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ORIGINAL ARTICLE

Recommendations for somatic and germline genetic testing of single pheochromocytoma and paraganglioma based on findings from a series of 329 patients

Maria Currás-Freixes,¹ Lucía Inglada-Pérez,^{1,2} Veronika Mancikova,¹ Cristina Montero-Conde,¹ Rocío Letón,¹ Iñaki Comino-Méndez,¹ María Apellániz-Ruiz,¹ Lara Sánchez-Barroso,¹ Miguel Aguirre Sánchez-Covisa,³ Victoria Alcázar,⁴ Javier Aller,⁵ Cristina Álvarez-Escolá,⁶ Víctor M Andía-Melero,⁷ Sharona Azriel-Mira,⁸ María Calatayud-Gutiérrez,⁹ José Ángel Díaz,¹⁰ Alberto Díez-Hernández,¹¹ Cristina Lamas-Oliveira,¹² Mónica Marazuela,¹³ Xavier Matias-Guiu,¹⁴ Amparo Meoro-Avilés,¹⁵ Ana Patiño-García,¹⁶ Susana Pedrinaci,¹⁷ Garcilaso Riesco-Eizaguirre,¹⁸ Constantino Sábado-Álvarez,¹⁹ Raquel Sáez-Villaverde,²⁰ Amaya Sainz de los Terreros,²¹ Óscar Sanz Guadarrama,²² Julia Sastre-Marcos,²³ Bartolomé Scolá-Yurrita,²⁴ Ángel Segura-Huerta,²⁵ María de la Soledad Serrano-Corredor,²⁶ María Rosa Villar-Vicente,²⁷ Cristina Rodríguez-Antona,^{1,2} Esther Korpershoek,²⁸ Alberto Cascón,^{1,2} Mercedes Robledo^{1,2}

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For numbered affiliations see end of article.

Correspondence to

Dr Mercedes Robledo, Hereditary Endocrine Cancer Group, Human Cancer Genetics Programme, Centro Nacional de Investigaciones Oncológicas, Melchor Fernández Almagro 3, Madrid 28029, Spain; mrobledo@cnic.es

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ABSTRACT

Background Nowadays, 65–80% of pheochromocytoma and paraganglioma (PPGL) cases are explained by germline or somatic mutations in one of 22 genes. Several genetic testing algorithms have been proposed, but they usually exclude sporadic-PPGLs (S-PPGLs) and none include somatic testing. We aimed to genetically characterise S-PPGL cases and propose an evidence-based algorithm for genetic testing, prioritising DNA source.

Methods The study included 329 probands fitting three criteria: single PPGL, no syndromic and no PPGL family history. Germline DNA was tested for point mutations in *RET* and for both point mutation and gross deletions in *VHL*, the *SDH* genes, *TMEM127*, *MAX* and *FH*. 99 tumours from patients negative for germline screening were available and tested for *RET*, *VHL*, *HRAS*, *EPAS1*, *MAX* and *SDHB*.

Results Germline mutations were found in 46 (14.0%) patients, being more prevalent in paragangliomas (PGLs) (28.7%) than in pheochromocytomas (PCCs) (4.5%) ($p=6.62 \times 10^{-10}$). Somatic mutations were found in 43% of those tested, being more prevalent in PCCs (48.5%) than in PGLs (32.3%) ($p=0.13$). A quarter of S-PPGLs had a somatic mutation, regardless of age at presentation. Head and neck PGLs (HN-PGLs) and thoracic-PGLs (T-PGLs) more commonly had germline mutations ($p=2.0 \times 10^{-4}$ and $p=0.027$, respectively). Five of the 29 metastatic cases harboured a somatic mutation, one in *HRAS*.

Conclusions We recommend prioritising testing for germline mutations in patients with HN-PGLs and T-PGLs, and for somatic mutations in those with PCC. Biochemical secretion and SDHB-immunohistochemistry

should guide genetic screening in abdominal-PGLs. Paediatric and metastatic cases should not be excluded from somatic screening.

