**

298   We have studied the role of two possible disease-modifying genes: *GNAS1* (18) (somatic) and *FGFR4*

299   (germline) (19). *GNAS1* mutations were absent in all the studied *AIP*mut positive somatotropinomas

300   (n=23), but were detected in 50% of the *AIP*mut negative familial somatotropinomas (5/10), 16.7% of

301   the *AIP*mut negative young-onset cases (1/6), and 26.3% of the unselected acromegaly cases studied

302   (5/19). The distribution of the *FGFR4* p.G388R SNP conserved the Hardy-Weinberg equilibrium (20)

303 and the genotype distribution was similar between patients (n=98) and carriers (n=108) (P=0.523).

304 The age at onset and at diagnosis, tumor size and frequency of extrasellar invasion were not

305 significantly different between the GG (wild-type) and GR/RR patients.

306

307 **DISCUSSION**

308

309 *AIP*muts are prevalent in young onset GH-excess patients (24%) and FIPA (17.1%), with more than

310 double frequency in patients with gigantism (46.7%) in our cohort, in concordance with other studies

311 (7;9;21;22). However, in contrast to previous reports, in this large and extensively studied cohort there

312 was no predominance of male patients among the *AIP*mut positive familial cases, and equal numbers

313 of male and female unaffected carriers were identified. Earlier studies (3;4;12;23) may have had an

314 ascertainment bias for families with cases of gigantism, a disease that is more prevalent in males, at

315 least partly due to the physiologically later puberty and therefore later cessation of growth in boys.

316

317 We have demonstrated that around a quarter of the individuals initially identified as unaffected

318 *AIP*mut carriers who underwent clinical screening tests were diagnosed with pituitary abnormalities.

319 Full clinical screening identified 28.1% of the carriers, with fewer tests understandably resulting in

320 fewer positive cases. Our data suggest that not all the *AIP*mut-associated pituitary adenomas have a

321 rapidly growing, aggressive phenotype. The follow-up of these patients allowed us to observe some

322 probably very early cases of acromegaly, where the current clinical scenario had not indicated

323 intervention at data closure. We cannot rule out that some of the small NFPAs are indeed

324 incidentalomas, similar to those frequently observed in *AIP*mut negative subjects of the general

325 population.

326

327 This frequency of prospective diagnosis may justify the clinical screening and, possibly, follow-up of

328 all the *AIP*mut positive unaffected carriers. Our data would support the assessment of all the newly

329 identified *AIP*mut carriers (clinical examination/history, PRL and IGF-1, as a minimum, up to a full

330 screening, including also an OGTT and contrast-enhanced pituitary MRI). Follow-up of the younger

12

331 family members should continue until at least the 30 years of age, preferably annually, with clinical

332 assessment and basal pituitary hormonal levels, leaving stimulation tests for cases with suspicion of

333 pituitary disease and a follow-up MRI if necessary (24;25). The cost-effectiveness and the possible

334 psychological burden of this approach will need future study. Stopping the follow-up should be

335 considered in older patients, given the low possibility of detecting new pituitary adenoma patients in

336 these individuals after the fifth decade of life (24;25). Once a case has been prospectively diagnosed,

337 the treatment and follow-up should proceed as for the general population of pituitary adenoma

338 patients, as there are no data to suggest a different type of treatment in *AIP*mut positive patients (26).

339

340 The genetic and clinical screening of *AIP*mut negative FIPA families is uncertain at this point.

341 Baseline screening and follow-up of obligate carriers could be considered, keeping in mind that the

342 age of onset is considerably older in these families. Education on possible signs and symptoms of

343 family members is a viable option in the routine setting. Patients with GH excess starting before the

344 age of five should be tested for the recently identified Xq26.3 chromosomal microduplications (11).

345 We expect that the identification of further genes implicated in the pathogenesis of FIPA in the next

346 years will allow us to tailor these recommendations in accordance with the clinical behavior of each

347 genetic entity.

348

349 The genetic screening of sporadic young-onset pituitary adenoma patients with no evidence of other

350 endocrine tumors should be focused on *AIP*muts in first instance in cases of GH excess (with or

351 without PRL co-secretion) and on *MEN1* mutations in cases of prolactinoma (9), as this can be the

352 first manifestation of MEN1 (27). Whether it would be advisable to continue screening young patients

353 with other diagnoses for *AIP*muts out of the setting of research studies needs longer follow-up.

354

355 To explain the variable clinical phenotype in our *AIP*mut positive patients, we evaluated the possible

356 influence of two disease-modifying genes, *GNAS1* and *FGFR4*. While somatic *GNAS1* mutations are

357 common in unselected somatotropinomas (4.4-59% of the cases) (28-35), we have not identified any

358 in adenomas from *AIP*mut positive patients, suggesting that germline *AIP*muts and somatic *GNAS1*

359   mutations are mutually exclusive in somatotropinomas. *GNAS1* mutations have rarely been studied in

360   pediatric patients with acromegaly and gigantism, and they seem to be an extremely infrequent

361   finding in this age-group (36;37). A recent study has shown no change in the AIP immunostaining in

362   sporadic somatotropinomas in the presence of *GNAS1* mutations (38). The characteristic phenotype of

363   adenomas containing the *GNAS1* mutations (small (32;39), highly responsive to the treatment with

364   SSAs, and more often densely granulated according to some (40), but not all studies (41)), seems to

365   be in contrast with the typical *AIP*mut positive tumor phenotype. On the other hand, in somatotroph

366   adenomas of *AIP*mut negative FIPA patients, half of the tested samples had *GNAS1* mutations. This

367   suggests that in *AIP*mut negative FIPA, somatic *GNAS1* mutations could exist in a similar frequency

368   as to in unselected somatotropinomas and possibly, in addition to a germline predisposing mutation,

369   may play a role in their pathogenesis.

370

371   The *FGFR4* gene SNP rs351855 (c.1162G>A, p.G388R), with a minor allele frequency of 0.3, is a

372   predictor of progression and poor prognosis in a variety of human neoplasms (42). A role for

373   rs351855 as a facilitator of somatotroph cell tumorigenesis has been recently proposed (19), and we

374   hypothesized that this variant could increase the penetrance and/or size and extension of *AIP*mut

375   positive pituitary adenomas. The screening for this SNP in our *AIP*mut positive patients failed to

376   show increase in size, extension or apoplexy, even though this association had previously been

377   suggested in sporadic acromegaly patients (19), and no earlier onset or higher penetrance were

378   observed. The lack of association with these two potentially disease-modifying genes suggests that

379   *AIP*mut-related pituitary adenomas are regulated by different pathogenic mechanisms than unselected

380   somatotropinomas.

381

382   We recognize the numerous limitations of our study. We chose an arbitrary age cut-off (≤ 30 years),

383   in concordance with previous *AIP*-related publications, but our data shows that only 13.2% of the

384   *AIP*mut positive patients had disease onset after the age of 30 years. Our patients were recruited from

385   different genetic backgrounds and this could have influenced the disease penetrance and presentation.

386   On the other hand, 19.7% of the *AIP*mut positive kindreds (24.3% of the *AIP*mut positive patients)

387   belong to a cohort with a founder *AIP*mut (p.R304*), originally from Northern Ireland (14). The

388   larger number of subjects screened in these families provided a higher number of carriers and chance

389   for detection of affected individuals. Additional genetic traits possibly co-segregating with this

390   founder mutation could modify the phenotype and thus introduce a bias into our results. Full genotype

391   and phenotype data were not available for all the families; therefore, we limited our penetrance

392   calculations to three large, well-characterized families. A better assessment of the prevalence of

393   pituitary apoplexy and extrapituitary adenomas in *AIP*mut positive patients would require a large

394   control group, screened *ad hoc*, which was beyond the scope of this study. Finally, the data about

395   therapeutic modalities was limited, hampering the analysis of the response to different treatments.

396

397   **CONCLUSIONS**

398

399   The analysis of this large cohort of FIPA patients allowed us establishing a number of novel aspects

400   of FIPA. A phenotype-genotype correlation was found with younger onset of disease in patients with

401   truncating *AIP*muts. We identified a surprisingly high percentage of somatic *GNAS1* mutations in the

402   *AIP*mut negative somatotropinomas, and their absence in *AIP*mut positive tumors. The lack of

403   influence of the germline *FGFR4* p.G388R variant on disease penetrance/severity suggests that

404   currently unknown factors drive penetrance and variable phenotype in *AIP*mut positive pituitary

405   adenomas. The presence of milder, more indolent disease in some *AIP*mut positive subjects has been

406   established. Genetic and clinical screening leads to the prospective identification of an unexpectedly

407   high proportion of affected patients in the originally apparently unaffected carrier group, resulting in

408   earlier diagnosis and treatment and, possibly, better long-term outcome (25). The recruitment of a

409   large study population with this uncommon disease has only been possible thanks to world-wide

410   collaboration.

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FIGURE LEGENDS

Figure 1. Patients with truncating vs. non-truncating *AIP*muts. a) Patients with truncating *AIP*muts present with a more aggressive phenotype, characterized by an earlier age at onset ($P=0.005$) and b) at diagnosis ($P=0.003$). c) This earlier disease onset results in a higher frequency of pediatric cases (n [total]= 131); in fact, the majority of the patients with truncating mutations present in childhood and adolescence. **, $P<0.01$.

Figure 2. Penetrance in screened *AIP*mut positive carriers (n [total]=160). The probability of detecting new cases of pituitary adenomas within apparently unaffected *AIP*mut carriers depends on the clinical assessment and the type of complementary biochemical/imaging studies included in the screening protocol (see text).

Table 1. Study population: demographics and general description

	Familial cohort	Sporadic cohort	Combined
Total individuals, no. (%)	1231 (71.4)	494 (28.6)	1725 (100)
Females, no. (%)	668 (54.3)	250 (50.6)	918 (53.2)
Current age, median (range, [IQR])	46.2 (2-97 [32-62])	35 (3-77 [26-42])	42.6 (2-97 [29-56])
Clinical status, no. (%):			
Affected	502 (40.8)	404 (81.8)	906 (52.5)
Unaffected	729 (59.2)	90 (18.2)	819 (47.5)
Affected males, no. (%)	219 (43.6)	203 (50.2)	422 (46.6)
Affected females, no. (%)	283 (56.4)	201 (49.8)	484 (53.4)
Diagnoses, no. (%):			
Acromegaly	170 (33.9)	203 (50.2)	373 (41.2)
Acromegaly/prolactinoma	17 (3.4)	12 (3)	29 (3.2)
Cushing's disease	24 (4.8)	21 (5.2)	45 (5)
FSHoma	2 (0.4)	1 (0.2)	3 (0.3)
Gigantism	44 (8.8)	65 (16.1)	109 (12)
Gigantism/prolactinoma	1 (0.2)	10 (2.5)	11 (1.2)
Mild acromegaly	2 (0.4)	-	2 (0.2)
NFPA	91 (18.1)	21 (5.2)	112 (12.4)
Pituitary tumor	17 (3.4)	2 (0.5)	19 (2.1)
Prolactinoma	134 (26.7)	67 (16.6)	201 (22.2)
TSHoma	-	2 (0.5)	2 (0.2)
GH excess patients, no. (%)	234 (46.6)	290 (71.8)	524 (57.8)
IQR: interquartile range. FSHoma: FSH secreting adenoma. TSHoma: thyrotropinoma. NFPA: non-functioning pituitary adenoma.			

