Germinal defects of $SDHx$ genes in patients with isolated pituitary adenoma

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Abstract

**Background:** The ‘3PAs’ syndrome, associating pituitary adenoma (PA) and pheochromocytoma/paraganglioma (PPGL), is sometimes associated with mutations in PPGL-predisposing genes, such as $SDHx$ or $MAX$. In ‘3PAs’ syndrome, PAs can occur before PPGL, suggesting a new gateway into $SDHx/MAX$-related diseases.

**Objective:** To determine the $SDHx/MAX$ mutation prevalence in patients with isolated PAs and characterize PAs of patients with $SDHx/MAX$ mutations.

**Design:** Genes involved in PAs ($AIP$/MEN1/$CDKN1B$) or PPGLs ($SDHx/MAX$) were sequenced in patients with isolated PAs. We then conducted a review of cases of PA in the setting of ‘3PAs’ syndrome.

**Results:** A total of 263 patients were recruited. Seven (likely) pathogenic variants were found in $AIP$, two in MEN1, two in $SDHA$, and one in $SDHC$. The prevalence of $SDHx$ mutations reached 1.1% (3/263). Of 31 reported patients with PAs harboring $SDHx/MAX$ mutations (28 published cases and 3 cases reported here), 6/31 (19%) developed PA before PPGL and 8/31 (26%) had isolated PA. The age of onset was later than in patients with $AIP$/MEN1 mutations. PAs were mainly macroprolactinomas and showed intracytoplasmic vacuoles seen on histopathology.

**Conclusions:** We discovered $SDHx$ mutations in patients bearing PA who had no familial or personal history of PPGL. However, the question of incidental association remains unresolved and data to determine the benefit of $SDHx/MAX$ genetic testing in these patients are lacking. We recommend that patients with isolated PA should be carefully examined for a family history of PPGLs. A family history of PPGL, as well as the presence of intracytoplasmic vacuoles in PA, requires $SDHx/MAX$ genetic testing of patients.

Introduction

Although pituitary adenomas (PAs) are benign tumors, they can be responsible for clinical features due to hormonal disturbances and symptoms of compression that are secondary to local invasion and that may lead to hypopituitarism. The reported prevalence of symptomatic PAs is as high as 1 in 1000 (1). PAs are most frequently sporadic diseases, but are inherited in approximately 5% of cases. In these cases, PAs may be isolated, as in familial isolated pituitary adenomas (FIPAs) due to $AIP$ mutation (AIP; OMIM 605555), or occur in a syndromic association, such as (1) multiple endocrine neoplasia 1 (MEN1; OMIM 131100), which predisposes patients mainly to primary hyperparathyroidism, endocrine duodeno-pancreatic tumors, and PA; and more rarely, (2) multiple endocrine...
neoplasia 4 (MEN4; OMIM 610755), which represents a MEN1-like syndrome; or (3) Carney complex (CNC; OMIM 160980) which has cutaneous manifestations, acromegaly, Cushing syndrome, myxoma and schwannoma (2). In syndromic forms or familial cases, patients may benefit from genetic screening to propose specific monitoring and genetic counseling.

Pheochromocytomas are neuroendocrine tumors arising from adrenal medulla cells. Paragangliomas originate from sympathetic or parasympathetic ganglia. The release of catecholamines by pheochromocytoma/paraganglioma (PPGL) can lead to episodes of hypertension, profuse sweating, headaches, panic attacks, arrhythmia, stroke, and death. The standard treatment is resection and management of PPGL requires highly specialized teams. Around 40% of PPGL are genetically determined (3). Among the 20 or so predisposing genes that have been identified, some lead to a syndromic form of the disease (VHL, NF1, RET, FH), while some lead to isolated forms (including SDHD, SDHC, SDHB, SDHA, SDHAF2, MAX, SLC25A11, TMEM127). Germinal mutations are found in SDHx genes (SDHD, SDHC, SDHB, SDHA and SDHAF2), encoding for the SDH subunits in half of the hereditary cases.

