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Original Article

3P association (3PAs): Pituitary adenoma and pheochromocytoma/paraganglioma. A heterogeneous clinical syndrome associated with different gene mutations

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ABSTRACT

Background: Pituitary adenomas (PA) associated with pheochromocytomas/paragangliomas (Pheo/PGL), also known as "the three P association" or "3PAs" could be the results of coincidence, but new evidence supports a common pathogenic mechanism in some patients. Our aim is to report the clinical data, surgical outcome, genetic findings of a large case series and review the current knowledge on this topic.
Methods and results: In a retrospective multicentre study, we compiled 10 patients with PAs (6 new unreported cases). Six patients were female with mean age of 51.6 ± 18.0 years. PA were: 6 acromegaly, 3 prolactinoma and 1 non-functioning PA (NFPA). Among the Pheo/PGL, 7 patients had a single tumour (4 Pheo and 3 PGL) and 3 patients had multiple or bilateral disease (2 PGL and 1 Pheo). Patients with GH-secreting PA and NFPA underwent surgery, while patients with prolactinoma received medical treatment (one patient required surgery). Unilateral adrenalectomy was carried out in all single Pheo and a bilateral procedure was performed in the patient with bilateral tumour. A single tumour was resected in two patients with multiple PGL. We found 3 germline pathogenic mutations: 2 in *SDHB* (c.166-170delCCTCA and a gross deletion involving exon 1) and 1 *SDHD* (p.P81L exon 3). Two variants of uncertain significance: 1 in *MEN1* (c.1618C > T; p.Pro540Ser) and 1 in *RET* (c.2556C > G, p.Ile852Met), and finally a RET^{M918T} somatic mutation in a Pheo tissue.
Conclusion: We actively suggest considering the possibility of hereditary disease in all cases with 3PA and performing a complete genetic study.

1. Introduction

Multiple endocrine neoplasia (MEN) syndromes are characterized by the presence of tumours affecting two or more endocrine glands in a single patient [1]. The main and early described forms were type 1 (MEN1) and type 2 (MEN2) MEN. Subsequently, other syndromes have been recognised, such as von Hippel-Lindau (VHL) disease, neurofibromatosis type 1 (NF1), Carney complex (CNC) and McCune-Albright syndrome (MAS) [2]. In recent years, other endocrine tumour associations have been reported. Pituitary adenomas (PA) and

Abbreviations: 3PAs, the three P association; CNC, Carney complex; MAS, McCune-Albright syndrome; MAX, MYC-associated factor X

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pheochromocytomas/paragangliomas (Pheo/PGL) are main components of MEN1 and MEN2, respectively. The presence of PA and Pheo/PGL in the same patient, also known as "the three P association" or "3PAs" is very rare in clinical practice [3]. To our knowledge, until February 2019, only 82 patients with 3PAs had been reported (4–7). This tumour association could be a result of coincidence, but at least in some cases, a common pathogenic mechanism might be involved [4].

In 2015, Xekouki and colleagues [3] confirmed that classical Pheo/PGL predisposing genes play a role in pituitary tumorigenesis. Germline mutations in genes coding succinate dehydrogenase (SDH) have been involved in the 3PAs development. SDH is a complex protein with dual function: it plays a role in the Krebs cycle, oxidizing succinate to fumarate, and it also forms the Complex II in the mitochondrial respiratory chain, having therefore a pivotal role for normal aerobic respiration [8]. SDH complex is composed of 4 protein subunits (SDHA, SDHB, SDHC and SDHD) and two assemble factors (SDHAF1 and SDHAF2). SDH subunits are encoded by nuclear genes and they act as tumour suppressor genes [9]. Defects in SDH genes triggers the inhibition of prolyl hydroxylases in the cytosol, and the consequent stabilization and activation of hypoxia inducible factor (HIF)-alpha, which favours tumour formation by activating angiogenesis, glucose metabolism and cell survival [10]. In addition, germline mutations in *MEN1*, *RET*, *VHL*, and more recently in suppressor MYC-associated factor X (*MAX*) gene are also identified in patients with 3PAs [5,6,11]. In this study we collected the clinical data, surgical outcomes and genetic tests results of 10 patients with 3PAs. We also review the most relevant and current information regarding clinical behaviour and genetic tests considered in patients with this unusual association of tumours.