Table 2. Screening for *AIP* mutations

	Familial cohort			Sporadic cohort			Combined		
	*AIP*mut positive familial	*AIP*mut negative familial	Total familial	*AIP*mut positive *simplex*	*AIP*mut negative sporadic	Total sporadic	*AIP*mut positive familial and *simplex*	*AIP*mut negative familial and sporadic	Total
Total number of kindreds, no. (%):	37 (17.1% of familial)	179 (82.9% of familial)	216 (34.8% of total)	34 (8.4% of sporadic)	370 (91.6% of sporadic)	404 (65.2% of total)	71 (11.5% of total)	549 (88.5% of total)	620 (100)
Total individuals, no. (%):	475 (38.6% of familial)	756 (61.4% of familial)	1231 (71.4% of total)	82 (16.6% of sporadic)	412 (83.4% of sporadic)	494 (28.6% of total)	557 (32.3% of total)	1168 (67.7% of total)	1725 (100)
Genetic status, no. (%):									
*AIP*mut negative patients	3 (0.6)	389 (51.5)*	392 (31.8)	-	370 (89.8)	370 (74.9)	3 (0.5)	759 (65)	762 (44.2)
*AIP*mut positive tested patients	95 (20)	-	95 (7.7)	34 (41.5)	-	34 (6.9)	129 (23.2)	-	129 (7.5)
At risk, but not tested	33 (6.9)	-	33 (2.7)	8 (9.8)	-	8 (1.6)	41 (7.4)	-	41 (2.4)
Not at risk	48 (10.1)	12 (1.6)	60 (4.9)	7 (8.5)	-	7 (1.4)	55 (9.9)	12 (1)	67 (3.9)
Obligate unaffected carriers, not tested	8 (1.7)	-	8 (0.6)	2 (2.4)	-	2 (0.4)	10 (1.8)	-	10 (0.6)
Predicted *AIP*mut positive patients	15 (3.2)	-	15 (1.2)	-	-	-	15 (2.7)	-	15 (0.9)
Unaffected *AIP*mut tested carriers	120 (25.3)	-	120 (9.7)	16 (19.5)	-	16 (3.2)	136 (24.4)	-	136 (7.9)
Unaffected and *AIP*mut negative	153 (32.2)	-	153 (12.4)	15 (18.3)	-	15 (3)	168 (30.2)	-	168 (9.7)
Unaffected relatives of *AIP*mut negative patients	-	355 (47)	355 (28.8)	-	42 (10.2)	42 (8.5)	-	397 (34)	397 (23)
Summary of *AIP*mut positive individuals, no. (%):									
Total *AIP*mut positive patients:†	110 (23.2)	-	110 (8.9)	34 (41.5)	-	34 (6.9)	144 (25.9)	-	144 (8.3)
Total unaffected *AIP*mut carriers:‡	128 (26.9)	-	128 (10.4)	18 (22)	-	18 (3.6)	146 (26.2)	-	146 (8.5)

* In *AIP*mut negative FIPA families, 199 patients were tested for *AIP*muts, the rest (n=190) were assumed to be negative.
†This is equal to the sum of tested *AIP*mut positive patients plus the predicted *AIP*mut positive patients.
‡ Sum of tested unaffected carriers plus obligate unaffected carriers.

1 **Table 3.** *AIP* **pathogenic or likely pathogenic mutations in the familial and sporadic cohorts**

Mutation (DNA level [protein level])	Mutation type	Pathogenic	Location in protein	Familial cohort (n=238)*	*Simplex* cohort (n=52)*	Combined (n=290)*	References/ SR‡
g.4856_4857CG>AA	Promoter	Yes†	Not in protein (5' UTR)	3 (1.3)	-	3 (1)	(3;12)/(SR30)
c.3G>A (p.?)	Start codon	Likely†	N-terminus	2 (0.8)	-	2 (0.7)	This paper
c.40C>T (p.Q14*)	Nonsense	Yes†	N-terminus	2 (0.8)	-	2 (0.7)	(1)/(SR31;32)
c.70G>T (p.E24*)	Nonsense	Yes†	N-terminus	9 (3.8)	-	9 (3.1)	(3)/(SR33)
c.74_81delins7 (p.L25Pfs*130)	Frameshift	Yes†	PPIase domain	10 (4.2)	-	10 (3.4)	(12)/(SR34)
c.100-1025_279+357del (ex2del) (p.A34_K93del)	Large genomic deletion	Yes†	PPIase domain	12 (5)	2 (4)	14 (4.8)	(SR35)
c.100-18C>T	Intronic	Likely	Not in protein (intron 1)	-	3 (6)	3 (1)	(3;7;10)/(SR31)
c.241C>T (p.R81*)	Nonsense	Yes†	PPIase domain	12 (5)	4 (8)	16 (5.5)	(3)/(SR30;36-38)
c.249G>T (p.G83Afs*15)	Splice site (cryptic splice site)	Yes†	PPIase domain	4 (1.7)	-	4 (1.4)	(12)
c.338_341dup (p.L115Pfs*16)	Frameshift	Yes†	PPIase domain	-	2 (4)	2 (0.7)	(6)
c.427C>T (p.Q143*)	Nonsense	Yes†	Between PPIase and TPR1 domains	-	1 (2)	1 (0.3)	This paper
c.469-2A>G (p.E158_Q184del)	Splice site	Likely	TPR1 domain	-	1 (2)	1 (0.3)	(5)/(SR39;40)
c.490C>T (p.Q164*)	Nonsense	Yes†	Between PPIase and TPR1 domains	3 (1.3)	-	3 (1)	(12)
c.570C>G (p.Y190*)	Nonsense	Yes†	TPR1 domain	9 (3.8)	-	9 (3.1)	This paper
c.662dupC (p.E222*)	Nonsense	Yes†	Between TPR1 and 2 domains	3 (1.3)	-	3 (1)	(12)
c.713G>A (p.C238Y)	Missense	Yes	TPR2 domain	4 (1.7)	-	4 (1.4)	(3)/(SR33)
c.783C>G (p.Y261*)	Nonsense	Yes†	TPR2 domain	4 (1.7)	-	4 (1.4)	(9)/(SR39;41;42)
c.787+9C>T	Intronic	Uncertain	Not in protein (intron 5)	-	1 (2)	1 (0.3)	This paper
c.804C>A (p.Y268*)	Nonsense	Yes†	TPR3 domain	19 (8)	3 (6)	22 (7.6)	(SR43;44)
c.805_825dup (p.F269_H275dup)	In-frame insertion	Yes	TPR3 domain	16 (6.7)	2 (4)	18 (6.2)	(3)/(SR30;39;45)
c.807C>T (p.(=))	Splice site (reduced transcript level)	Yes	TPR3 domain	7 (2.9)	4 (8)	11 (3.8)	(3;5;7;10;12)/ (SR46;47)
c.811C>T (p.R271W)	Missense	Yes	TPR3 domain	-	1 (2)	1 (0.3)	(2;7;12)/(SR48)
c.816delC (p.K273Rfs*30)	Frameshift	Yes†	TPR3 domain	-	1 (2)	1 (0.3)	This paper
c.868A>T (p.K290*)	Nonsense	Yes†	TPR3 domain	-	1 (2)	1 (0.3)	This paper
c.872_877delTGCTGG (p.V291_L292del)	In-frame deletion	Yes	TPR3 domain	-	1 (2)	1 (0.3)	This paper
c.910C>T (p.R304*)	Nonsense	Yes†	C-terminal α-helix	88 (37)	16 (31)	104 (35.9)	(1-3;5;7;9;12;14)/ (SR39;49-51)
c.911G>A (p.R304Q)	Missense	Yes	C-terminal α-helix	20 (8.4)	3 (6)	23 (7.9)	(3;5;7;9;12)/ (SR31;39;52;53)
c.967delC (p.R323Gfs*39)	Frameshift	Yes†	C-terminal α-helix	-	4 (8)	4 (1.4)	This paper
c.976_977insC (p.G326Afs*?)	Frameshift	Yes†	C-terminal α-helix	-	1 (2)	1 (0.3)	This paper
c.978dupG (p.I327Dfs*?)	Frameshift	Yes†	C-terminal α-helix	-	1 (2)	1 (0.3)	This paper
c.1-?_993+?del- (whole gene deletion)	Large genomic deletion	Yes†	Absence of the whole protein	11 (4.6)	-	11 (3.8)	(12)

* Number of positive individuals for each mutation, considering the *AIP*mut positive tested individuals, the obligate carriers and the predicted *AIP*mut patients.
† Truncating mutation. ‡ Supplemental references (see Supplemental Material).
PPIase, peptidylprolyl isomerase. TPR, tetratricopeptide repeat.