INTRODUCTION

Pheochromocytomas (PCCs) and paragangliomas (PGLs), together abbreviated to PPGLs, are neuroendocrine tumours originating from neural crest-derived chromaffin cells and arising in the adrenal medulla and extra-adrenal paraganglia, respectively. PCCs and PGLs localised in the thorax (T-PGLs) and abdomen (A-PGLs) are derived from sympathetic lineage and tend to be functional by secreting catecholamines. Head and neck PGLs (HN-PGLs) are derived from parasympathetic lineage and are usually non-secreting.

Although rare, with a reported prevalence of 0.2–0.6% in patients with arterial hypertension,¹ PPGLs show the highest degree of heritability of all human neoplasias.^{2,3} Since the description of the *NF1* gene in 1990,⁴ numerous discoveries related to the genetic background of PPGLs have been made, and it is now known that around 70% of PPGLs are associated with a mutation in one of the 22 known PPGL-related genes, either germline only (in *SDHC*, *KIF1β*, *SDHAF2*, *TMEM127*, *SDHA* or *FH*),² somatic only (in *HRAS*),² germline or somatic (in *NF1*, *RET*, *VHL*, *SDHD*,⁵ *SDHB*⁶ or *MAX*)² or somatic and somatic mosaicism (in *EPAS1*).² Mutations in other genes, such as *MEN1*,⁷ *EGLN1*, *EGLN2*,^{2,8} *MDH2*⁹ and *IDH1*,² have been reported in single cases or families suggesting that their contribution to the disease is

modest. In addition, somatic mutations in *ATRX*,¹⁰ *BRAF* and *TP53*¹¹ have been described, but their role is yet to be established.

Systematic genetic screening of all PPGL-related genes is an expensive and time-consuming task and so algorithms are required to guide genetic testing based on the presence of associated pathologies or clinical or molecular features. Information on indicators of hereditary disease (such as positive family history, syndromic features or metastatic, extra-adrenal, multifocal or bilateral presentation), age at diagnosis, tumour location, biochemical phenotype and particularly SDHB/SDHA-immunostaining, makes it possible to prioritise genes for mutational screening.^{1 3 5 12–16} The current clinical practice guidelines for PPGLs¹ recommend that for cases with indicators of low heritability (unilateral PCC without syndromic features or metastatic presentation, or PPGLs without a family history of the disease), the decision to perform germline genetic testing be balanced between the cost of testing and the psychological impact on the patient and their family of not having a test that might explain why they have the disease. These cases have been referred to as sporadic-PPGLs (S-PPGLs).

It has been reported that 11–24% of S-PPGLs harbour a germline mutation in a PPGL-related gene,^{14 15 17–20} and therefore algorithms focused specifically on S-PPGLs have been also proposed.^{14 15 17–20} However, none of them contemplate testing for somatic mutations, which seem to also be relevant in S-PPGLs.¹ Somatic mutations can cause metastatic^{21–23} and paediatric²¹ cases, as well as PPGLs diagnosed before the age of 40 years.^{21 24–26} Despite this knowledge, in routine clinical practice S-PPGLs have been excluded from comprehensive genetic screening beyond *SDHB* mutations, and even that is not always performed. Gene panels using next-generation sequencing (NGS) are a promising genetic diagnostic tool because they are faster and cheaper than the classically used Sanger sequencing.^{11 23 27–30} However, assessing the large amount of sequence data and variants of unknown significance generated, and applying appropriate biological interpretation of these results, remains a challenge. Thus, their use is yet to be optimised in daily clinical practice for all PPGL cases. Additionally, as a diagnostic tool, gene panel testing needs to be complemented with the study of gross deletions with multiplex ligation-dependent probe amplification (MLPA) or multiplex PCR, and with Sanger sequencing of the exonic regions that cannot be correctly sequenced by NGS.³

Therefore, information about germline and somatic mutations in S-PPGLs is still scarce, and there is no recommendation about whether germline or tumour DNA should be prioritised for testing. Therefore, while currently available algorithms may be useful, they require updating.