2. Materials and methods

In the present work we have compiled 10 patients with 3PAs syndrome in a retrospective multicentre study that was carried out to analyse the clinical characteristics, treatment outcome and genetic test results. Patients were diagnosed and treated in 7 tertiary Spanish hospitals (Hospital Universitari de Bellvitge, Barcelona; Hospital Universitario de la Ribera, Valencia; Hospital Universitario San Cecilio, Granada; Hospital Universitari Parc Taulí, Barcelona; Hospital Universitario Central de la Defensa, Madrid; Hospital Universitario Virgen Macarena, Sevilla; Hospital Universitario Ramón y Cajal, Madrid). The variables evaluated were sex, age at diagnosis of both tumours, clinical manifestation, medical or surgical outcomes and the results of genetic tests performed. This is a retrospective analysis of our usual everyday work. Patients provided informed consent for the use of specimens and clinical data in accordance with the guidelines of the institutional ethics committees. This manuscript has been approved for its publication by the Clinical Research Ethics Committee of Bellvitge University Hospital (reference PR050/19).

Functioning PA was suspected in patients with pituitary hypersecretion symptoms and sellar mass in magnetic resonance imaging (MRI). Acromegaly was considered in patients with proper clinical findings, elevated serum insulin-like growth factor 1 (IGF-1) and lack of suppression of serum growth hormone (GH) after 75 g glucose load (GH > 1 ng/ml). Prolactin-secreting PA was diagnosed in patients with symptoms of hyperprolactinemia and serum prolactin level ≥ 100 ng/ml. Non-functioning pituitary adenoma (NFPA) was diagnosed in patient with sellar mass in MRI, no symptoms and biochemical findings of hormone excess. PA was confirmed following pathological evaluation of the pituitary specimen after the surgical tumours removal. Pheo/PGL was suspected in patients with high catecholamine and/or their metabolites levels in plasma or 24 h urine and related symptoms. In asymptomatic patients, Pheo/PGL was considered when characteristic features in computed tomography (CT), MRI, ^{123} I-metaiodobenzylguanidine (MIBG) scintigraphy or somatostatin receptor scintigraphy (Octreoscan®) were present. In patients who underwent surgery, Pheo/PGL was confirmed with the histological evaluation of the tumour.

DNA was extracted from peripheral blood samples following a standard method (FlexiGene DNA Kit, Qiagen, Hilden, Germany). Genetic screening on germline DNA was performed using a TruSeq Custom Amplicon 1.5 kit system (Illumina, San Diego, CA, United States), which included genes of interest for this study (*RET, VHL, NF1, MAX, TMEM127, SDHA, SDHB, SDHD, SDHC, SDHAF2, MDH2, FH, EPAS1, HRAS, KIF1B, MEN1, EGLN1*, *EGLN2*), and a MiSeq platform as previously described [12]. Two additional genes related to PA, aryl hydrocarbon receptor-interacting protein (*AIP)* and *CDKN1B* (*p27Kip1*), were analysed by Sanger sequencing in those patients negative for the targeted NGS assay describe above and with DNA available. In those cases with FFPE sample available, the genetic screening was performed using a second panel that included the *RET, VHL, NF1, MAX, TMEM127, SDHA, SDHB, SDHD, SDHC, SDHAF2, MDH2, FH, EPAS1,* and *HRAS* genes [12]. Testing for gross deletions in *VHL*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *SDHA* and *MAX* was done by MLPA (MRC-Holland), according to manufacturer's protocol. In addition, we evaluated the SDHB staining by immunohistochemistry according to previously described [13].

3. Results

A total of 10 patients (6 females) with 3PAs were included in the present compilation. Six patients are new and unreported (cases 1–6) and 4 patients were previously reported by us elsewhere (cases 7–10) [14,15]. Main characteristics of new cases are shown in Table 1 and a summary of all patients of the compilation are displayed in the Supplementary Table 1. In the complete series, mean age at first tumour diagnosis was 51.6 ± 18.0 years (range 36–73). Pituitary disease was firstly detected in 6 patients, while Pheo/PGL was the initial manifestation in 4 cases. Five patients had GH secreting PA, 3 of which were microadenomas. In one patient (case 5) with clinical features and biochemical diagnosis of acromegaly, MRI revealed no sellar lesion. Serum GH–*releasing hormone* (*GHRH*) was not available and radiological studies, including thoracic, abdominal and pelvic CT, MIBG scintigraphy and Octreoscan® were unable to locate a neuroendocrine tumour. During this radiological screening, a left Pheo was detected. After adrenalectomy, acromegaly persisted and Pheo histology for GHRH was negative. In the remaining four PA, 3 cases were prolactinomas, two of them macroprolactinomas and 1 patient had NFPA (Fig. 1).