The association between PA and PPGL was first described by Iversen in 1952 (4). This association can occur during MEN1 syndrome or independently (5, 6, 7, 8, 9). This condition, called ‘3PAs’ syndrome (for pituitary adenoma/pheochromocytoma/paraganglioma association) by Xekouki, can be described as the co-occurrence of PA and PPGL without other features of MEN1 syndrome (5, 6, 10). This association is rare, with fewer than 100 cases published in 2019. The ‘3PAs’ syndrome may be associated with germline mutations in genes responsible for predisposition to non-syndromic PPGL, such as genes encoding for SDH subunits or MAX (6, 7, 8, 9).

The objective of this study was to assess the involvement of the main non-syndromic PPGL-predisposing genes in patients with isolated PAs and to study the PA characteristics of patients with SDHx/MAX mutations. To this end (1), we determined the prevalence of germline mutations in MEN1, CDKN1B, and AIP and in SDHA, SDHB, SDHC, SDHD, SDHAF2 (herein called SDHx genes) and MAX in a large series of patients for which genetic testing was performed for sporadic or familial isolated PA (2). We reviewed the literature for published cases of PA in the setting of ‘3PAs’ syndrome to determine if patients with PA and genetic mutations in PPGL-predisposing genes show phenotypic differences compared to patients with AIP, MEN1, PRKAR1A, or CDKN1B mutation and those with non-genetically determined PAs.

Patients and methods

Subjects

All patients who underwent genetic testing in the context of an isolated PA, without other endocrine lesions, at the molecular laboratory of Marseille Conception Hospital between November 2016 and December 2018 were included. Written informed consent from all patients for genetic analysis was obtained during one-on-one genetic counseling. This study was approved by the ethics committee of Aix-Marseille University (approval number: 2018-13-12-004).

Next-generation sequencing (NGS)

Genomic DNA was extracted from blood lymphocytes (standard EDTA samples) using Qiagen DNA Midi Kit (Qiagen). Exons and 20 bp flanking introns of AIP (NM_003977.2), MEN1 (NM_130799.2), CDKN1B (NM_004064), SDHA (NM_004168.2), SDHB (NM_003002.2), SDHC (NM_003001.3), SDHD (NM_003002.2), SDHAF2 (NM_017841.1) and MAX (NM_002382.3) were sequenced by next-generation sequencing (NGS) using the QiSeq library (Qiagen) according to the manufacturer’s instructions. This library included unique molecular indices that are added to each DNA fragment before amplification, in order to reduce the sequencing error rates and to suppress PCR duplicates. Libraries were sequenced on MiSeqDX (Illumina). The alignment and variant calling were performed using the Biomedical Genomics Workbench 5.0.1 (Qiagen) and based on human genome GRCh37/hg19. Annotation was done using VariantStudio v2.2 (Illumina), according to the HGVS guidelines (11). All regions were sequenced with coverage of depth superior to 30x after removing PCR duplicates.

We then performed copy number variation (CNV) analysis using the CNV detection tools from the Biomedical Genomics Workbench, based on the relative depth of coverage of specimen.

Each variant was classified according to the guidelines of the American College of Medical Genetics and Genomics (ACMG) into one of the five following classes (12):

Class 1: benign variant (BV)
Class 2: likely benign variant (LBV)
Class 3: variant of uncertain significance (VUS)
Class 4: likely pathogenic variant (LPV)
Class 5: pathogenic variant (PV)

In silico predictions were performed using Alamut Visual software (Interactive Biosoftware, Rouen, France), including the conservation level, SIFT, PolyPhen-2, and the study of the splicing impact. The population data from population database (gnomAD database, https://gnomad.broadinstitute.org/, last visited November 2019) and from inherited disease databases, ClinVar, LOVD, and HGMD, were collected. Variants with a frequency greater than 5% in the population were not retained. All PVs and LPVs were confirmed by Sanger sequencing (primers and protocols are available upon request).

Exploration of SDHx mutations in pituitary adenomas

To examine the role of the SDHx germinal mutation in PAs, both SDHB immunohistochemistry (IHC) and research of loss of heterozygosity (LOH) were performed on formalin-fixed paraffin-embedded (FFPE) slides obtained from surgically removed samples of the pituitary adenoma, where this was available.