This study aimed to assess the prevalence of somatic and germline mutations in S-PPGLs, and to propose an algorithm that includes a recommendation about the type of material to be used in distinct scenarios.

METHODS

Patients and tumours

The inclusion criteria for patients with S-PPGL included: (1) the presence of a single PPGL (focal and unilateral); (2) the absence of syndromic features of neurofibromatosis type 1, multiple endocrine neoplasia type 1 and type 2 and von Hippel–Lindau syndrome in the patient and their relatives and (3) the absence of a family history of PPGL. The diagnosis was based on pathological study and plasma or urinary catecholamines/metanephrines; plus imaging tests for parasympathetic PGLs.

A blood sample was collected from eligible patients and a complete clinical questionnaire was completed at Spanish public hospitals (see online supplementary materials and methods).

A total of 329 unrelated Spanish index cases with S-PPGLs were recruited between 1997 and 2014: 60.8% were PCCs and 39.2% were PGLs. Among PGLs, 47.3% were HN-PGLs, 10.1% were T-PGLs, 41.9% were A-PGLs and the location of one PGL was not specified. The median age at onset was 46 (IQR: 35–59) years and 58% were women.

Physicians were recontacted to request a frozen or formalin-fixed paraffin-embedded (FFPE) tumour sample from patients with negative germline genetic screening. Of 99 tumour samples collected, 75 were FFPE and were evaluated for SDHB-immunohistochemistry (SDHB-IHC) using anti-SDHB rabbit polyclonal antibodies (Sigma-Aldrich Corp). SDHA-IHC was performed on tumours testing negative on SDHB-IHC, as previously described.³¹ Staining was considered positive when mitochondrial-specific granular labelling was identified, which suggests the absence of a mutation in the SDH genes.³² Frozen tumours were not tested for SDHB/SDHA-IHC because this technique was not optimised for this type of sample. All patients provided informed consent.

DNA extraction and mutation testing

DNA was extracted from blood following a standard method³³ and from frozen/FFPE tissue using the DNeasy kit (Qiagen Inc.), following the manufacturer's instructions.

Germline DNA from each patient was tested by Sanger sequencing for mutations in *RET* (ENST00000355710, exons 10, 11, 13–16), *VHL* (ENST00000256474, all exons, plus the promoter region), *SDHA* (ENST00000264932), *SDHB* (ENST00000375499), *SDHC* (ENST00000367975), *SDHD* (ENST00000375549), *SDHAF2* (ENST00000301761), *TMEM127* (ENST00000258439), *MAX* (ENST00000358664) and *FH* (ENST00000366560). Testing for gross deletions in *VHL*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *TMEM127*, *MAX* and *FH* was done by MLPA (MRC-Holland) or multiplex PCR, as previously described.^{34–36}

The study of somatic mutations was carried out based on biochemical secretion and SDHB-IHC, the latter only if an FFPE sample was available. Hence, *RET* was studied in adrenergic secreting tumours, *VHL* in noradrenergic secreting tumours and *SDHB* if SDHB-IHC showed negative staining or could not be determined. *SDHC* and *SDHD* were only tested if SDHB-IHC was negative and SDHA-IHC was positive. *EPAS1* (ENST00000263734), *H-RAS* (ENST00000451590), *MAX*, *RET* and *VHL* were studied for somatic mutations in tumours from cases for which no SDHB-IHC or biochemical secretion data were available, or when secretion was not detected, in the latter scenario because it was not known whether this was due to the tumour being non-secreting or exhibiting paroxysmal secretion. Because the predominant secretion pattern has not been clearly established for *EPAS1* and *HRAS*, and because *MAX*-mutated cases present both types of secretion, all tumours (except one with negative SDHB-IHC) were studied for somatic mutations in these three genes. Thus, the somatic study included *HRAS* (exons 2–3), *EPAS1* (exon 12) and *MAX* in 98 tumours, *VHL* (promoter region as well as exons 1–3) in 80 tumours, *RET* (exons 10, 11 and 16) in 59 tumours, *SDHB* (all exons) in 23 tumours and *SDHC* and *SDHD* (all exons) only in 1 tumour. Finally, we studied somatic mutations in *NF1* (ENST00000358273, all exons, using primers described³⁷) in adrenergic PPGL in which frozen tumour sample was available (five tumours). *NF1* was analysed in one of the tumours by NGS as part of another study