Regarding Pheo/PGL, 7 patients had a single tumour (4 Pheo and 3 PGL), and in 3 cases the tumour was multiple or bilateral (2 PGL and 1 Pheo). Interestingly, 3 of 5 Pheo were asymptomatic and diagnosis was incidental. Especially relevant was the case 5, who was admitted to the hospital due to a severe arterial hypertension with Takotsubo-pattern cardiomyopathy. One patient (case 1) showed a symptomatic PGL of the organ of Zuckerkandl. In the three patients with neck PGL, the tumour was non-functional. Some cases exhibited a third endocrine disease, 3 cases had multinodular goitre (cases 3, 5, and 10) and 1 case primary hyperparathyroidism (case 10).

All patients with GH-secreting PA underwent transphenoidal surgery. In 4 of them, tumour resection was apparently complete, but in 3 patients acromegaly persisted or recurred after surgery (patient 2, 3 and 4). These 3 patients and one patient with subtotal extirpation were managed with medical treatment (somatostatin analogues (SA), dopaminergic agonist (DA) or pegvisomant). The patient with acromegaly and no tumour found (case 5) was initially treated with SA. Later, because of SA resistance, DA and pegvisomant were prescribed. All patients with prolactinomas, received DA and one of them (case 8) underwent surgery because of bromocriptine intolerance. Surgery was also performed in the patient with NFPA obtaining a subtotal resection. A subcentimeter residual lesion remained stable during the follow up. Unilateral adrenalectomy was carried out in all single Pheo and a bilateral procedure in the patient with bilateral tumour. Regarding PGL, a single tumour was resected in two patients affected with multiple PGL, in case 8 the largest cervical mass was removed and in case 7 the mediastinal tumour was unresectable as it was too close to vascular

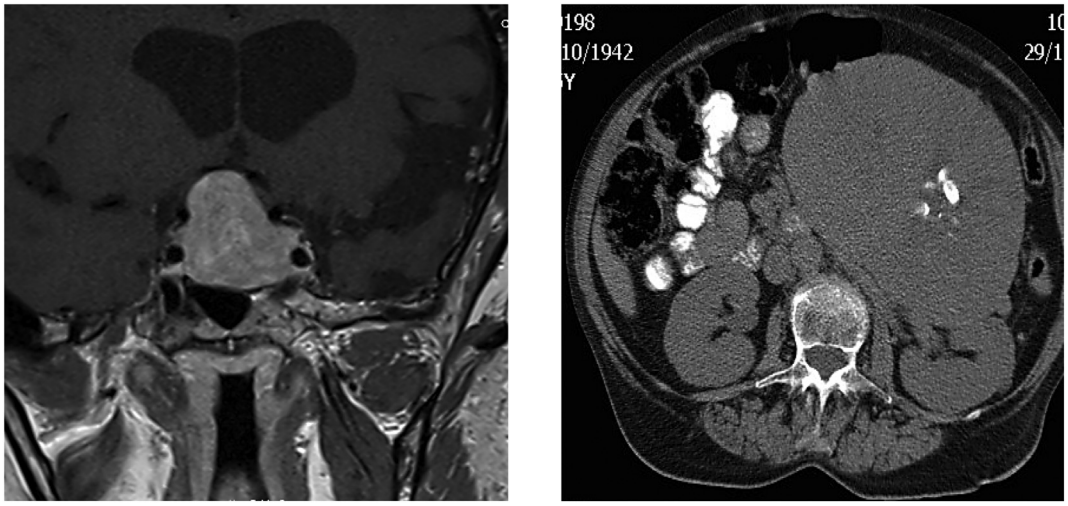

Fig. 1. A (Case 1): Coronal T1 weighted gadolinium-enhanced MRI corresponding with a non-functioning pituitary adenoma. B (Case 4): CT displaying a left pheochromocytoma.

structures.