SDHB IHC analysis

The investigation of the loss of SDH complex expression in neoplastic cells was performed using a commercially available polyclonal antibody against SDHB (Sigma Aldrich, HPA002868) at a dilution of 1:150. If any component of the SDH complex is damaged, the entire SDH complex becomes unstable, releasing the SDHB subunit into the cytoplasm where it is rapidly degraded (13). The staining protocol used (XT UltraView DAB v3, Benchmark IHC/ISH module) included pre-treatment with cell conditioner 1, incubation with primary antibody at 37°C, followed by incubation with Prep Kit 517 solution for 32 min, followed by counterstaining with hematoxylin for 8 min.

Sanger sequencing for examination of LOH in tumors

DNA was extracted from samples using QIAamp DNA FFPE tissue kit (Qiagen). Using the AmpliTaq Gold 360 Master Mix (ThermoFisher Scientific), DNA was amplified by PCR targeting exon 5 of SDHC or exon 13 of SDHA (primers available upon request). After purification, the PCR products were sequenced using the Sanger method on a AB3500XL genetic analyser (ThermoFisher Scientific).

Comparison of patients with ‘3PAs’ syndrome and ‘non-3PAs’ syndrome based on published data

The characteristics of patients who presented with PA and SDHx/MAX (likely) pathogenic variants were compared to patients with AIP, MEN1, PRKAR1A, CDKN1B-related PA and to patients with non-genetically determined PAs. The patients with non-genetically determined PAs came from the cohort reported by Daly et al. from a Belgian population (1). The AIP cases corresponded to 64 published cases with that phenotype (a list of references is available upon request). The MEN1 cases were extracted from the UMD-MEN1 Database (14), the Carney complex cases were from a literature review published by Cuny et al. (15), and the MEN4 cases were from reviews conducted by Alrezk et al. and Fredericksen et al. (16, 17).

Statistical analysis

Statistical analyses were performed using Prism v6.0 (GraphPad Software). Patient characteristics were compared using the two-tailed Fisher’s exact test for qualitative variables and the non-paired, non-parametric Mann-Whitney U-test for quantitative values.

Results

A total of 263 patients were included (Table 1 and Supplementary Table 1, see section on supplementary materials given at the end of this article). The mean age at diagnosis of PA was 29.3 years (min: 8; max: 78), and the mean age at genetic screening was 36.1 years (min: 8; max: 79). The occurrence of PA was sporadic in 227 patients (86.3%), while 36 patients presented with a familial history of PA (13.7%). By NGS sequencing, we found 295 single nucleotide variants, among which 7 variants were classified as pathogenic, 5 as likely pathogenic, and 7 as VUS (Fig. 1, Table 1 and Supplementary Tables 2, 3). We did not find CNV. Five PV and LPV were found in patients with a familial history of PA out of 36 and 7 in patients with sporadic PA out of 227. The probability to find a (likely) pathogenic variant was higher in cases of patients with a family history of PA compared to those with no history (odds ratio: 5.069, 95% CI: 1.69–15.79, P=0.014). Among the sporadic cases, five mutations occurred in patients younger than 30 years (5/133) and two occurred in patients older than 30 years. Among the 12 PVs and LPVs, we found 7 variants in AIP, 2 in MEN1, 2 in SDHA, and 1 in SDHC (Supplementary Table 2). The medical
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Clinical Study

Table 1  Clinical characteristics of the patients included in this study.