(data not shown); the mutation was confirmed by Sanger sequencing in tumour DNA and ruled out in germline DNA.

The details summarising the steps of the study are shown in online supplementary figure S1. The assessment of the pathogenicity of the variants found is described in online supplementary materials and methods.

Statistical analysis

We used Pearson's χ^2 test, or Fisher's exact test when necessary, to compare proportions. Two-sided p values < 0.05 were considered statistically significant. Statistical analyses were performed using IBM SPSS statistics V.17.0 (IBM, Armonk, New York, USA) and R software V.2.7.2 (<http://www.r-project.org/>) was used to generate figure 1.

RESULTS

Clinical characterisation

The clinical characteristics of cases are detailed in table 1. Clinical and genetic data from the 329 patients included in the study are shown in online supplementary table S1. The clinical presentation of S-PPGLs was mainly symptomatic (71.4%). Adrenergic symptoms were the predominant clinical presentation in PCCs (68.1%), T-PGLs (37.5%) and A-PGLs (47.7%), while local mass symptoms were more common in HN-PGLs (79.4%). PCCs and A-PGLs were predominantly secreting tumours (96.1% and 94.6%, respectively), HN-PGLs were more often non-secreting tumours (80%) and T-PGLs were a more even mix of both. Although the proportion of the secreting HN-PGL seems to be higher than previously described,³⁸ it

is important to note that we did not have this information for all patients. In these patients with secreting HN-PGL, additional PPGLs were ruled out using images techniques. Fifteen paediatric cases (diagnosed at or under the age of 18 years) were recruited, all derived from sympathetic lineage: seven PCCs, one T-PGL and seven A-PGLs, and most (83.3%) presenting with adrenergic symptoms. Twenty-nine cases had developed metastases, which were more common in PGLs (14.0%) than in PCCs (5.5%; $p=0.008$), and in T-PGLs (38.5%) than in HN-PGLs (8.2%) or A-PGLs (13.0%) ($p=0.012$ and $p=0.046$, respectively).

Genetic characterisation

Genetic analysis revealed mutations in 89 (27.1%) of 329 S-PPGLs; 46 were germline (14%) and 43 were somatic (43.4% of the 99 tumours tested). A summary of the assessment of each variant found (mutation vs variant of unknown significance) is shown in online supplementary table S2. Germline mutations were more prevalent in PGLs (37/129, 28.7%) than in PCCs (9/200, 4.5%) ($p=6.62 \times 10^{-10}$). The most frequently germline-mutated gene in S-PPGLs was *SDHB* (29/46, 63.0%). This result was expected due to the existence of founder effects affecting this gene in the Spanish population.²⁰ The number of mutations in other genes was six for *SDHD*, two for *SDHC*, two for *RET* and one for *VHL* mutation. Moreover, as previously published,^{2 3 23 25 27-29 39-41} the contribution of mutations in 'new' PPGL-genes was minor: three in *SDHA* (0.9%), one in *SDHAF2* (0.3%), two in *TMEM127* (0.6%) and no *FH* and *MAX* mutations.