2

3   **Figure 1**

4

Age at onset

Age at diagnosis

a

b

Distribution of age at onset
by *AIP*mut type

c
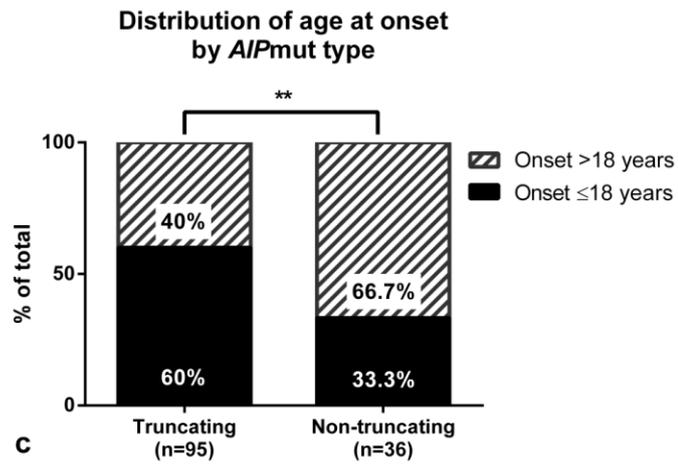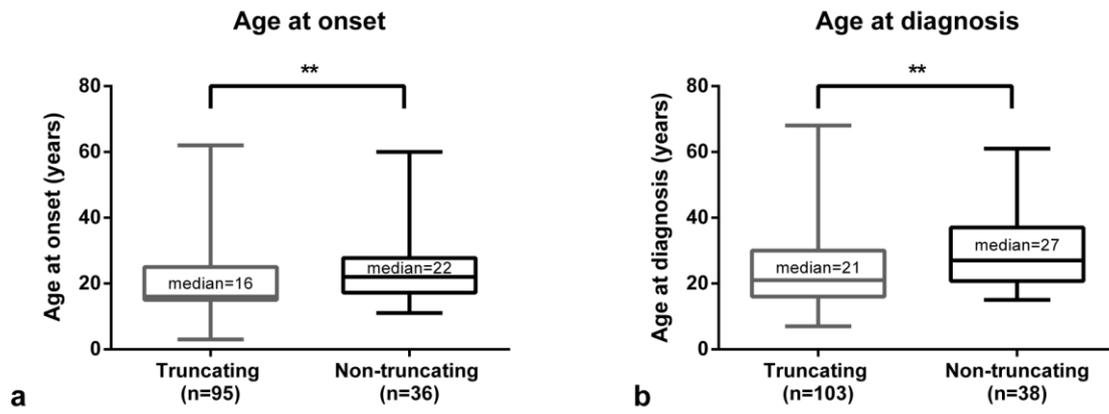
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Disease penetrance in *AIP*mut positive carriers according to screening tests
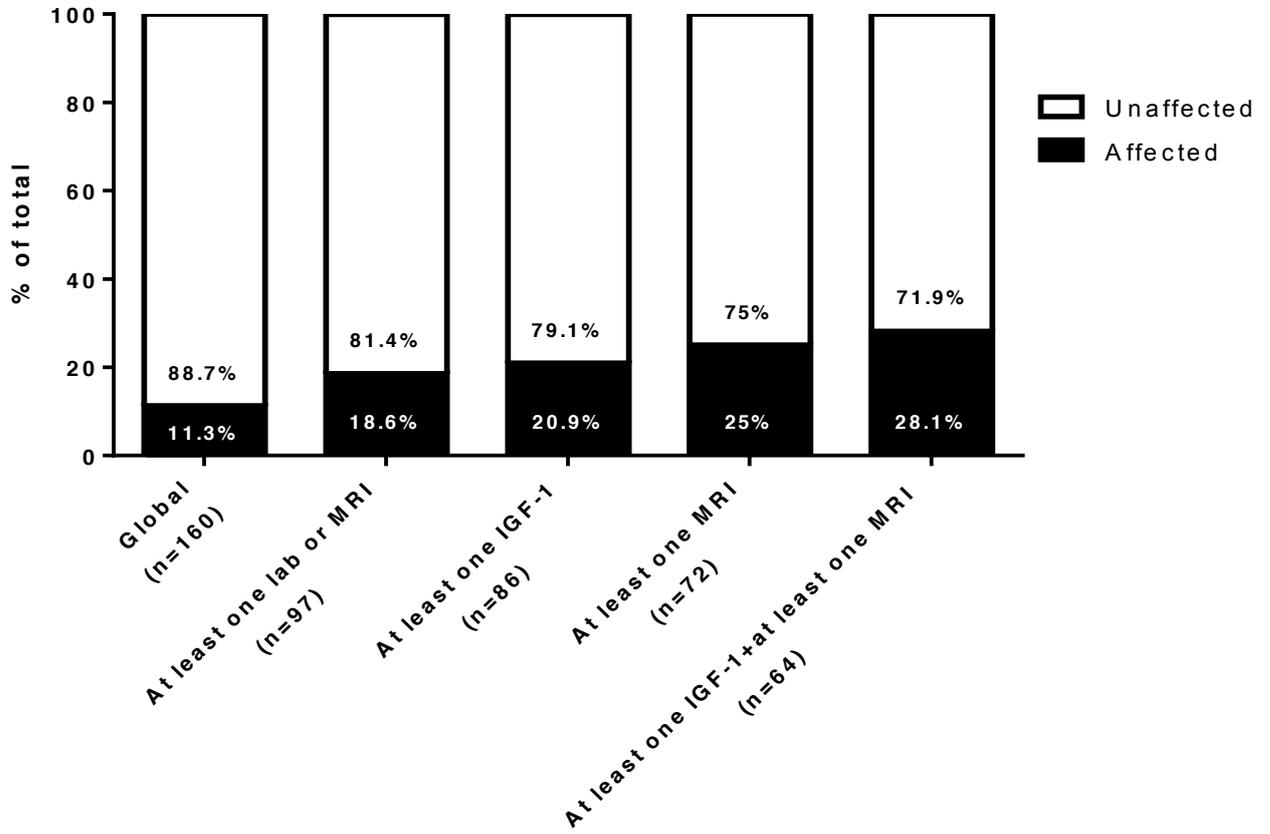
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10 **Figure 2**

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Study population

At recruitment, relevant clinical and biochemical data were collected at each participating center using a standard datasheet designed for this study (available on request) and all the information was entered into our central database. Data about the follow-up, treatments and current status of the patients were prospectively requested and collected from the collaborating centers and directly from the patients. Data about the historical cases were collected from family members and from hospital archives, when available. With a few exceptions, genetic screening results were directly sent to our center and entered in the database. The available data did not allow a comprehensive analysis of the response to specific therapeutic modalities.

We identified subjects 'at risk' (those with the possibility of inheriting an *AIP*mut), 'obligate carriers' (based on their position in family tree, *AIP*muts were verified when possible) and 'unaffected carriers'. Therefore, in our analysis the term 'unaffected carrier' includes all the relatives of *AIP*mut-positive patients without clinical manifestations of a pituitary adenoma and with either a genetic screening positive for the *AIP*mut present in the proband or with a position in the family tree defining them as 'obligate carriers'. Additionally, the analysis of the family trees led to the identification of some affected individuals as 'predicted *AIP*mut-positive patients', defined as individuals with an established clinical diagnosis of pituitary adenoma in whom the genetic screening could not be carried out due to unavailability of a DNA sample, but in whom the presence of the mutation was assumed based on both the phenotype and the position in the family tree. Therefore, the term '*AIP*mut-positive patient' will refer to both 'predicted *AIP*mut-positive patients' and '*AIP*mut-positive patients' in whom the presence of the mutation was verified. Subjects 'not at risk' of inheriting an *AIP*mut were defined based on their position in the family tree. In the sporadic cohort, the *AIP*mut-positive patients

with no apparent familial history of pituitary disease were also referred as 'simplex' cases as they can be considered the first case of a potentially hereditary disease.

Genetic and clinical screening

Pituitary adenoma patients and their apparently unaffected relatives were screened for *AIP*muts using Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA), as described in Supplemental Material. We have divided the *AIP* variants into five classes according to the likelihood of pathogenicity, as recommended by Plon *et al*. (SR1): definitely pathogenic, likely pathogenic, uncertain, unlikely pathogenic and not pathogenic. All the unaffected individuals with positive genetic screening for *AIP*muts were advised to undergo clinical, biochemical and image screening tests for the early diagnosis of possible pituitary disease, on an annual basis or as appropriate. The recommendations for screening were based on the published experience of our group (24) and others (26). Additional genetic tests were performed in subjects with no pituitary adenomas, but with other clinical features indicative of such tests (screening for mutations in *BRCA1* and *2* and *TP53* was performed in members of a family with breast cancer, osteosarcoma and a neuroendocrine tumor of the colon), as well as and in a randomly selected cohort of *AIP*mut-negative FIPA probands, searching for mutations in other genes via direct sequencing and MLPA (*BRCA1* and *2*, *CDKN1B*, *MEN1*, *TP53*, *PRKAR1A)* or via a next-generation sequencing panel *(MAX, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, TMEM127*, and *VHL*) (SR2).

Genomic DNA was obtained from blood (Ilustra DNA Extraction Kit BACC2, GE Healthcare, Little Chalfont, UK) or saliva (Oragene-DNA [collection] and prepIT-L2P [extraction] kits, DNA Genotek, Ontario, Canada) samples. The detection of the *AIP* gene variants and dosage was performed at the Molecular Genetics Laboratory, Royal Devon and Exeter, NHS Foundation Trust for the great majority of the samples, as previously described (3;12). Although the genetic tests were performed in one of the largest Genetics laboratories in the world, with appropriate quality controls, we cannot rule out that mutations were not identified in a small number of cases, due to either technical problems or due to location of mutations in areas not analyzed (such as intronic regions). The pathogenicity of the

detected variants was assessed using the Pathogenic Or Not-Pipeline (PON-P) and Alamut 2.2.1 *in silico* prediction programs, as well as considering the scientific literature concerning clinical and experimental data on the previously reported variants. Only those variants considered as definitely or likely pathogenic (SR1) were included in the study. Additionally, we included one novel intronic variant with no experimental data available, for which the prediction software could not exclude pathogenicity. The variants described in this paper are listed by their position in the DNA, with the corresponding change at the protein level in parentheses, according to the nomenclature guidelines of the Human Genome Variation Society (HGVS) version 1.0 (SR3) and the changes proposed for the version 2.0 (http://www.hgvs.org/mutnomen/). The nomenclature was verified using the Mutalyzer 2.0.beta-21 software (http://www.lovd.nl/mutalyzer/). The positions in the DNA are based on the GRCh37/hg19 assembly of the human genome and the human *AIP* reference sequence (Locus Reference Genomic code LRG_460 (SR4), based on NG_008969.1 and NM_003977.2). Array comparative genomic hybridization analysis was performed in a group of patients with gigantism, and patients positive for Xq26 microduplications (11) were excluded from further analysis.

Disease-modifying genes

Genomic DNA (gDNA) samples from 98 *AIP*mut-positive patients (55 males/43 females) and 108 unaffected *AIP*mut carriers (56 males/52 females) were subjected to PCR, using previously described primers (SR5) and screened for the *FGFR4* p.G388R (rs351855) single nucleotide polymorphism (SNP). Additionally, gDNA was extracted from paraffin-embedded somatotropinomas for 23 *AIP*mut-positive patients (familial and simplex), ten *AIP*mut-negative FIPA patients and six *AIP*mut-negative sporadic patients and cDNA was obtained from 19 frozen somatotropinomas from unselected acromegaly cases (control group, 13 males and six females, age at diagnosis 37-77 years). All these samples were screened for mutations in the *GNAS1* codons 201 and 227 using previously described primers for gDNA (SR6), and the primers 5'-CAAGCAGGCTGACTATGTGC-3' and 5'-ACCACGAAGATGATGGCAGT-3' for cDNA. The sequence analysis of the *FGFR4* and *GNAS1* PCR products was carried out by Sanger sequencing (BigDye Terminator v 3.1 kit in and ABI 3730 capillary sequencer, Applied Biosystems, Foster City, CA, USA).

SUPPLEMENTAL RESULTS AND DISCUSSION

Clinical and histopathological features

Gender distribution

Among the familial patients, there was a significantly different gender distribution of the affected individuals between the *AIP*mut-positive and negative subgroups (*P*=0.0015, Supplemental Figure 3a), showing a predominance of females in the *AIP*mut-negative families. This difference is unlikely to be due to a selection bias, as the gender distribution was not significantly different between affected and unaffected individuals in the whole study population (*P*=0.8581), or, in the familial cohort, between unaffected *AIP*mut-positive and negative individuals (*P*=0.4421, Supplemental Figure 3b), or between *AIP*mut-positive affected and unaffected individuals (*P*=0.1367). We did not see a difference in gender distribution between the *AIP*mut-positive and negative sporadic patients either (*P*=0.1605, Supplemental Figure 3c).

Age

Familial patients

FIPA *AIP*mut-positive patients were younger at disease onset (Supplemental Figure 4a) compared with *AIP*mut-negative FIPA patients. In the *AIP*mut-positive subgroup, the earliest age at onset was three years, while in the *AIP*mut-negative families a female patient with Cushing's disease had the earliest disease onset at seven years. Most of the *AIP*mut-positive FIPA patients (71.7% [71/99]) developed their pituitary adenomas during the second and third decades of life (10-29 years), whereas only 39.2% (121/309) of the *AIP*mut-negative FIPA patients had their first signs/symptoms of pituitary adenoma during the same stage of life (*P*<0.0001, Supplemental Figure 4a and b). The age at diagnosis was also significantly different (*P*<0.0001): 68.2% (75/110) of the *AIP*mut-positive FIPA patients were diagnosed at ≤30 years of age, whereas the diagnosis was established in only 36.7% (116/316) of the *AIP*mut-negative patients by that age. The earlier disease presentation was also reflected in a much higher frequency of pediatric cases (disease onset at ≤18 years of age,

Supplemental Figure 4c) in the *AIP*mut-positive FIPA families, compared with the *AIP*mut-negative FIPA families (44.1 vs. 11.8%, *P*<0.0001). These distributions were calculated taking into consideration the prospectively diagnosed *AIP*mut-positive patients; however, the statistical analysis results were not significantly different when those patients were excluded.