<table>
<thead>
<tr>
<th></th>
<th>Sporadic cases</th>
<th>Familial cases</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;30 years</td>
<td>&gt;30 years</td>
<td></td>
</tr>
<tr>
<td>Number of patients (n)</td>
<td>133</td>
<td>94</td>
<td>263</td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td>22.2 (9–30)</td>
<td>39.4 (31–78)</td>
<td>32.3 (8–77)</td>
</tr>
<tr>
<td>Sex ratio (male/female)</td>
<td>58 M/75 F (0.77)</td>
<td>62 M/32 F (1.9)</td>
<td>17 M/19 F (0.9)</td>
</tr>
<tr>
<td>Size of pituitary adenoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macro adenoma</td>
<td>95</td>
<td>70</td>
<td>187 (71.1%)</td>
</tr>
<tr>
<td>Micro adenoma</td>
<td>15</td>
<td>4</td>
<td>25 (9.5%)</td>
</tr>
<tr>
<td>NA</td>
<td>23</td>
<td>20</td>
<td>51 (19.4%)</td>
</tr>
<tr>
<td>Secretion of pituitary adenoma (n, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRL</td>
<td>52</td>
<td>20</td>
<td>14 (32.7%)</td>
</tr>
<tr>
<td>GH</td>
<td>31</td>
<td>36</td>
<td>5 (27.4%)</td>
</tr>
<tr>
<td>NFPA</td>
<td>8</td>
<td>6</td>
<td>8 (22.4%)</td>
</tr>
<tr>
<td>ACTH</td>
<td>21</td>
<td>3</td>
<td>2 (9.9%)</td>
</tr>
<tr>
<td>Mixed</td>
<td>4</td>
<td>8</td>
<td>1 (4.2%)</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>1</td>
<td>4</td>
<td>0 (1.9%)</td>
</tr>
<tr>
<td>TSH</td>
<td>1</td>
<td>1</td>
<td>0 (0.8%)</td>
</tr>
<tr>
<td>NA</td>
<td>15</td>
<td>16</td>
<td>6 (14.1%)</td>
</tr>
<tr>
<td>Number of variants, all genes (n)</td>
<td>148</td>
<td>115</td>
<td>32 (295)</td>
</tr>
<tr>
<td>VUS</td>
<td>4</td>
<td>3</td>
<td>0 (7)</td>
</tr>
<tr>
<td>LPV</td>
<td>0</td>
<td>2</td>
<td>3 (5)</td>
</tr>
<tr>
<td>PV</td>
<td>5</td>
<td>0</td>
<td>2 (7)</td>
</tr>
</tbody>
</table>

CTH, adrenocorticotrophic hormone; F, female; FSH, follicular-stimulating hormone; GH, growth hormone; LH, luteinising hormone; LPV, likely pathogenic variant; M, male; NA, not available; NFPA, non-functional pituitary adenoma; PRL, prolactin; PV, pathologic variant; TSH, thyroid-stimulating hormone; VUS, variant of uncertain significance. Macroadenoma is defined by a diameter >10 mm; microadenoma is defined by a diameter <10 mm.

histories of the patients with three SDHx mutations are described subsequently and in Table 2.

Case presentation

Case 1

A 17-year-old previously healthy male patient presented with a recent history of severe right vision loss, clinically measured on the monoyer scale (right eye: 4/10, left eye: 10/10). MRI revealed a large (>10 mm) cystic pituitary mass, and his prolactin level was measured at 91 μg/L (reference range: 4.1–15.34 μg/L). The patient underwent transphenoidal surgical debulking because of the visual defect. The post-operative examination was without complications and demonstrated a marked improvement in vision. The post-operative prolactin secretion was measured at 104 μg/L, probably explained by an incomplete resection. Post-operative MRI displayed post-operative reorganization without visible residue, resolved 6 months later. The histopathological examination of the resected tissue was consistent with a prolactinoma showing aggressive criteria (grade 1b under the new classification) (18). No neoplastic cells demonstrated vacuolated cytoplasm. The Ki-67 labeling index was 5%, and P53 was expressed in 10% of cells. Cabergoline was initiated at a dose of 0.5 mg weekly, producing prolactin normalization (3 μg/L at 3 months).

A SDHC: c.405+1G>T, p.(?) heterozygous pathogenic variant was demonstrated. This variant was present in the population database at a weak allele frequency (Minor Allele Frequency – MAF <0.001%). This variant has been reported as causing the deletion of exon 5 and a shift in the reading frame (19). SDHC IHC analysis of the PA was positive (Fig. 1 and Table 2), while the search for LOH in the tumor was negative. A whole-body CT and investigation of serum and urinary catecholamines did not show signs of PPGL. The SDHC variant was present in the father, who is asymptomatic at the age of 46. Careful examination revealed no lesions, and plasma methoxyamines were normal. Imaging exploration has not to date been carried out.