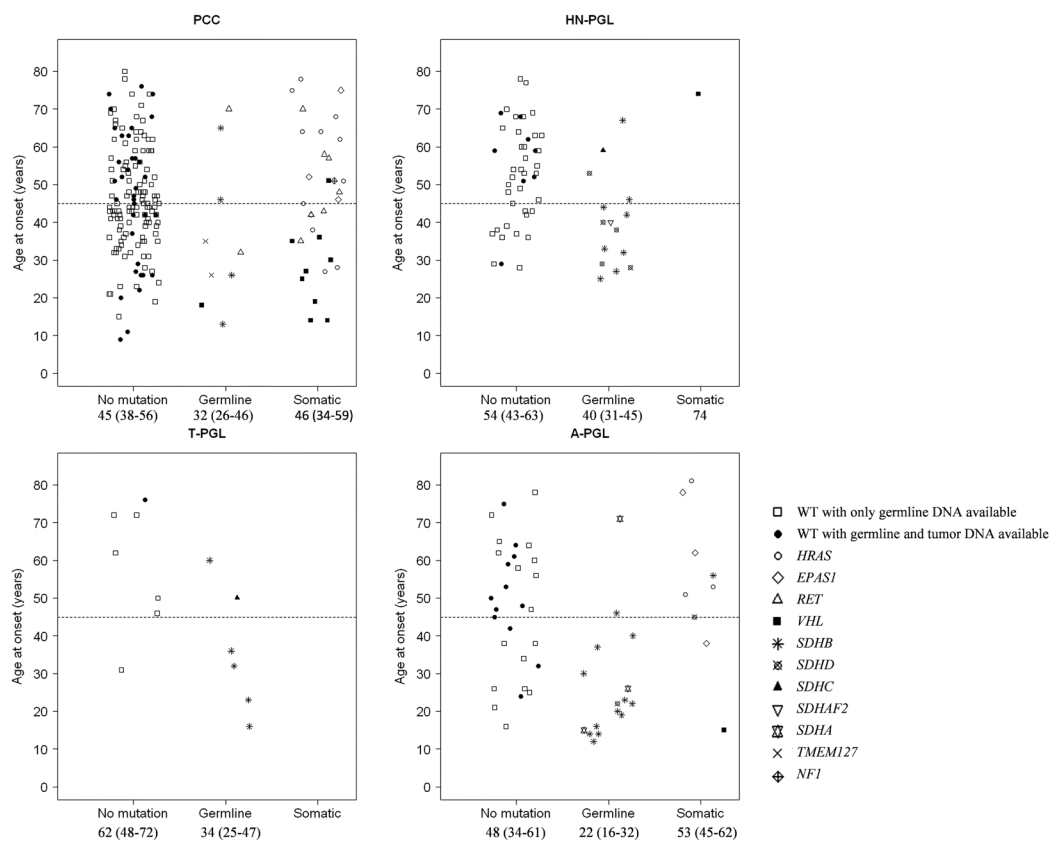


Figure 1 Age at disease onset by tumour location and genetic mutation status (germline mutation, somatic mutation, no mutation found in germline DNA study or no mutation found in germline plus tumour DNA study). The median and IQR of age in years at disease onset are shown for each group. A-PGL, abdominal-paraganglioma; HN-PGL, head and neck-paraganglioma; PCC, pheochromocytoma; T-PGL, thoracic-paraganglioma; WT, wild type.

Table 1 Clinical characteristics by tumour location

Location	N	Gender		Age at onset in years Median (IQR)	Clinical presentation		Biochemical secretion		Metastatic cases
		Male	Female		Incidentaloma (image/surgery)	Symptomatic (adrenergic/local mass)	Biochemical secretion		
							No	Yes	
PCC	200	88	112	45 (36–57)	41 (36/5)	100 (96/4)	5	124	11
p Value ^{PCC vs PGL}				NS				2.3×10^{-12}	0.0083
PGL	129	50	79	48 (33–60)	24 (13/11)	62 (26/36)	35	47	18
HN-PGL	61	21	40	52 (39–61)	5 (2/3)	29 (2/27)	28	7	5
p Value ^{HN-PGL vs T-PGL}				NS			NS		0.012
p Value ^{HN-PGL vs A-PGL}				0.0057			3.6×10^{-11}		NS
T-PGL	13	3	10	50 (32–62)	4 (2/2)	4 (3/1)	5	5	5
p Value ^{T-PGL vs A-PGL}				NS			0.0028		0.046
A-PGL	54	26	28	44 (24–59)	15 (9/6)	29 (21/8)	2	35	7
Unlocated PGL	1		1	62	Unknown		Unknown		1
Total cases	329	138	191	46 (35–59)	65 (49/16)	162 (122/40)	40	171	29