Sporadic patients

Even though our sporadic cohort included only young-onset pituitary adenoma patients, a significant younger age at onset was still found within this young group in the *AIP*mut-positive simplex patients in comparison with the *AIP*mut-negative ones (median 16 [IQR 14.8-22.3] vs. 22 [IQR 16-26] years, *P*=0.0054, Supplemental Figure 4d), and there was a higher proportion of pediatric cases within the *AIP*mut-positive subgroup (58.8% vs. 35.9%, *P*=0.0085). Nevertheless, while the youngest age at onset in the *AIP*mut-positive simplex patients was nine years, 3% (11/369) of the *AIP*mut-negative patients had disease onset before the nine years of age, with a minimum age of three years.

Clinical diagnoses

GH excess patients accounted for 57.8% (524/906) of the total affected individuals in the entire cohort: 46.6% (234/502) of the familial and 71.8% (290/404) of the sporadic cases. Patients with GH excess, prolactinomas and NFPAs were present in both *AIP*mut-positive and negative subgroups, but Cushing's disease, functioning gonadotropinomas and TSHomas were not found in patients bearing *AIP*muts.

Familial patients

We classified the FIPA families as 'homogeneous', when all the affected individuals within the family had the same diagnosis (GH excess was considered as a single category), or 'heterogeneous', when different diagnoses were found in the same family (17). Around one half of the families in our cohort were homogeneous FIPA families (families with only one pituitary adenoma type) in both the *AIP*mut-positive (48.6%) and negative (52.5%) subgroups (Supplemental Table 4). The most common family type in both subgroups (according to the diagnostic categories found in the affected members)

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was the pure GH excess family, but it was significantly more frequent within the *AIP*mut-positive FIPA families (*P*=0.0249). The most common diagnoses in *AIP*mut-positive and negative families were the different categories of GH excess; nevertheless, these cases were significantly more frequent in the *AIP*mut-positive subgroup, with at least one case of GH excess in 91.9% (34/37) of the *AIP*mut-positive and in 53.1% (95/179) of the *AIP*mut-negative FIPA families (*P*<0.0001, Supplemental Figure 4e). There was a higher frequency of PRL co-secretion among the *AIP*mut-positive patients with acromegaly or gigantism, compared with the *AIP*mut-negative ones (*P*=0.0158, Supplemental Figure 4f). In the *AIP*mut-negative FIPA patients the most frequent diagnosis was acromegaly, in 35.3% (137/389) of the patients, with prolactinoma in the second place of frequency (30.9%, 120/389). In sharp contrast to *AIP*mut-positive families, where 31% (35/113) of the patients had gigantism, only 2.1% (8/389) of the *AIP*mut-negative FIPA patients had this diagnosis.

Sporadic patients

In the sporadic cohort, all the *AIP*mut-positive simplex patients harbored GH-secreting adenomas (vs. 69.2% of the *AIP*mut-negative cases), as proven by the clinical diagnosis and immunohistochemistry (IHC) report. The predominance of GH excess cases in both groups could be due to a selection bias, as the previously reported association between *AIP*muts and acromegaly/gigantism could have influenced the referral of these patients for the study.

Histopathology

Familial patients

The IHC analysis of the operated pituitary adenomas confirmed the clinical/biochemical picture in the vast majority of the cases, reporting a predominance of somatotropinomas and mammosomatotroph adenomas in FIPA patients, more evident in the *AIP*mut-positive subgroup (*P*= 0.0304, Supplemental Figure 5a and b). There was a unique case of a double adenoma (one tumor positive for GH and another one for PRL) and one unusual case of somatotroph hyperplasia in a patient with gigantism within the *AIP*mut-positive patients. None of the few *AIP*mut-positive clinically NFPA cases were

gonadotroph or null cell adenomas. In contrast, in the *AIP*mut-negative FIPA families, 48.3% of the NFPAs analyzed were reported as gonadotropinomas and 31% were null cell adenomas (based on negative immunostaining for GH, ACTH, PRL, TSH, LH and FSH). There was a similar prevalence of plurihormonal tumors in both subgroups (17.4% in the *AIP*mut-positive and 10.5% in the *AIP*mut-negative families, *P*=0.2763). Seventy five percent of all the plurihormonal tumors in both subgroups had positive GH staining. There was a significant difference among the *AIP*mut-positive and negative FIPA patients involving the granulation pattern in GH positive adenomas. All the *AIP*mut-positive FIPA patients for whom this parameter was available (22/22) had sparsely granulated adenomas, while 43.8% (7/16) of the *AIP*mut-negative patients harbored densely granulated adenomas (*P*<0.0001, Supplemental Figure 5c); this difference could correspond to the response to the treatment with SSA, as suggested by previous reports (SR7). We found no difference in the proportion of patients with Ki-67 index ≥3% between the two subgroups (global 28.1%, *P*=1.0000).

The presence of two different types of pituitary adenomas in the same gland is infrequent (2.3% of all the cases and 3.3% of the cases of Cushing's disease) (SR8). Multiple pituitary adenomas have been previously described in a few cases of MEN1 and FIPA (not screened for *AIP*muts) patients (SR9-13). Although somatotroph hyperplasia has been described before in the setting of *AIP*muts (10;SR14), this finding does not seem to be particularly frequent, as in our cohort it was found only in one patient with acromegaly and PRL co-secretion.

There was a marked predominance of sparsely granulated GH-secreting adenomas among the *AIP*mut-positive patients, compared with the *AIP*mut-negative ones. Patients with sparsely granulated tumors are usually younger at diagnosis than those with a densely granulated pattern (SR15;SR16), have increased invasiveness (SR7;SR15-17) and reduced response to the treatment with SSA (SR7;SR17), though the strength of these associations has been variable among different studies. The mechanism proposed for this effect in sporadic adenomas implies a reduced expression of the somatostatin receptor subtype 2 (SSTR2) (SR18;SR19). Since the expression of the SSTR2 and other somatostatin receptor subtypes is not reduced in somatotropinomas from *AIP*mut-positive patients,

other molecular mechanisms must be involved in the association of these mutations with decreased responsiveness to SSAs and a sparsely granulated pattern, such as ZAC1 activation (SR20;SR21) or an impaired inhibitory G protein subunit function in these tumors (SR22).

Sporadic patients

All the *AIP*mut-positive patients with available histopathology results (n=14) had GH positive pituitary adenomas by IHC, 28.6% of them (n=4) were mammosomatotroph adenomas (Supplemental Figure 5d). In contrast, the *AIP*mut-negative subgroup (n=89) included corticotropinomas (7.9%), null cell adenomas (3.4%), plurihormonal tumors (13.5%), prolactinomas (12.4%), somatotropinomas (32.6%), mammosomatotroph adenomas (29.2%), as well as a TSHoma (1.1%, Supplemental Figure 5e). In the *AIP*mut-positive subgroup, one third (2/6) of the somatotroph adenomas with available cytokeratin staining had a densely granulated pattern and the rest were sparsely granulated. The distribution was similar in the *AIP*mut-negative subgroup, where 31.6% of the GH adenomas presented a densely granulated pattern (6/19) and 68.4% were sparsely granulated. Additionally, one *AIP*mut-negative patient had a somatotropinoma with a mixed granulation pattern.

Pituitary adenoma size and extension

Familial patients

We compared size and extension of pituitary adenomas between *AIP*mut-positive and negative FIPA patients (Supplemental Figure 6), and for this purpose, the prospectively diagnosed *AIP*mut-positive patients were excluded from the analysis. Despite macroadenomas being predominant in both FIPA patient groups, the *AIP*mut-positive FIPA patients had larger tumors, demonstrated by a larger maximum diameter (P=0.0404, Supplemental Figure 6a) and a higher prevalence of macroadenomas (P<0.0001, Supplemental Figure 6b). The proportion of giant (maximum diameter ≥40mm) adenomas (6.3% in *AIP*mut-positive and 3% in *AIP*mut-negative patients) was not significantly different (P=0.1766). There was a higher frequency of extrasellar extension in *AIP*mut-positive FIPA patients with pituitary adenomas (P=0.004, Supplemental Figure 6c). Three of the *AIP*mut-negative, but none of the *AIP*mut-positive patients, harbored tumors with extensive invasion (defined as involvement of

intracranial areas beyond the perisellar region); two of them had somatotropinomas and the third one harbored a gonadotropinoma. None of the patients in our cohort had evidence of metastases to justify a diagnosis of pituitary carcinoma.

Sporadic patients

In the sporadic cohort, the maximum diameter of the tumors and the proportion of giant adenomas were similar between *AIP*mut-positive and negative sporadic cases (*P*=0.6965 and 0.7859, respectively). All the *AIP*mut-positive patients had macroadenomas (29/29) vs. 86.3% (283/328) of the *AIP*mut-negative subgroup, and the presence of extrasellar extension was more common in the former group (95% vs. 58.9%, *P*=0.0011).

Apoplexy of the pituitary adenoma

Excluding the prospectively diagnosed patients, symptomatic apoplexy of the pituitary adenoma occurred in 8.3% of the *AIP*mut-positive cases (9.1% of the familial cases, including three families with two cases per family, and 5.9% of the sporadic patients) and in only 1.3% of the patients in the *AIP*mut-negative subgroup (*P*<0.0001) and this difference remained significant when only the familial cases were analyzed (10.6% of the *AIP*mut-positive vs. 2.3% of the *AIP*mut-negative patients, *P*=0.0002, Supplemental Figure 6d). Eight (72.7%) of the *AIP*mut-positive patients with a history of pituitary apoplexy had a diagnosis of gigantism, and in three of them (27.2%) apoplexy was the manifestation that led to the diagnosis of pituitary disease (Supplemental Figure 6e). There were no significant differences in the age at onset/diagnosis or in the tumoral size between the *AIP*mut-positive patients that developed pituitary apoplexy and those who did not have this complication. Out of ten *AIP*mut-negative pituitary adenoma patients with a history of apoplexy, six had NFPA, two had acromegaly, one had gigantism and the specific diagnosis was unknown in the last patient.

The original description of multiple cases of pituitary adenoma apoplexy in *AIP*mut-positive patients (3) was later confirmed in other studies (4;12;25;SR14;SR23;SR24) as well as now in this larger cohort. Although the prevalence of 8.3% does not seem to be higher than the prevalence reported in

populations of unselected pituitary adenomas (7.9%) (SR25), in the latter study patients were older (mean age 60.5 years) and harbored NFPAs, while in our cohort the majority had gigantism and the rest, acromegaly or prolactinoma, with a mean age at diagnosis of 23.4 years. Our three familial apoplexy families, together with a recently reported family with three apoplexy cases (SR24) provide support for the phenotype of young-onset, familial apoplexy in *AIP*mut-positive patients. To our knowledge, there are no previously known genetic causes of familial pituitary adenoma apoplexy, and this remains an uncommon finding. The mechanism why *AIP*mut-positive cases are more prone to apoplexy needs further study.

GH excess patients

With the purpose of analyzing a relatively homogeneous population of patients, we compared the main clinical features of the *AIP*mut-positive and negative GH excess patients from both cohorts, excluding the prospectively diagnosed patients. Similar to the whole study population, the GH excess *AIP*mut-positive patients had an earlier disease onset and diagnosis, had significantly more apoplexy cases (8.4 vs. 1.2%, $P<0.0001$) and a higher frequency of sparsely granulated tumors (91.7 vs. 57.1%, $P=0.0073$). In the *AIP*mut-positive subgroup there is a preponderance of males (60.7% [65/107]), in contrast with the gender distribution found in patients with all the diagnostic categories. PRL co-secretion was more common in *AIP*mut-positive patients (14 vs. 5.9%, $P=0.0046$). There were no differences in tumor size, frequency of extrasellar extension, or giant tumors, though most of the tumors in both subgroups (89.5%) were macroadenomas. There was no significant difference in the number of therapeutic modalities employed between the two subgroups, but there were fewer patients cured or controlled in the *AIP*mut-negative subgroup (41/66 vs. 86/192, $P=0.0151$). Given that the *AIP*mut-positive patients had a significantly longer follow-up duration, we decided to evaluate the current status (i.e. effect of the therapies) only in patients with zero to five years of follow-up. In this subset of patients, there was no significant difference in the percentage of cured or controlled patients between the *AIP*mut-positive (57.1%) and the *AIP*mut-negative (41.7%) subgroups.