Case 2

A 42-year-old male patient, with no personal or family history of endocrine disease, consulted due to an 18-month history of erectile dysfunction and decreased libido. The primary hormonal assessment found a low testosterone level (0.64 ng/mL) and a pituitary profile showing FSH of 1.3 IU/L, LH of 2.7 IU/L and prolactin of 84 μg/L, thus supporting a central origin. Cranial MRI showed a pituitary macroadenoma with a moderate

https://eje.bioscientifica.com
family history of PA, with macroprolactinoma in his family but without any history of PPGL. His brother died during surgery to remove a massive pituitary adenoma. Prolactin secretion was well-controlled under cabergoline (PRL < 10 μg/L), but due to side effects a change to treatment with bromocriptine was made. Subsequently, prolactin levels were poorly controlled due to poor adherence to therapy (16.6 to 40 μg/L). Neurosurgical removal was performed and histopathological analysis of the tumor did not show cytoplasmic vacuolization. Analysis of PPGL genes found a heterozygous missense variant in exon 13 of SDHA (c.1753C>T, p.(Arg585Trp)). This variant is present in the population database (gnomAD MAF: 0.0025%); however, in silico analysis predicted a deleterious impact and studies have reported this variant as pathogenic (20). Therefore, this variant was classified as likely pathogenic. SDHB IHC analysis was positive (Fig. 1 and Table 1), and the search for LOH by Sanger sequencing was negative. Family members were not available to allow a genetic family survey to be conducted.

**Literature review and phenotypic features in ‘3PAs’ syndrome**

After reclassification of the variants using ACMG criteria (12) and excluding patients with VUS or (likely) benign variants, we found 23 published cases of ‘3PAs’ syndrome with SDHx or MAX (likely) pathogenic variants (Supplementary Table 4) (5, 6, 7, 8, 9, 21, 22, 23, 24, 25, 26, 27, 28, 29). Among the 23 cases, 19 patients had SDHx (likely) pathogenic variants: 2 in SDHA, 9 in SDHB (39%, 9/23), 2 in SDHC, and 6 in SDHD (26%, 6/23); four patients had MAX pathogenic variants. PA occurred before PPGL in six cases (6/23, 26%). Moreover, we also found five published cases with an isolated PA and a mutation in SDHx (one in SDHA and four in SDHB); all of these had a family history of PPGLs (Supplementary Table 5) (6, 30, 31, 32).

Overall, the SDHx/MAX patients with PA included those harboring PPGL (n = 23, Supplementary Table 4), those with a family history of PPGL (n = 5, Supplementary Table 5), and those without PPGL context (n = 3 from this study). Of these 31 SDHx/MAX patients, 6 had a family history of PA (Supplementary Table 4 and our case #3), and among these only one without PPGL history (our case #3). Cases included 16 females and 14 males (sex ratio: 1.1/1, the sex was not specified for one patient). Diagnosis of PA was on average at 42.4 years of age (range: 16(min) – 72 (max) years). There were 23 macroPAs, representing 74% of cases and 4 microPAs (13%). The
Table 2  Characteristics of patients harboring an isolated pituitary adenoma and SDHx/MAX likely pathogenic or pathogenic variants in this study: genetic exploration and functional analysis.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Age at PA diagnosis</td>
<td>17</td>
<td>42</td>
<td>37</td>
</tr>
<tr>
<td>Size of PA</td>
<td>Macro</td>
<td>Macro</td>
<td>Micro</td>
</tr>
<tr>
<td>Secretion of PA</td>
<td>PRL</td>
<td>PRL</td>
<td>PRL</td>
</tr>
<tr>
<td>Familial PA</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Familial PPGL</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>PRL at diagnosis (μg/L)</td>
<td>91</td>
<td>84</td>
<td>55</td>
</tr>
<tr>
<td>Medical treatment</td>
<td>Cabergoline (postoperative)</td>
<td>Cabergoline</td>
<td>Cabergoline</td>
</tr>
<tr>
<td>Surgery</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Results of AIP, MEN1, CDKN1B genetic testing</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Gene</td>
<td>SDHC</td>
<td>SDHA</td>
<td>SDHA</td>
</tr>
<tr>
<td>Variant</td>
<td>c.405+1G&gt;T, p.(?)</td>
<td>c.757_758del, p.(Val253Cys*67)</td>
<td>c.1753C&gt;T, p.(Arg585Trp)</td>
</tr>
<tr>
<td>Classification*</td>
<td>PV</td>
<td>LPV</td>
<td>LPV</td>
</tr>
<tr>
<td>Histopathological examination</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Hormonal status</td>
<td>PRL+</td>
<td>PRL+</td>
<td>PRL+</td>
</tr>
<tr>
<td>% of PS3-positive cells</td>
<td>5%</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>IHC SDH</td>
<td>Positive</td>
<td>-</td>
<td>Positive</td>
</tr>
<tr>
<td>LOH</td>
<td>Negative</td>
<td>-</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Htz, heterozygous; LOH, Loss of heterozygosity; LPV, likely pathogenic variant; M, Male; macro, macroadenoma, defined by a diameter >10 mm; micro, microadenoma, defined by a diameter <10 mm; PRL, prolactin; PV, pathogenic variant; SDHB IHC, SDHB immunohistochemistry. *Classification using ACMG guidelines for classification of sequence variants (12).