A-PGL, abdominal PGL; HN-PGL, head and neck PGL; N, number of cases; NS, not statistically significant; PCC, pheochromocytoma; PGL, paraganglioma; T-PGL, thoracic-PGL; vs, versus.

Among the 99 tumour samples, 68 were PCCs and 31 PGLs. Among the 75 FFPE samples, all except one showed positive SDHB-IHC. Somatic mutations were more prevalent in PCCs (48.5%) than in PGLs (32.3%; $p=0.13$). *HRAS* was the gene most often somatically mutated (15.3% of the 98 tumours tested), followed by *VHL* (11/80, 13.8%), *RET* (8/59, 13.6%), *EPAS1* (6/98, 6.1%), *SDHB* (1/23, 4.3%) and *NF1* and *SDHD* (one case each). A summary of the genetic testing is presented in online supplementary figure S2.

Tumour location

To make a recommendation about which type of sample should be prioritised for genetic screening of the known PPGL-related genes, and to avoid an overestimation of the frequency of patients with somatic mutations, not only the 99 germline-negative cases with tumour material available were included in the analysis, but also the 46 germline-positive patients. The remaining 184 germline-negative cases were excluded from this study since tumour material was not available and thus, somatic screening was not feasible. Statistically significant differences were found between PCCs and PGLs regarding the proportion of somatic mutation carriers versus germline mutation carriers ($p=6.67 \times 10^{-8}$). In this subset of patients, somatic mutations were found in 4.2% of HN-PGLs, 0% of T-PGLs, 24.3% of A-PGLs and 42.9% of PCCs. Among all locations, HN-PGLs and T-PGLs were mainly associated with germline mutations ($p=2.0 \times 10^{-4}$ and $p=0.027$, respectively) (table 2).

The most frequently mutated gene in PCCs was *HRAS*, while *SDHB* was the major contributor in PGLs regardless of their location, followed by *SDHD* in HN-PGLs, even though the involvement of this gene has been mainly related to multiple PPGLs.² In A-PGLs, *SDHA*, *EPAS1* and *HRAS* were mutated with similar frequency (three cases each). Genetic results by tumour location, and clinical characteristics by gene mutated, are detailed in table 3.

Utility of predominant secretion of PPGLs to guide genetic screening

HRAS-mutated, *RET*-mutated and *NF1*-mutated S-PPGLs presented predominantly adrenergic secretion. *EPAS1*-mutated, *VHL*-mutated, *SDHB*-mutated and *SDHA*-mutated S-PPGLs had, as expected, noradrenergic secretion. Only two *TMEM127*-mutated cases were found, one with adrenergic and the other with noradrenergic secretion. The case in our series with a mutation in *SDHAF2* showed mainly adrenergic secretion. The result from an assessment of genes mutated by location, for adrenergic and noradrenergic secreting tumours, suggested that in case of noradrenergic secreting tumours, *VHL* should be tested before *SDHB* ($p=3.51 \times 10^{-5}$) and *SDHD* ($p=7.1 \times 10^{-4}$) in PCCs, *SDHD* before *VHL* ($p=0.0095$) in HN-PGLs and *SDHB* before *VHL* ($p=0.0024$) in thoracic plus abdominal-PGLs (TA-PGLs) (see online supplementary table S3). However, no statistically significant differences were found in adrenergic tumours between the proportions of *HRAS*-mutated and *RET*-mutated cases for each location (table 3).