Gigantism

This study included 120 patients with gigantism, 45 of them, (37.5%) were part of FIPA families and 75 (62.5%) presented as sporadic patients. Overall, 46.7% (56/120) of the patients with gigantism were *AIP*mut-positive. Males were predominant among *AIP*mut-positive and negative patients (global 67.5%), as expected for gigantism cases. Childhood-onset GH excess resulting in gigantism was more prevalent among the *AIP*mut-positive patients (48.3% [56/116]) than GH excess with no pathological body height, while the opposite pattern was observed in the *AIP*mut-negative subgroup (only 16.7% [64/408] had gigantism, $P<0.0001$). Sixty percent of the *AIP*mut-positive families had at least one patient with gigantism. The frequency of *AIP*muts was much higher in the gigantism cases occurring in a familial setting (Supplemental Figure 7a), where 82.2% (37/45) of the patients were *AIP*mut-positive, in comparison with the sporadic cohort, where *AIP*mut-positive patients accounted for only 25.3% (19/75) of the patients ($P<0.0001$). Familial gigantism, defined as the occurrence of two or more gigantism cases due to pituitary adenoma in the same family, occurred only in *AIP*mut-positive FIPA families (9/37 families, 24.3%, Supplemental Figure 7b). Four of these families harbored the p.R304* *AIP*mut, and the *AIP*muts g.4856_4857CG>AA, p.Q164*, p.269_H275dup, p.E24* and a whole gene deletion accounted for one family each. *AIP*mut-positive gigantism patients were taller than their *AIP*mut-negative counterparts if we considered the criterion of height >3SD over percentile 50 but not when considering >2SD over midparental height (Supplemental Figure 7c and d).

There was no difference in the age at diagnosis (global median 18 [IQR 15-23]) between the *AIP*mut-positive and negative gigantism subgroups. Differences in the frequency of disease onset and diagnosis during the first decade of life did not reach statistical significance (onset: *AIP*mut-positive 9.1% vs. *AIP*mut-negative 9.5%; diagnosis: 3.6% vs. 1.6%). There were no significant differences in the parameters of tumor size and extension either (maximum diameter, frequency of giant adenomas and extrasellar invasion). However, it is worth noting that the vast majority of the tumors in both subgroups were macroadenomas (global 91.5%), and most of them displayed extrasellar invasion (77.6%). A small percentage of the patients had PRL co-secretion at diagnosis (9.2% global, not significantly different between *AIP*mut-positive and negative patients). There were no significant differences in the number of treatments received or the frequency of controlled patients between the

two subgroups. Overall, 43.2% of all the patients with gigantism have currently active or only partially controlled disease.

Extra-pituitary neoplasms in *AIP*mut-positive individuals

To explore the possibility of a syndromic presentation, we looked for additional neoplasms in the affected and unaffected *AIP*mut-positive individuals (n=290). We found a total of ten cases of eight different extra-pituitary neoplasms (osteosarcoma, breast cancer, neuroendocrine tumor of the colon, gastrointestinal stromal tumor [GIST], glioma, meningioma, non-Hodgkin's lymphoma and spinal ependymoma) in nine subjects (four patients and five unaffected *AIP*mut carriers, Supplemental Table 5), accounting for 3.1% of the *AIP*mut-positive individuals studied. *AIP*mut-positive GH excess patients accounted for 44.4% (4/9) of the individuals with extra-pituitary neoplasms, while the rest were unaffected *AIP*mut-positive carriers. We note that the association of these tumors with *AIP*muts could be coincidental.

An increased risk of malignancy among unselected pituitary adenoma patients has been previously reported (SR26;SR27). We have also found neoplasms within the *AIP*mut-positive individuals with no pituitary adenomas, where hormonal excess, especially GH, does not play a role. Further analyses are needed to establish whether there is a possible association between *AIP*muts and these neoplasms. Recently, germline *AIP*muts have been associated with three cases of parathyroid adenomas (two middle aged women in the setting of non-familial, isolated hyperparathyroidism and a young male with acromegaly) (SR28;SR29). An MEN-1 like phenotype was an exclusion criterion in our study, therefore, it was not possible to assess this novel pathogenic association, and none of our patients or carriers developed hyperparathyroidism during the follow-up.

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SUPPLEMENTAL FIGURES AND TABLES

Supplemental Table 1. Definition of the clinical diagnostic categories used in our study.

Diagnosis		Criteria
Cushing's disease		Evidence of ACTH-depending hypercortisolemia with proven pituitary adenoma, in accordance to the diagnostic protocol of each institution
Clinically functioning FSH-secreting pituitary adenoma (FSHoma)		Raised serum FSH levels for age and gender and evidence of gonadal stimulation in a patient with a pituitary adenoma
GH excess	Acromegaly	Raised IGF-1 levels and unsuppressed GH during an oral glucose tolerance test (OGTT), with cut-offs according to the protocol of each institution
	Acromegaly/prolactinoma	Diagnosis of acromegaly with concurrent hyperprolactinemia
	Mild acromegaly*	Mild clinical features attributed to acromegaly, fulfilling the criterion of raised IGF-1 levels but not the lack of suppression of GH during an OGTT, or normal IGF-1 but lack of suppression of GH during an OGTT (16)
	Gigantism	Any of the following categories in a patient with a pituitary adenoma: (i) abnormally high growth velocity in children or teenagers with abnormal IGF-1 and OGTT, (ii) height >3SD above the mean height for age, (iii) >2SD over the calculated midparental height, using country-specific growth charts when possible
	Gigantism/prolactinoma	Diagnosis of gigantism with concurrent hyperprolactinemia
Clinically nonfunctioning pituitary adenoma (NFPA)		Pituitary adenoma in the absence of clinical or biochemical evidence of pituitary hypersecretion
Pituitary tumor		Cases of pituitary tumor where the diagnosis could not be specified, due to unavailability of histopathological specimens, clinical and/or biochemical data
Prolactinoma		Hyperprolactinemia in the presence of a pituitary adenoma and unlikely to be purely due to a stalk effect, based on either histopathology results or the relation between PRL levels and tumor size
Thyrotropinoma (TSHoma)		Hyperthyrotropinemia in a patient with a pituitary adenoma, with clinical and/or biochemical hyperthyroidism and no other demonstrable causes of raised TSH
* This category is important in our study, as we detected acromegaly via biochemical screening of *AIP*mut-positive carriers, often not presented (yet) clinically.		

Supplemental Table 2. Other genes tested.

	Familial cohort			Sporadic cohort			Combined, no. (%)
	*AIP*mut-positive, no. (%)	*AIP*mut-negative, no. (%)	Total familial, no. (%)	*AIP*mut-positive, no. (%)	*AIP*mut-negative, no. (%)	Total sporadic, no. (%)	
BRCA1	1 (14.3)	2 (0.7)	3 (1)	-	-	-	3 (0.8)
BRCA2	1 (14.3)	2 (0.7)	3 (1)	-	-	-	3 (0.8)
CDKN1B	-	20 (6.5)	20 (6.4)	-	1 (2.4)	1 (2.4)	21 (5.9)
GPR101	-	-	-	-	8 (19)	8 (19)	8 (2.2)
MAX	-	23 (7.5)	23 (7.3)	-	-	-	23 (6.5)
MEN1	3 (42.9)	51 (16.6)	54 (17.2)	-	33 (78.6)	33 (78.6)	87 (24.4)
PRKAR1A	-	23 (7.5)	23 (7.3)	-	-	-	23 (6.5)
RET	-	23 (7.5)	23 (7.3)	-	-	-	23 (6.5)
SDHA	-	23 (7.5)	23 (7.3)	-	-	-	23 (6.5)
SDHAF2	-	23 (7.5)	23 (7.3)	-	-	-	23 (6.5)
SDHB	-	23 (7.5)	23 (7.3)	-	-	-	23 (6.5)
SDHC	-	23 (7.5)	23 (7.3)	-	-	-	23 (6.5)
SDHD	-	25 (8.1)	25 (8)	-	-	-	25 (7)
TMEM127	-	23 (7.5)	23 (7.3)	-	-	-	23 (6.5)
TP53	2 (28.6)	-	2 (0.6)	-	-	-	2 (0.6)
VHL	-	23 (7.5)	23 (7.3)	-	-	-	23 (6.5)
Total	7	307	314	0	42	42	712

-, no individuals in this category.

Supplemental Table 3. *AIP* nonpathogenic mutations in the familial and sporadic cohorts.

Variant (DNA level [protein level])	Variant type	Pathogenic	Location in protein	Familial cohort[*] (N=19)	Sporadic cohort[*] (N=37)	Combined[*] (N=56)	References/ SR[‡]
c.47G>A (p.R16H)	Missense	No	N-terminus	0	2	2	(2;5;7)/ (SR31;39;54-58)
c.132C>T (p.(=))	Synonymous	No	PPIase domain	0	3	3	(5)/(SR59)
c.144C>T (p.(=))	Synonymous	No	PPIase domain	0	1	1	(SR53;59-61)
c.516C>T (p.(=))	Synonymous	No	Between PPIase and TPR1 domains	8	13	21	(5;12)/(SR56;58; 59;61-63)
c.573C>T (p.(=))	Synonymous	No	TPR1 domain	0	0	0	This paper
c.579G>T (p.(=))	Synonymous	No	TPR1 domain	1	0	1	This paper
c.682A>C (p.K228Q)[†]	Missense	No	Between TPR1 and 2 domains	2	16	18	(5)/(SR58;59;63)
c.831C>T (p.(=))	Synonymous	Unlikely	TPR3 domain	1	0	1	This paper
c.891C>A (p.(=))	Synonymous	No	TPR3 domain	0	2	2	(5)/(SR59)
C.896C>T (p.A299V)	Missense	Unlikely	TPR3 domain	5	0	5	(12)/(SR31)
c.906G>A (p.(=))	Synonymous	No	C-terminal α-helix	2	0	2	(SR31;59)

* Number of positive individuals for each mutation, considering the *AIP*mut-positive tested individuals, the obligate carriers and the predicted *AIP*mut patients.
†There is a Q at this position in the *AIP* reference sequence, but we consider K as the wild type amino acid, due to its higher prevalence in the population screened so far (Stals K., unpublished data).
PPIase, peptidylprolyl isomerase, TPR, tetratricopeptide repeat.