SDHB germline pathogenic mutations were found in 2 patients. The c.166-170delCCTCA mutation in case 1, and a gross deletion in case 7. The c.166-170delCCTCA carrier had a twin brother who died a few years earlier due to a metastatic Pheo (clinical information about this brother was not available). Another brother showed a negative test. A *SDHB* gross deletion involving the exon 1 and the promoter of the gene was detected in case 7. In this family, the index case was her brother who had undergone surgery for functioning para-aortic PGL. In case 8, a *SDHD* (p.P81L, exon 3) mutation was identified. A cervical PGL was diagnosed to her brother who was a carrier of the same mutation. Finally, two variants of uncertain significance (VUS) were detected: the first one affecting *MEN1* (c.1618C > T; p.Pro540Ser) in case 9, and the second in *RET*, c.2556C > G, p.Ile852Met, in case 3. The genetic screening on germline was negative for the remaining 5 patients (Supplementary Table 1). Paraffin embedded tumour tissue from patients 2, 3 and 4 was available. SDHB immunohistochemistry (IHC) showed granular and cytoplasmic staining positive in all of them, ruling out the presence of germline and somatic *SDH* mutations. DNA tumour quality from patients 2 and 3 was appropriate for making a genetic screening using NGS and a custom panel as previously described [12]. In case 10, a RET^{M918T} somatic mutation was identified in Pheo tissue.

4. Discussion

Even though the overall estimated prevalence of PAs (autopsy and radiologic studies) could reach 16% [16], clinically relevant tumours are less frequent. The prevalence of symptomatic PAs is around 1 case in 1000–1500 individuals [17–20]. Only 5% of PAs appear in a family setting. *MEN1* and *AIP* mutations are the most common in these patients. Others PA familial syndromes are related to mutations in *SDH*, *CDKN1B* (MEN4), *CNC*, *MAS*, *GPR101* duplication (X-linked acrogigantism syndrome) and seldom in DICER1 syndrome [21]. On the other hand, Pheo/PGL are more infrequent tumours found with an estimated prevalence ranging from 1:2500 to 1:6667 [22,23]. Nowadays, it is accepted that 30–40% of Pheo/PGL occur in the setting of hereditary disease [24] and somatic mutations can be detected in 25–30% of sporadic tumours [25]. Germline mutations in *SDH* genes are the most prevalent hereditary syndromes in patients with Pheo/PGL [26]. Other syndromic forms include: MEN2A, MEN2B, VHL, NF1 and CNC [2]. Bearing in mind the above mentioned and considering the statistical probability of developing both tumours, the presence of PA and Pheo/PGL in the same patient should make the clinician strongly consider the possibility of a hereditary disease.

In a recent update of patients with PA and Pheo/PGL reported in

Table 1
Clinical characteristic, treatment performed and results of genetic study of 6 new unreported 3PAs patients.

Case	Sex	Tumour association	Age (yrs)	Clinical features	Other endocrine diseases	Treatment	Form of disease	Genetic study	Pheo/PGL IHC study
1	M	Zuckerkandl organ PGL	40	Arterial hypertension, family history Pheo	None	SR	Familial	*SDHB* mutation (c.166-170delCCTCA)	NA
		NFPA	45	Incidental		TSS		(patient index case)	
2	M	Right Pheo	56	Incidental (abdominal CT)	None	SR	Sporadic	No germline findings (*)	Positive
		GH secreting PA	60	Acromegaly features		TSS, SA			
3	M	GH secreting PA	73	Acromegaly features	Multinodular goitre	TSS, SA, DA	Sporadic	*RET germline VUSc.2556C > G, p.Ile852Me.* (*)	Positive
		PGL (neck)	84	Incidental (cervical US)		None			
4	F	GH secreting PA	54	Acromegaly features	None	TSS, SA, RDT	Sporadic	No germline findings (*)	Positive
		Left Pheo	63	NA		SR			
5	F	Acromegaly	58	Acromegaly features	Multinodular goitre	SA, DA, PGV	Sporadic	No germline findings (*)	NA
		Left Pheo	58	Incidental (abdominal CT)		SR			
6	F	Prolactinoma	NA	NA	None	DA	Sporadic	No germline findings (*)	NA
		Vagal PGL	54	NA		SR			

CT, computed tomography; DA, dopaminergic agonist; F, female; GH, growth hormone; IHC, immunohistochemical staining; M, male; MEN, multiple endocrine neoplasia; NA, Not available; NFPA, non-functioning pituitary adenoma; PA, pituitary adenoma; PGL, paraganglioma; PGV, pegvisomant; Pheo, pheochromocytoma; RDT, Radiotherapy; SA, somatostatin analogues; SR, Surgical resection; TSS, transphenoidal surgery; US, ultrasound; VUS, variant of uncertain significance; yrs., years old; (*), Gene mutation tested: *RET, VHL, NF1, MAX, TMEM127, SDHA, SDHB, SDHD, SDHC, SDHAF2, MDH2, FH, EPAS1, HRAS, KIF1B, MEN1, EGLN1*, *AIP*, *CDKN1B* (*p27Kip1*), *EGLN2, MLPA*. MLPA: gross deletions were not detected in *VHL*, *SDHs* and *MAX*.