Paediatric presentation

Data on age at presentation by tumour location and genetic mutational status are summarised in figure 1. The median age at onset for germline mutation carriers was lower than that for somatic mutation carriers and cases without a mutation.

Driver mutations were more frequently found in paediatric than in adult cases (73.3% vs 25.2%, $p=0.00020$). Germline mutations were found in 53.3% of children, involving *SDHB* in

Table 2 Summary of genotype profile by tumour location for cases with germline and tumour DNA available

Location	Total N=145	Germline mutation N=46	p Value Germline vs somatic mutation	Somatic mutation N=43	Total mutated N=89	p Value Mutated vs non-mutated	Non-mutated N=56
PCC	77	9		33	42		35
PGL	68	37	6.67×10^{-8}	10	47	NS	21
HN-PGL	24	15	2.0×10^{-4}	1	16	NS	8
T-PGL	7	6	0.027	0	6	NS	1
A-PGL	37	16	NS	9	25	NS	12
TA-PGL	44	22	0.0078	9	31	NS	13

A-PGL, abdominal PGL; HN-PGL, head and neck PGL; NS, not statistically significant; PCC, pheochromocytoma; PGL, paraganglioma; T-PGL, thoracic-PGL; TA-PGL, thoracic plus abdominal-PGL; vs, versus.

six cases (75%) and *SDHA* and *VHL* in one case each. In addition, three somatic mutations were found in the five tumours available from the paediatric cases with negative germline screening (60%), all of them in *VHL*. Proportionally less adult cases (12.5%) presented germline mutations ($p=0.00030$), while somatic mutations were found in a similar percentage (41.9%) to that for paediatric patients ($p=0.65$).

Of paediatric S-PPGL with a bona fide diagnosis (patients with germline mutation and those with negative germline screening and tumour available), 4 (23.1%) presented a somatic mutation, similar to the 37 (29.8%) somatic mutations found in S-PPGL cases older than 18 years old with a bona fide diagnosis ($p=0.15$). If only paediatric and adult cases with a bona fide diagnosis were taken into account, no statistically significant differences in the proportion of mutated cases were observed ($p=0.068$).

Minor but not less important metastatic-related players beyond *SDHB* gene

Of the 29 metastatic cases, 19 (65.5%) carried a driver mutation. Germline mutations were found in 14 (48.3%), most commonly in *SDHB* (71.4%), followed by *SDHD* (14.3%). No mutations were found in other genes associated with a higher rate of metastases, such as *MAX* and *FH*.^{35 41}

However, in one metastatic S-PPGL we found a somatic mutation in *HRAS*, a gene that has not previously been reported to be involved in metastatic PCCs. In addition, as previously described, there were metastatic cases with germline mutations in *SDHA*, *SDHC* or with somatic mutations in *EPAS1*, *RET*, *VHL* and *SDHD*.⁴²

DISCUSSION

Mutation analysis

Since 2002, several reports have been published on genetic screening in S-PPGLs, with the estimated prevalence of hereditary cases ranging from 11.3% to 24%.^{14 15 17–20 43} However, it is difficult to compare these findings because the criteria used to define S-PPGLs varied between studies; some included cases with multifocal¹⁹ or bilateral^{18 19} tumours, or cases with family history,¹⁹ while others focused on benign tumours¹⁷ or secreting tumours^{18 19} or included cases diagnosed within a specific age range.⁴³ Further, only germline mutations in the PPGL-related genes known at the time (*RET*, *VHL*, *SDHB*, *SDHD* and *SDHC*) were considered. That said, in our 2009 study, we found germline mutations in 135 (14%) of the cases studied, and this report shows the same percentage of hereditary cases, despite having increased the sample size (from 135 to 329) and five additional genes having been included in the analysis.¹⁵ This is likely explained by the limited contribution of the 'novel'

PPGL-genes: *SDHA* (<1%²), *SDHAF2* (<1%⁴⁰), *TMEM127* (0.9%³⁹), *MAX* (1.1%²⁵) and *FH* (0.83%⁴¹).^{27–29}