most frequent types were prolactinoma (19/31, 61%), GH-secreting adenoma and NFPA (5/31, 16% for both) (Supplementary Table 6).

Next, we compared these SDHx/MAX patients with those from published cases of genetically determined PAs (i.e. due to mutations in AIP, MEN1, CDKN1B or PRKAR1A) and non-genetically determined PAs. The age of occurrence of PAs in the SDHx/MAX patients (mean: 42.4 years, range: 16–72) was later than that for AIP patients (mean: 25.9 years, range: 10–60, P<0.001), or MEN1 patients (mean: 34.2 years, range: 7–82, P=0.024), or PRKAR1A patients (mean 31 years, range: 16–55, P=0.007) (Fig. 2 and Supplementary Table 6).

The proportion of prolactinomas was identical in SDHx/MAX patients and in the control population from the cohort published by Daly et al. (66% vs 61%) (Supplementary Table 6) (1). However, the PAs of the 31 SDHx/MAX patients were larger (23/31 macroadenomas vs 29/68 in control population P=0.002). In fact, macroprolactinomas were more frequent in the SDHx/MAX population (15/31 vs 11/68 P=0.0013). The SDHx/MAX patients also presented a tendency to show an older age at PA diagnosis than in the control population (42.4 years vs 34.5 years) though this was not statistically significant (P=0.08).

Discussion

Though mutations in MEN1, AIP, CDKN1B, and PRKAR1A genes have been identified as causal factors in familial PA, in most cases, no genetic cause was identified. For example, AIP mutations were found in only 20% of FIPA cases (33). In France, according to the TENgen guidelines (Oncogenetic Network in Neuroendocrine Tumors), mutations in AIP, MEN1, and CDKN1B genes are investigated in patients with PA who have (1) familial presentation, (2) syndromic association, or (3) isolated and sporadic pituitary macroadenoma occurring before the age of 30. PRKAR1A is investigated only in those patients with a typical syndromic association. Of note, in our cohort, several patients aged older than 30 years, with sporadic and isolated PA, had genetic testing on the express request of the endocrinologist due to notably aggressive or drug-resistant PAs, which are features of MEN1- and AIP-related PAs. In our cohort, the prevalence of mutations in AIP and MEN1 was consistent with the prevalence reported in the literature for patients with PAs and using similar inclusion criteria (AIP: 1.1–11.7%, MEN1: 0–3.4%) (34, 35, 36, 37, 38, 39).