literature from the first patient described by Iversen in 1952 [27], Xekouki et al. [7] summarized a total of 82 patients. Thirty-one patients (37.8%) had identified PA or Pheo/PGL predisposing genes mutation. Among all 3PAs, 22 patients (26.8%) had personal or family history suggested of hereditary endocrine syndrome, 37 patients (45.1%) were isolated and no information related to family history was available in 23 patients (28%). It is important to note that in many cases reported, genetic testing was not undergone or it was unavailable. The concurrence of PA and Pheo/PGL in the same patient could be explained by tumours arising coincidentally, or due to a shared pathogenesis. O'Toole et al. [4] proposed some hypotheses: 1) Pheo/PGL predisposing mutations also causing PAs; 2) PA predisposing mutations also causing Pheo/PGL; 3) two different mutations in the same patient or family causing both neoplasms; 4) Pheo/PGL producing an ectopic hormone secretion and mimics a PA; 5) a novel gene mutation causing both types of tumours.

Dénes et al. [11] studied 39 patients with sporadic ($n = 19$) and familial ($n = 20$) Pheo/PGL associated to PA and they found a heterogeneous genetic background. They identified 11 germline mutations in 5 different genes: five *SDHB*, one *SDHC*, one *SDHD*, two *VHL* and two *MEN1*. They also found 4 germline VUS in 3 distinct genes: two *SDHA*, one *SDHB* and one *SDHAF2*. The authors integrated all cases with confirmed gene mutation and PA (their own series and cases reported in literature) counted: 20 cases with *SDH* mutations (two *SDHA*, eight *SDHB*, two *SDHC* and eight *SDHD*); 5 patients with *RET*; 2 with *VHL* and 6 with *MEN1* mutations. They demonstrated loss of heterozygosity (LOH) at the *SDHB* locus in the PA samples and LOH at the *MEN1* locus in the Pheo samples. Although not concluded, these issues suggest the pathogenic role of these genes in these non-classically specific tissues [11]. More recently, *MAX* gene mutations have been identified in patients with PA and Pheo/PGL, expanding the genetic setting of this tumours association. Roszko et al. [5] reported a patient with prolactinoma, bilateral Pheo and primary hyperparathyroidism who tested positive for germline *MAX* mutation. Also, Daly et al. [6] described three patients with Pheo/PGL and PA with positive germline heterozygous exon deletions of *MAX*, two patients with single deletions (exon 3 and 4) and one case with multiple deletion (exon 1–3 and intron 3). In our series, germline pathogenic mutations in *SDHB*, *SDHD* were found in 3 out of 10 cases, as well as two VUS in *RET* and *MEN1*, even including in the analysis a long list of genes related either PA or Pheo/PGL considering a series of 3PA patients.

Although concurrent mutations involving two different genes have been described [28] in our study this possibility was ruled out as NGS was able to simultaneously interrogate mutations in all relevant genes described so far for this clinical association. In our series, among those patients negative for pathogenic mutation for germline genetic screening, it was possible to perform IHC in Pheo/PGL for SDHB in 3 cases (patients 2, 3 and 4), and discard the involvement of *SDH* genes as responsible of the disease. An ectopic hormone secretion produced by Pheo/PGL simulating a PA is also a very rare clinical situation. Only two cases had been reported before the patient with co-secreting GHRH Pheo resulting in a clinical and biochemical acromegaly published by Mumby et al. in 2014 [29–31]. In our series, one patient (case 5) had Pheo and acromegaly. An ectopic GHRH secretion was considered, but unfortunately serum GHRH determination was not available and GHRH was negative in Pheo specimen.