Supplemental Table 4. Classification of FIPA families by diagnoses

	*AIP*mut-positive	*AIP*mut-negative	Total
Total families, no.:	37	179	216
Diagnoses:			
Cushing's disease only, no. (%)	-	3 (1.7)	3 (1.4)
Cushing's disease + FSHoma, no. (%)	-	1 (0.6)	1 (0.5)
Cushing's disease + NFPA, no. (%)	-	1 (0.6)	1 (0.5)
Cushing's disease + NFPA + pituitary tumor, no. (%)	-	1 (0.6)	1 (0.5)
Cushing's disease + prolactinoma, no. (%)	-	5 (2.8)	5 (2.3)
FSHoma + prolactinoma, no. (%)	-	1 (0.6)	1 (0.5)
Cushing's disease+ GH excess, no. (%)	-	7 (3.9)	7 (3.2)
GH excess only, no. (%)	16 (43.2)	44 (24.6)	60 (27.8)
GH excess + NFPA, no. (%)	8 (21.6)	12 (6.7)	20 (9.3)
GH excess + NFPA + prolactinoma, no. (%)	1 (2.7%)	3 (1.7)	4 (1.9)
GH excess + pituitary tumor, no. (%)	-	5 (2.8)	5 (2.3)
GH excess + pituitary tumor + prolactinoma, no. (%)	-	1 (0.6)	1 (0.5)
GH excess + prolactinoma, no. (%)	9 (24.3)	30 (16.8)	39 (18.1)
NFPA only, no. (%)	2 (5.4)	17 (9.5)	19 (8.8)
NFPA + pituitary tumor, no. (%)	-	7 (3.9)	7 (3.2)
NFPA + prolactinoma, no. (%)	1 (2.7)	10 (5.6)	11 (5.1)
Pituitary tumor + prolactinoma, no. (%)	-	1 (0.6)	1 (0.5)
Prolactinoma, no. (%)	-	30 (16.8)	30 (13.9)

* The category "GH excess" includes the following diagnoses: acromegaly, acromegaly/ prolactinoma, gigantism, gigantism/ prolactinoma and mild acromegaly.
-, no families in this category.
FSHoma, FSH secreting adenoma. NFPA, nonfunctioning pituitary adenoma.

Supplemental Table 5. Extrapituitary neoplasms in *AIP*mut-positive individuals.

Pituitary diagnosis	Cohort	Gender	*AIP*mut	Extrapituitary neoplasm
Unaffected	Familial	Male	c.910C>T (p.R304*)	Osteosarcoma and neuroendocrine tumor of the colon †
Unaffected	Familial	Female	c.910C>T (p.R304*)	Breast cancer†
Unaffected	Familial	Female	c.910C>T (p.R304*)	Breast cancer†
Acromegaly	Familial	Male	c.805_825dup (p.F269_H275dup)	GIST
Acromegaly	Familial	Male	c.241C>T (p.R81*)	GIST*
Unaffected	Sporadic	Male	c.910C>T (p.R304*)	Glioma
Acromegaly	Familial	Female	c.241C>T (p.R81*)	Meningioma*
Gigantism	Familial	Male	c.74_81delins7 (p.L25Pfs*130)	Non-Hodgkin's lymphoma
Unaffected	Familial	Female	c.100-1025_279+357del (ex2del) (p.A34_K93del)	Spinal ependymoma
* Brother and sister. † Brother and 2 sisters. GIST, gastrointestinal stromal tumor.				

AIPmut types in the familial and sporadic cohorts

a
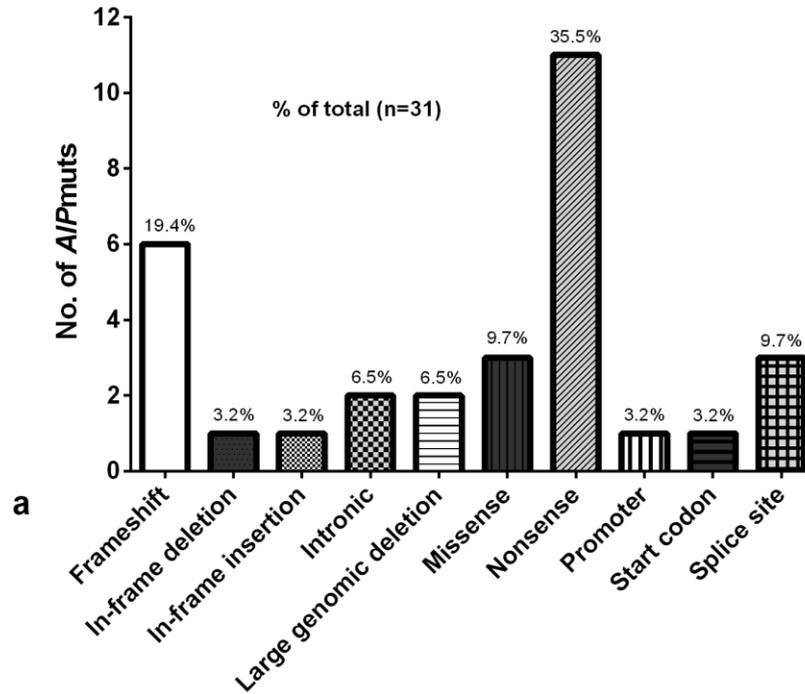
Positivity for AIPmut per age at onset

b
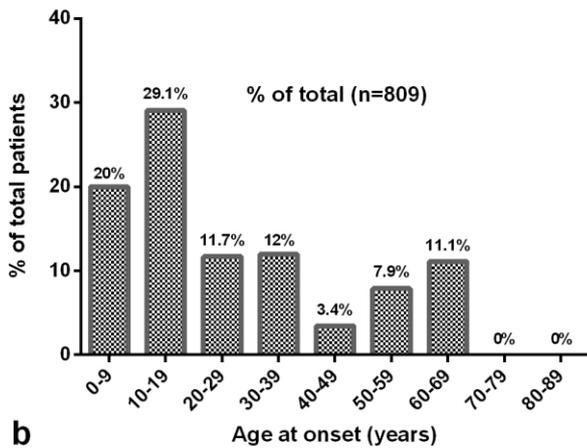
Distribution of AIPmut-positive patients per age at onset