Recently, a novel syndromic association termed ‘3PAs’ and involving PAs and PPGLs has been described in some
cases associated with germline mutations in SDHx/MAX genes (5, 6, 7, 9, 22). A literature review on patients with SDHx/MAX mutations showing ‘3PAs’ syndrome found six patients in whom the PAs were present in patients with PPGLs, while five patients had an isolated PA (Supplementary Tables 4 and 5), suggesting a new gateway into SDHx/MAX-related diseases. These data raise several issues regarding: (1) the incidence of SDHx/MAX mutations in patients with isolated PAs, (2) the characteristics of patients with isolated PAs harboring these mutations and, consequently, (3) whether mutations in SDHx/MAX genes might be looked for in patients with isolated PAs.

Herein, we highlight for the first time the co-occurrence of SDHx germine mutations in patients with an isolated PA and without personal or familial history of PPGL. In our study, among 263 patients with isolated PAs, we found 3 (likely) pathogenic variants in SDHx genes: 2 in sporadic cases of PA and 1 in a patient with a strong family history of PAs. The prevalence rate of mutations was 1.1%. In 2015, Xekouki et al. studied the prevalence of SDHx germine mutations in a cohort of 168 patients with PA, including 143 patients with isolated and sporadic PAs, 3 patients with sporadic ‘3PAs’ syndrome, and 22 patients with familial ‘3PAs’ syndrome (5). Three SDHx mutations were identified in patients with familial ‘3PAs’ syndrome.

No mutations were found in the patients presenting with isolated PAs, probably because the cohort included a high proportion of patients with ACTH-secreting PAs (118/168, 70%).

It should be noted that the presence of SDHx (likely) pathogenic variants in patients with PAs might also be due to a fortuitous association. In a study by Hoekstra et al., nonsense SDHA mutations were found at a frequency of 0.5% in a healthy population (40). Moreover, potentially (likely) pathogenic variants in SDHx/MAX genes, such as loss of function variants, are registered in population databases including gnomAD. There may, therefore, be a risk of an incidental association between pituitary adenoma and the presence of SDHx germline mutations. However, we did not identify any SDHx/MAX (likely) pathogenic variants in a cohort of 239 patients who presented with hyperparathyroidism and who had no personal or family history of PPGL and PA (personal data). These data favor a non-random association between SDHx/MAX (likely) pathogenic variants and PAs.

It is also true that the involvement of SDHx/MAX genes in PA tumorigenesis remains unclear. We have shown here that the age of onset of PA in patients with SDHx/MAX mutations is higher than that for other tumour suppressor genes and is equivalent to that of controls. The loss of expression of SDH within tumor tissue was not established in all patients, as was the case for cases 1 and 3 in our study. In published cases, among the 24 patients with PA and germline mutations in SDHx, SDHB IHC and LOH testing in tumor samples were done for ten cases, but a loss of staining/LOH was not observed in two of these, which does not support the hypothesis of Knudson’s double hit in these cases. Nevertheless, the Sdhb+/− murine model is consistent with the involvement of SDHx mutation in pituitary tumorigenesis (5). At 12 months age, the Sdhb+/− mice developed a hyperplastic adenohypophysis, as is classically found in AIP-deficient mice (41) and in human PAs due to AIP, PRKAR1A mutations or Xq26 microduplication (42, 43, 44). The adenohypophyseal cells of Sdhb+/− mice showed several intranuclear abnormalities and strong cytoplasmic and nuclear HIF-1α staining consistent with the activation of the pseudohypoxia pathway in SDHx-mutated tumors (5, 9, 45, 46, 47). Concerning MAX, there are no publications reporting PA associating germline MAX mutation and negative MAX IHC and/or LOH or second somatic MAX mutation in PA tissue. Nevertheless in humans, data on the increased risk of PA in patients with SDHx/MAX mutations is required to reach a conclusion on the need for pituitary gland monitoring in symptomatic and
Asymptomatic carriers of SDHx/MAX mutations. Induced pluripotent stem cells (iPS) modified with the SDHx/MAX mutation and differentiated into pituitary cells may help to clarify the mechanisms of pituitary tumorigenesis in this syndrome.