In patients with 3PAs without *SDH* mutations, a possible explanation is a sequencing screening missed SDHx promoter and other intronic or genomic defects that may not be detectable by the technology we are currently applying. Also, epigenetic changes, mosaicism, pituitary hyperplasia due to ectopic hypothalamic hormone secretion or another gene different to SDH could be implicated [3,7,32]. In our series, these potential genetic defects were discarded at least in the 3 patients with negative genetic screening and tumour available, as SDHB IHC showed positive staining. Taking into account the series reported in the literature and those cases described herein, there is a substantial group of 3PA patients without an identified germline mutation, suggesting that further 3PA genes are still to be identified. Efstathiadou et al. [33] described a case with the coexistence of PA, neck PGL, papillary thyroid cancer and Cowden-like syndrome. Boguszewski et al. [34] reported a patient with GH secreting macroadenoma and coexisting with left Pheo, gastrointestinal stromal tumours (GISTs) and thyroid follicular adenoma. The genetic analysis performed in both cases (including all SDH subunits) was negative and authors suggested that these cases could represent a new variant of MEN syndrome with a yet unidentified genetic mutation [33,34]. In our series, the coexistence of 4 endocrine diseases in one patient (case 10) with no germline mutations found also supports this hypothesis.

SDH germline mutations were initially thought to cause only hereditary Pheo/PGL syndrome [35]. However, nowadays germline in *SDH* mutations have been related with other different tumours like GISTs, renal cell carcinomas or PA [36,37]. In addition, few other tumours such as papillary thyroid carcinoma, Hodgkin lymphoma, pancreatic neuroendocrine tumour, adenomatoid tumour and many others, have been reported in *SDH* mutation carriers [38–41]. Nonetheless, if concurrence of these tumours in patients with *SDH* germline mutations is by chance or causal linked, is uncertain [36].

In 2008 López-Jiménez et al. [42] reported the first patient with PGL associated with PA and *SDH* mutation (*SDHC* in this case). Xekouki et al. [43] published in 2012 the case of a patient with multiple PGL and GH secreting PA. They found a novel germline *SDHD* pathogenic mutation, specifically *SDHD* LOH with down regulation of protein and increased HIF1 levels in the GH secreting PA [43]. In the following year, another PA case arising in the context of germline *SDH* mutation (this time *SDHA*) was reported [44]. The loss of SDHA protein expression was observed in both the PGL (proband) and PA (proband son) [44]. All these findings suggested the possibility of pathogenic relation between SDH defects and pituitary tumorigenesis.

Xekouki et al. [3] studied 146 patients with sporadic and 22 patients with familial PA. Among patients with sporadic PA, they found three cases that also had Pheo or PGL (not previously known). None had family history of endocrine tumours and genetic testing for *SDH* germline mutations was negative. However, among patients with familial PA, four cases also had Pheo or PGL, and three of them (75%) had *SDH* mutation. Utilizing the Sdhb $^{+/-}$ mouse model, they also found increased HIF1 expression in adenohypophyseal cells and mitochondrial and nuclear abnormalities (previously observed under hypoxic environments in human tumours cells), and they suggested that pituitary hyperplasia in SDH deficient cell could be the first step leading to the neoplasm development. They concluded that *SDH* mutations are rare in sporadic PA. This issue was well documented by Gill et al. who found only one *SDH* case with mutation among 309 pituitary adenomas [45]. For this reason, a systematic genetic screening is not warranted in patients with sporadic PA. However, a genetic study should be indicated for patients with PA and coexistent Pheo/PGL or family history of Pheo/PGL [3,37].

PA in patients with 3PAs are prolactinomas, GH-secreting adenomas and NFPA. The majority of them are large and locally aggressive or refractory to treatment. In patients reported in literature, 75% needed two or more therapeutic interventions [9,35]. Especially notable for the tumour aggressiveness was the patient recently reported by Tofton et al. [46]. They described the first pituitary carcinoma (foramen magnum metastatic deposit and extra-medullary cervical lesion) in a patient with a PGL history and *SDHB* mutation. In our series, the 3 female patients with prolactinoma had macroadenomas; which is uncommon in women of childbearing age. However, and contrary to the usual findings, 3 of 5 patients with GH secreting PA had microadenomas. In all of these cases, Pheo/PGL was previously detected and patients were under endocrinologist surveillance, which allowed the diagnosis at an early stage of the disease.

In summary, PA associated with Pheo/PGL, is a rare tumour association in clinical practice. So far, germline mutations in all *SDH*

subunits (A-D), *MEN1*, *RET*, *VHL* and *MAX* have been reported in these patients. We actively suggest considering the possibility of hereditary disease in all cases with this tumour association and performing a complete genetic study including the above mentioned screening.

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Declaration of Competing Interest

No authors have any competing interest to declare.

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