c
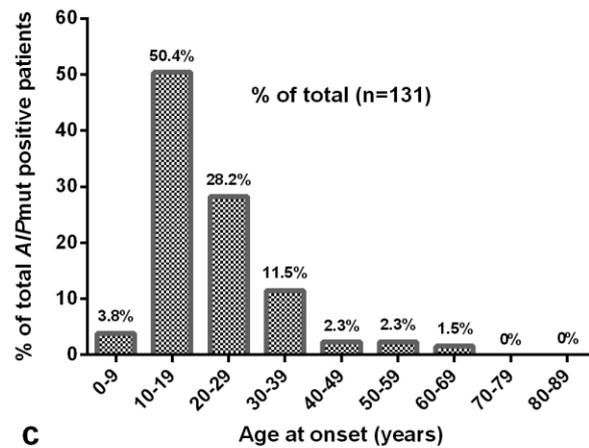
Supplemental Figure 1. *AIP*mut types and frequency according to age at disease onset in the familial and sporadic cohorts (whole study population). a) Number of *AIP*muts per mutation type, note the predominance of nonsense mutations. b) The probability of finding an *AIP*mut was higher when testing patients with disease onset during the second decade of life; c) in concordance, three quarters of all the *AIP*mut-positive patients had disease onset during the second and third decades of life.
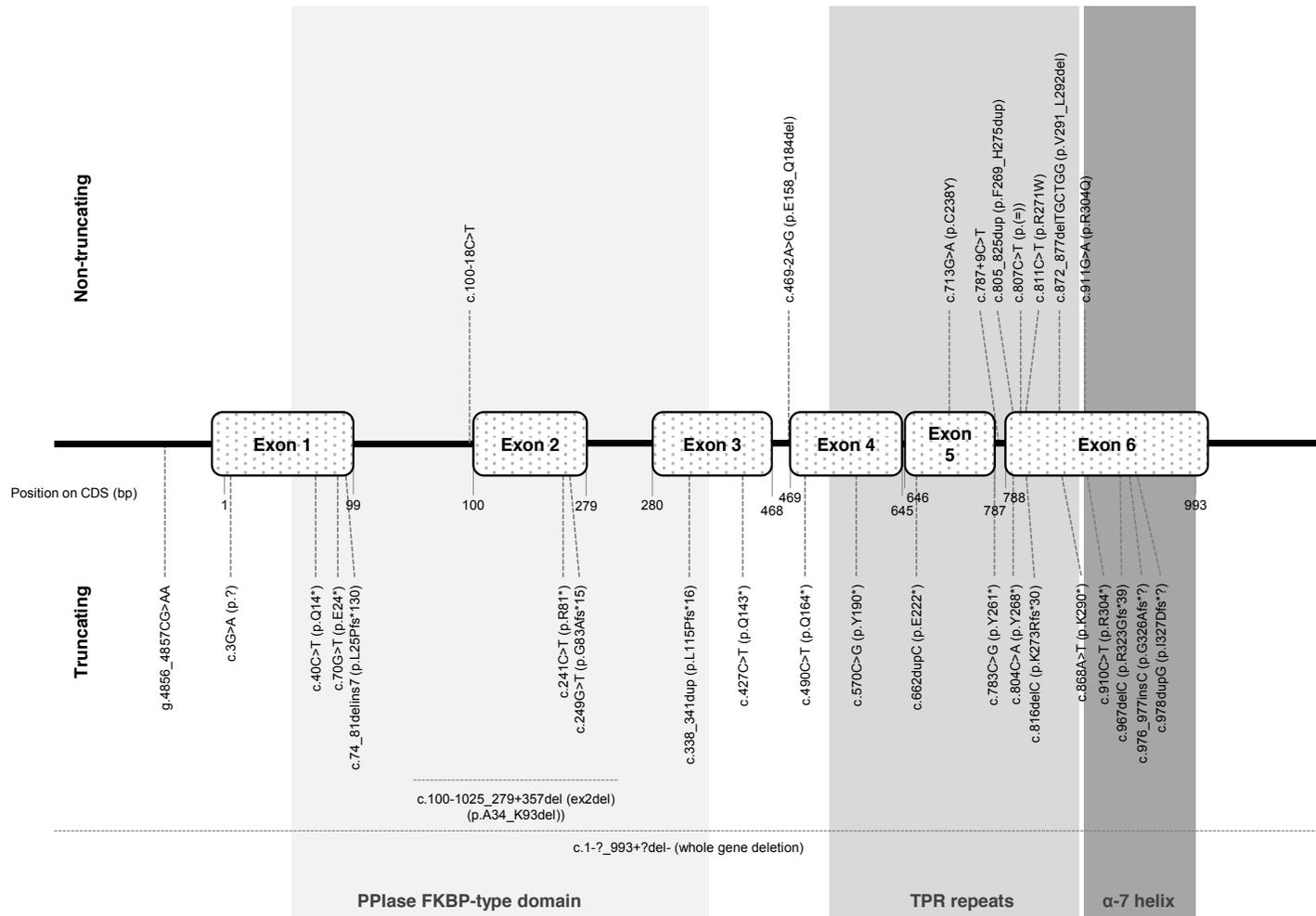
Supplemental Figure 2. *AIP*muts detected in the study population and their position in the *AIP* gene. Shadowed areas indicate the protein domains codified by each region of the gene. Mutations producing a truncated or missing protein are shown at the bottom of the scheme, and nontruncating mutations are at the top. Even though we identified variants throughout the whole *AIP* gene, mutations tended to cluster in the genomic regions encoding the tetratricopeptide repeat (TPR) domains and the C-terminal α-helix of the protein. Furthermore, the mutations located at the N-terminal extreme and inside the peptidylprolyl isomerase (PPIase) domain were essentially truncating mutations, resulting in short and unstable proteins, lacking the TPR domains. As expected based on previous data (26;SR64), the commonest mutation in both cohorts was c.910C>T (p.R304*), found in 33.3% of the *AIP*mut patients and in 35.9% of all the *AIP*mut-positive individuals (affected plus unaffected carriers). There were no exclusive associations of specific *AIP*muts with particular diagnoses. However, 77.4% of all the mutations (24/31) were found in cases of gigantism (with or without prolactin (PRL) co-secretion), being this the diagnosis with the highest number of associated *AIP*muts. Furthermore, all the mutations were found in at least one patient with GH excess, supporting this diagnostic category as the most frequent *AIP*mut pathogenic association. Patients with diagnosis of NFPA harbored 29% (9/31) of the *AIP*muts found in the study, and 22.6% of them (7/31) were detected in prolactinoma cases.
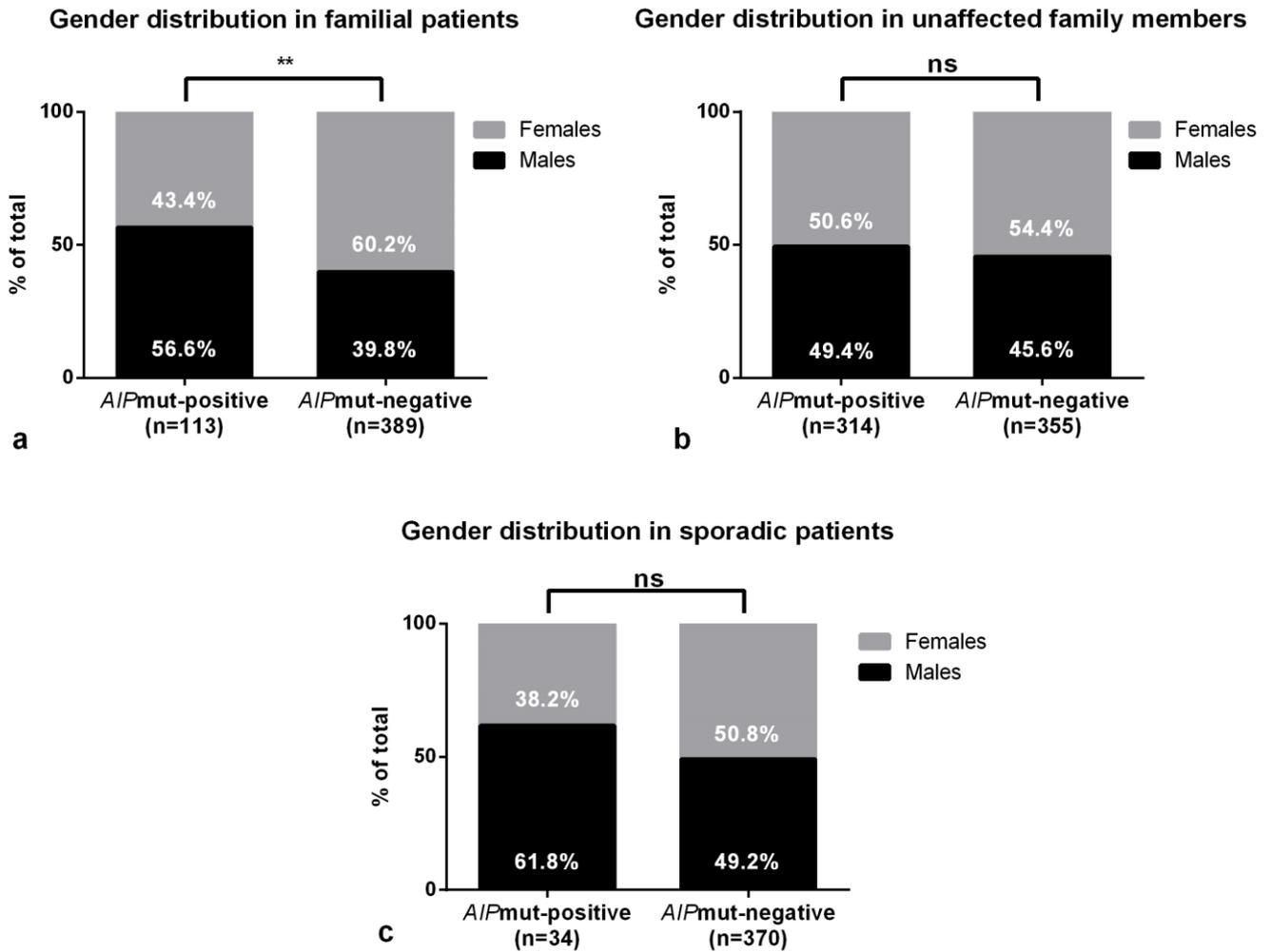
Supplemental Figure 3. Gender distribution in FIPA families and sporadic patients: a) Gender distribution was different between the *AIP*mut-positive and negative FIPA patients, due to a predominance of female patients within the *AIP*mut-negative families. b) This difference cannot be explained by a selection bias towards one specific gender, as there were similar numbers of males and females within the unaffected family members (excluding 'not at risk' individuals) of *AIP*mut-positive and negative FIPA families. c) The gender distribution was not significantly different between *AIP*mut-positive and negative patients, despite a slight prevalence of males in the *AIP*mut-positive subgroup. ns, not significant, **, *P*<0.01.
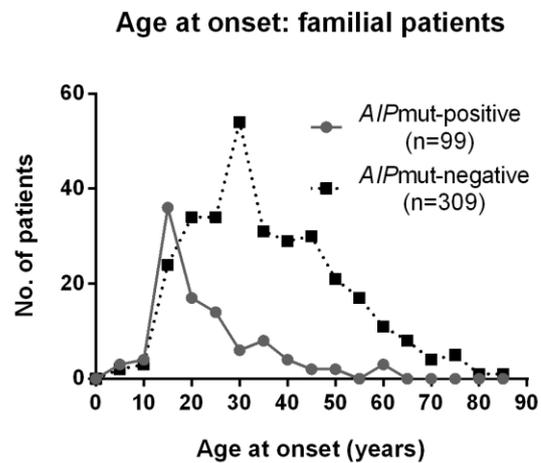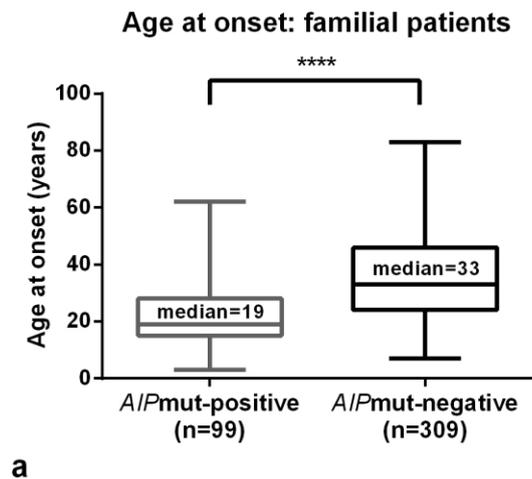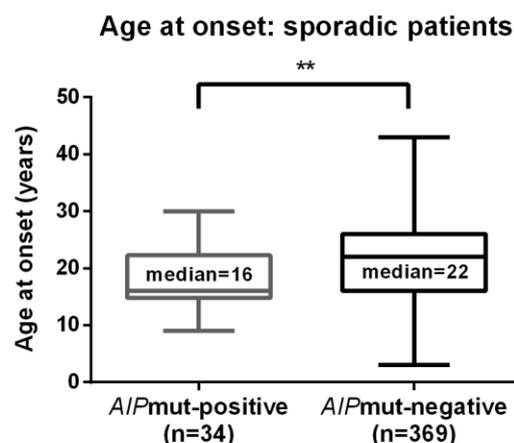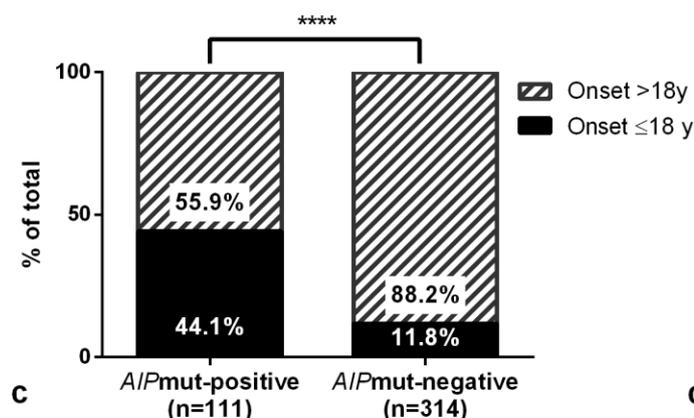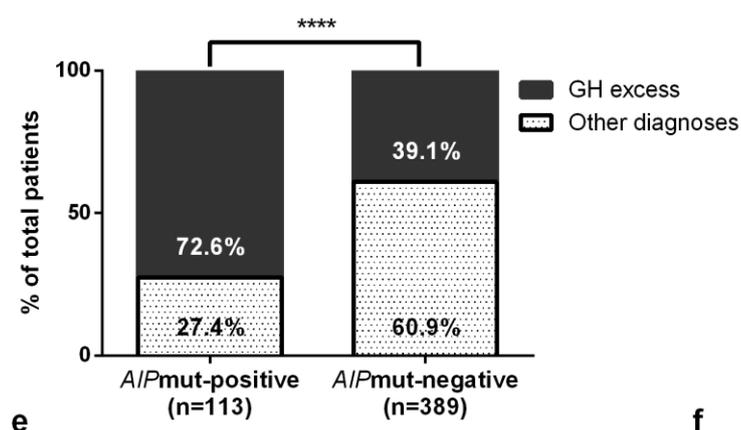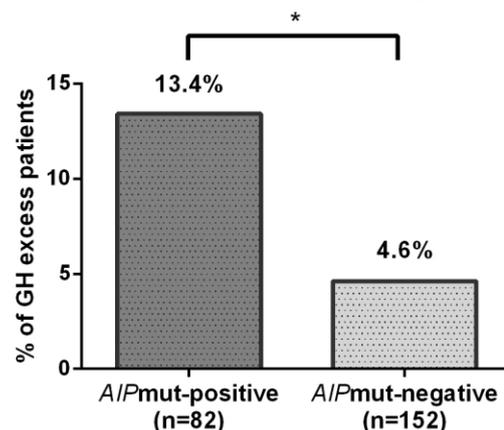
Supplemental Figure 4. Clinical features in FIPA families and sporadic patients: a) *AIP*mut-positive familial patients were younger at disease onset (*P*<0.0001), b) as most of them developed symptoms after the age of 10 and before the age of 40. c) There was a higher frequency of pediatric cases (n [total]=425) in the *AIP*mut-positive FIPA families, compared with the *AIP*mut-negative FIPA families. d) In the sporadic group, although all these patients were ≤30 years at disease onset, *AIP*mut-positive individuals were significantly younger at disease onset than the *AIP*mut-negative ones. e) GH excess and f) presence of GH and PRL co-secretion were significantly more frequent in *AIP*mut-positive familial patients. *, *P*<0.05, **, *P*<0.01, ****, *P*<0.0001.