On the other hand, SDHx/MAX genetic testing for patients with PA should be decided while also taking into account (1) the low penetrance of SDHx-related manifestations, (2) the possible anxiety generated by this information for the patient and their family, (3) the exposure to ionizing radiation related to lifetime monitoring, and (4) the cost of clinical follow-up.

Of the three variants identified in patients with isolated PA, two occurred in SDHA and one in SDHC. In the PPGL population, SDHx germline mutations accounted for approximately 15% of all cases and for half of the familial cases. SDHB and SDHD mutations have been reported to be the most common, SDHA and SDHC mutations being less frequent (48). On a large series of SDHA-PPGL patients, the penetrance was calculated at 10% at 70 years of age (49), while Benn et al. and Maniam et al., using a Bayesian statistical approach, indicated an overall penetrance of 1.7% (95% CI: 0.8–3.8%) and 0.1–4.9%, respectively (50, 51). In the same study, Benn et al. calculated the SDHC penetrance at 8.3% (95% CI: 3.5–18.5%) (50). Consequently, the absence of PPGL in our patients with SDHA or SDHC mutations, as well as in their families, is not unexpected since the penetrance of SDHA/SDHC-related PPGL is low and the age of disease onset is late. Indeed the age at PPGL diagnosis in SDHC- and SDHA-related patients is usually 35–40 years. In our three patients, initial exploration did not reveal PPGL but a longer follow-up is required to conclude the absence of PPGL.

Therefore, until proven otherwise, these three patients are at risk of developing PPGL later and should benefit from long-term monitoring.

Indeed, data are lacking to determine if the increased risk of PPGL in families with SDHx mutations, without a family history of PPGL, is the same as that of a family with a history of PPGL, and if these patients require the same level of monitoring, especially for SDHA asymptomatic carriers. However, taking into account the current state of our knowledge, it seems obvious that the presence of SDHx/MAX (likely) pathogenic variants in patients with isolated PAs justifies screening for PPGLs via careful clinical examination, full-body imaging, and the measurement of urinary catecholamine levels (40). Additionally, the monitoring data of patients with SDHB, SDHC, SDHD, and SDHAF2 mutations, as secondary findings in clinical exome and genome sequencing from the ACMG, should certainly provide some answers (52). In any case, patients with a PA must be carefully examined not only for their family history of PA, but also for a history of PPGL, particularly for those patients bearing macroprolactinomas. Taking into account the published data, in cases of a family history of PPGL, genetic screening for SDHx/MAX is an absolute requirement for a family member bearing an isolated PA. For those patients with an isolated PA and any family history of PPGL, the benefit of SDHx/MAX genetic testing remains to be assessed.

A rare condition that requires SDHx/MAX genetic testing in patients with PA appears to be the presence of intracytoplasmic vacuoles in tumoral cells, a particular histological phenotype that has been reported in SDHx-related PAs (7) (Supplementary Table 3). These vacuoles do not appear to be fragments of mitochondria or endoplasmic reticulum (6) and most likely represent autophagic bodies, due to pseudohypoxia (47, 53, 54). In our patients, these vacuoles were not observed, but taking into account the literature, as in renal carcinoma (48), vacuolization of the cytoplasm should lead to SDHB (+/−SDHA) IHC and SDHx genetic analysis being carried out.

In conclusion, we report for the first time SDHx mutations in patients bearing PA without any family or personal history of PPGL. The prevalence rate of 1.1% is similar to that of MEN1 in this indication, raising the question of whether systematic genetic screening for SDHx/MAX is necessary for such patients. However, the question of incidental association remains unresolved. Data are lacking to determine the benefit of SDHx/MAX genetic testing of patients with isolated PA who have any family history of PPGL. Conversely, data on the increased risk of PA is needed to reach a conclusion on the pituitary gland monitoring in symptomatic and asymptomatic carriers of SDHx/MAX mutations. Meanwhile, we recommend a careful examination of patients with isolated PAs, not only on their family history of PAs but also on any history of PPGLs. Lastly, a family history of PPGL, as well as the presence of intracytoplasmic vacuoles in the PA, requires SDHx/MAX genetic testing for PA patients.

**Supplementary materials**
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**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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