Histopathology: *AIP*mut-positive families

2.2% Double GH&PRL
2.2% Fibrous tissue
4.4% Apoplexy
17.4% Plurihormonal
45.7% Somatotropinoma
23.9% GH/PRLoma
2.2% PRLoma
2.2% GH hyperplasia

IHC plurihormonal	No.
GH/LH/FSH	1
GH/PRL/ACTH/FSH	1
GH/PRL/α-SU	2
GH, PRL and LH	1
GH/PRL/LH/FSH	1
PRL/TSH/ACTH/LH/FSH	1
PRL/TSH/LH/FSH	1

a

n=46

Histopathology: *AIP*mut-negative families

1.3% Spindle cell oncocytoma
10.5% ACTHoma
11.8% Null cell
38.2% Somatotropinoma
10.5% Plurihormonal
23.7% GH/PRLoma
4% PRLoma

IHC plurihormonal	No.
ACTH/α-SU	1
GH/PRL/ACTH	1
GH/PRL/ACTH/LH/FSH	1
GH/PRL/FSH	1
GH/PRL/TSH	1
GH/PRL/TSH/LH/FSH	1
GH/TSH	1
PRL/TSH/ACTH/LH/FSH	1

b

n=76

Granulation pattern in FIPA

****

% of GH-positive tumours

■ Densely
▨ Sparsely

56.2%
100%
43.8%

*AIP*mut-positive (n=21)
*AIP*mut-negative (n=16)

c

Histopathology: *AIP*mut-positive simplex patients

28.57% GH/PRLoma
71.4% Somatotropinoma

d

n=14

Histopathology: *AIP*mut-negative sporadic patients

1.1% TSHoma
7.9% Corticotropinoma
3.4% Null cell
32.6% Somatotropinoma
13.5% Plurihormonal
12.4% PRLoma
29.2% GH/PRLoma

IHC plurihormonal	No.
ACTH/PRL	1
GH/ACTH positive	2
GH/LH positive	1
GH/PRL/ACTH	1
GH/PRL/ACTH/LH/FSH	1
GH/PRL/LH/FSH	2
GH/PRL/TSH	1
GH/TSH	1
GH/TSH/ACTH/LH	1
TSH/FSH	1

e

n=89

Supplemental Figure 5. Histopathological diagnoses in FIPA families and sporadic patients. The distribution of the IHC diagnoses was different between *AIP*mut-positive (a) and negative (b) familial patients, though GH positive tumors predominated in both subgroups. c) The analysis of the granulation pattern reported sparsely granulated tumors in all the *AIP*mut-positive and in 43.8% of the *AIP*mut-negative familial adenomas (*P*<0.0001). d) *AIP*mut simplex patients had GH positive adenomas (with or without positive PRL staining), while e) the *AIP*mut-negative sporadic patients had a variety of other tumor types. PRLoma, prolactinoma; GH/PRLoma, mammosomatotroph adenoma; ns, not significant; ****, *P*<0.0001.
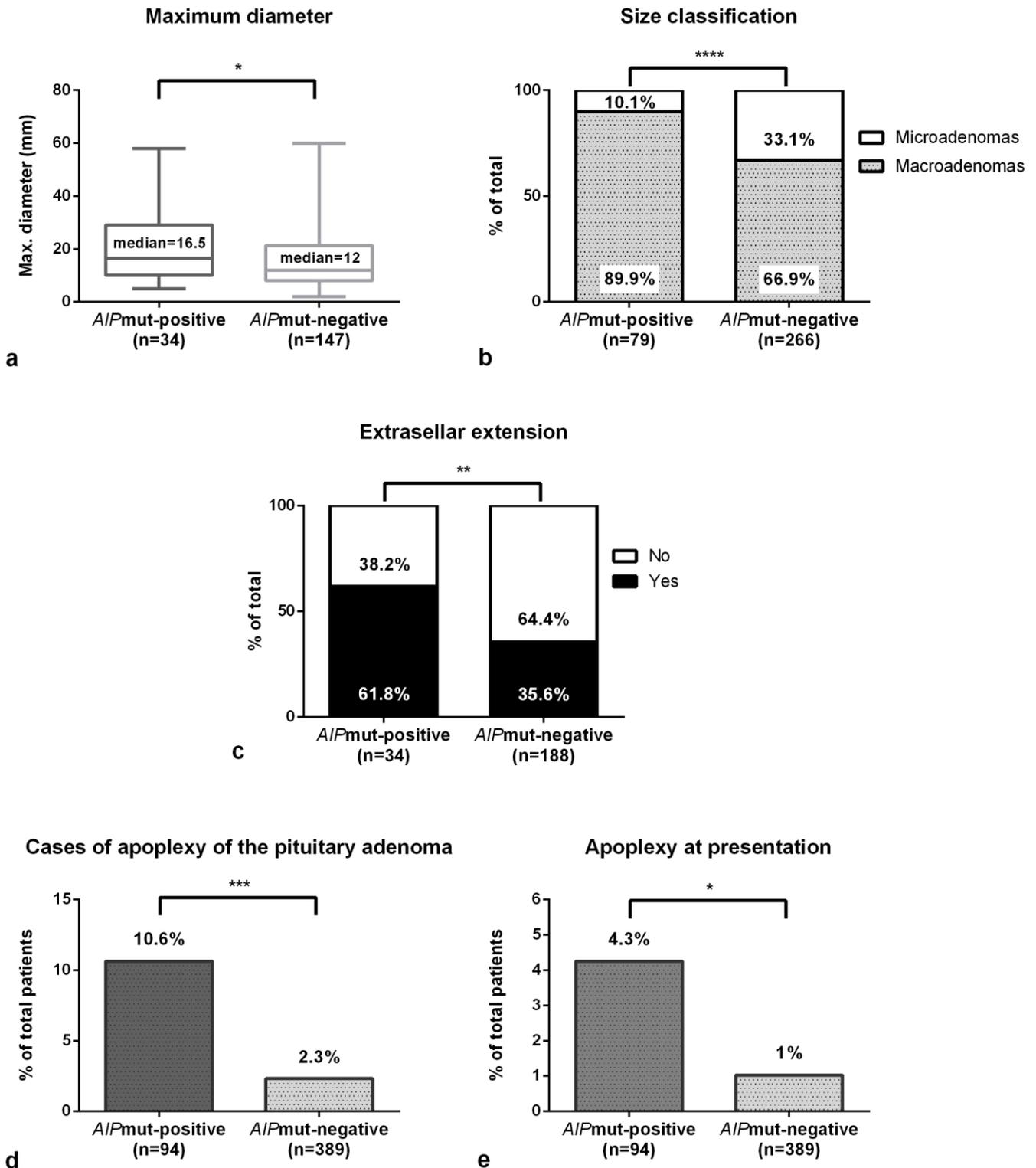
Supplemental Figure 6. Tumor size and and pituitary apoplexy in FIPA families (excluding prospectively diagnosed _AIP_mut-positive patients): _AIP_mut-positive vs. _AIP_mut-negative patients. a) Pituitary adenomas were larger in _AIP_mut-positive familial patients (P=0.040), b) what was reflected in a higher frequency of macroadenomas (P=0.0001). c) In concordance with this, there was a higher frequency of extrasellar extension within _AIP_mut-positive patients (P=0.004). d) The occurrence of symptomatic apoplexy of the pituitary adenoma was significantly more common among the _AIP_mut-positive families, occurring in 10.6% of these patients (vs. 2.3% of the _AIP_mut-negative FIPA patients, (P=0.0002), including one phenocopy NFPA patient. e) Apoplexy was the first sign of pituitary disease in 4.3% of the _AIP_mut-positive familial patients, but only in 1% of the _AIP_mut-negative ones. * $P<0.05$, **, $P<0.01$, ***, $P<0.001$, ****, $P<0.0001$.
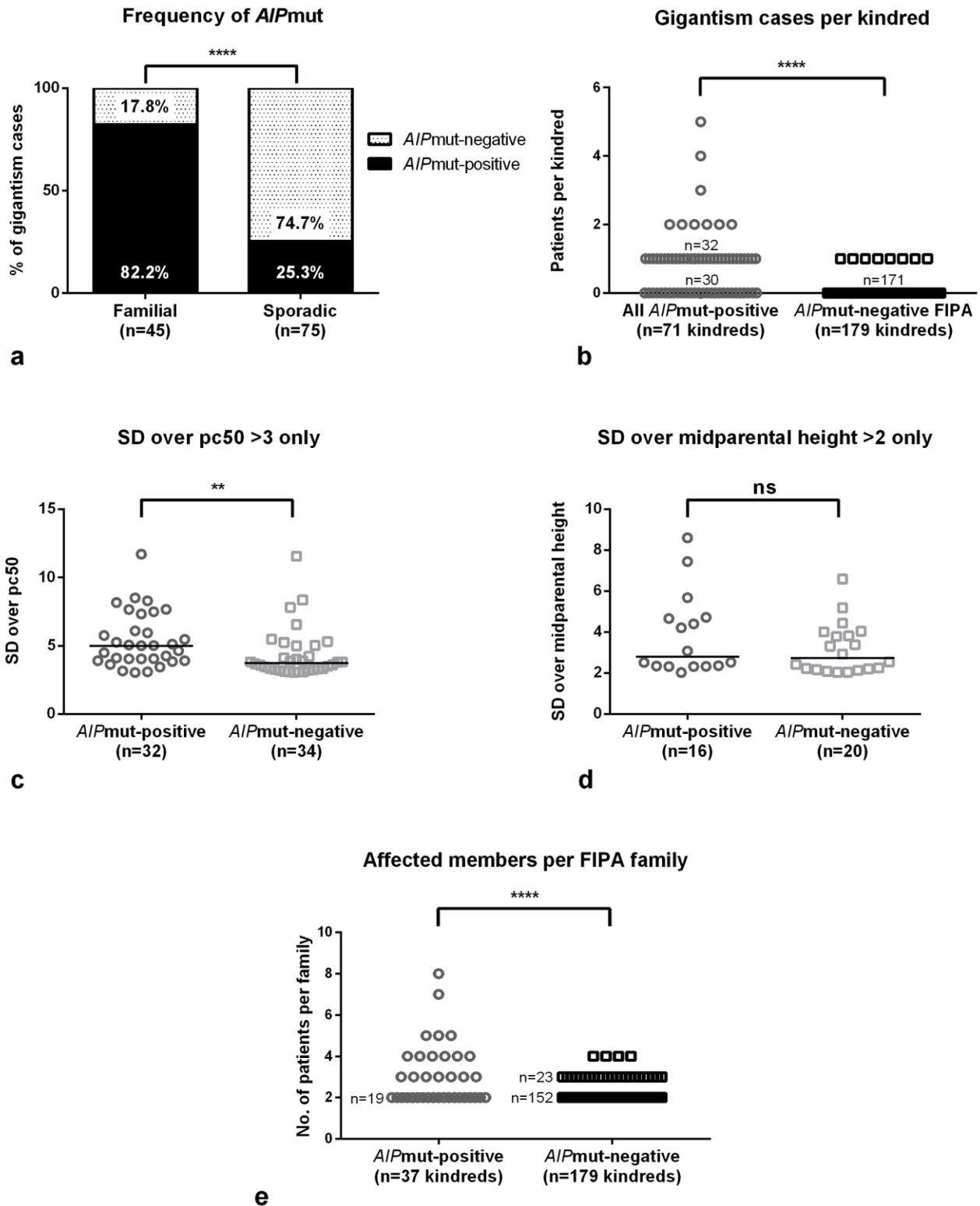
Supplemental Figure 7. Characteristics of gigantism cases (familial n=45, sporadic n=75) and penetrance. a) The great majority of the gigantism cases occurring in a familial setting were *AIP*mut-positive vs. only one quarter of those cases presenting sporadically (*P*<0.0001). b) In our study population, all the kindreds including more than one case of gigantism carried *AIP*muts (this graph includes all the *AIP*mut-positive kindreds, FIPA and simplex patients, and the *AIP*mut-negative FIPA families). c) Considering only those patients fulfilling the criterion of height >3SD over percentile 50, *AIP*mut-positive patients were taller at diagnosis than the *AIP*mut-negative ones (*P*=0.0164); however, d) there was no significant difference in height when the comparison was done among patients fulfilling the criterion of >2SD over midparental height. e) In average, there were more affected individuals per family in the *AIP*mut-positive families (*P*<0.0001). ns, not significant, * *P*<0.05, ** *P*<0.01, ****, *P*<0.0001.