In recent years, somatic mutations in S-PPGLs have also been reported, highlighting the importance of working with tumour samples to provide a genetic diagnosis.^{11 21–26 29 30 44} Our study shows a higher frequency of somatic mutations than the reported 36%,²² probably because we included the study of *HRAS* and *EPAS1*, which seems to be relevant to S-PPGL. This higher frequency of mutations was seen for *RET*, *VHL* and *HRAS*; the previously reported prevalences were 5–5.1%, 8.5–9.2% and 6.9–10%, respectively.^{11 21 22 24 26} On the other hand, our study found a similar frequency of *EPAS1* mutations (6.1%) to that previously reported 7.9%.⁴⁴

In 2012, two independent studies found somatic *NF1* mutations in 24% and 41% of PPGL patients, predominantly in PCCs and one A-PGL.^{22 23} For three of the cases, the mutation was found to be in the germline, all had mild features and none had previously been identified as syndromic *NF1* patients.²² We found one somatic mutation in *NF1* among the five adrenergic frozen tumours available (20%). This lower percentage is probably due to the limited number of tumours analysed for somatic *NF1* mutations. The use of NGS could resolve the difficulties of studying *NF1* by Sanger sequencing (large size, the absence of identified hot spots, high cost and time of delivery), and its future use will probably explain a large number of cases with unknown driver mutations.

Only one case had a somatic mutation in *SDHB* and *SDHD*. Finally, *MAX* was not somatically involved in our series, which is consistent with previous reports (1.65–2.5% frequency).^{22 25} Similar to the COMETE cohort study, where somatic genetic assessment was guided by findings from genome-wide expression studies,^{21 22} our somatic study was, in part, guided by the fractionated biochemical secretion observed for each tumour, highlighting the importance of having access to secretion data. In this regard, it has been already demonstrated that mutation screening of *SDHB* is unnecessary in metastatic tumours secreting epinephrine.⁴⁵

Although germline mutations in the *HRAS* and *EPAS1* genes have been reported to be associated with the 'Costello syndrome' and 'familial erythrocytosis type 4', respectively, no case with PPGL has been reported in families with those syndromes.^{2 24 26 46} However, elevated urine catecholamine metabolites have been described in some patients with Costello syndrome⁴⁷ and *EPAS1* mutations have been found as a mosaic in germline DNA extracted from leucocytes and buccal cells in two patients with polycythaemia and PPGLs.^{48 49} Although we have not ruled out the presence of these mutations in germline DNA, none of the cases with somatic mutations in *EPAS1* or *HRAS* showed any of the associated syndromic features.

Table 3 Mutations by gene, tumour location and clinical characteristics

Location	Number of cases (number of malignant cases)																
	HRAS		RET		VHL		SDHB		SDHD		SDHC		SDHA		NF1		
	Ger.	Som.	Ger.	Som.	Ger.	Som.	Ger.	Som.	Ger.	Som.	Ger.	Som.	Ger.	Som.	Ger.	Som.	
PCC	12 (1)	2 (0)	2 (0)	8 (1)	3 (1)	1 (0)	9 (0)	4 (2)	0	0	0	0	0	0	0	2 (0)	1 (0)
HN-PGL	0	0	0	0	0	0	1 (1)	8 (2)	0	5 (1)	1 (0)	1 (0)	0	1 (0)	0	1 (0)	0
TA-PGL	3 (0)	0	0	0	3 (0)	0	1 (0)	17 (6)	1 (0)	1 (1)	1 (1)	3 (1)	0	0	0	0	0
T-PGL	0	0	0	0	0	0	0	5 (4)	0	0	0	0	0	0	0	0	0
A-PGL	3 (0)	0	0	0	3 (0)	0	1 (0)	12 (2)	1 (0)	1 (1)	0	3 (1)	0	0	0	0	0
Gender: Male/Female	5/10	2/0	2/0	5/3	0/6	0/1	7/4	18/11	1/0	4/2	0/1	1/2	1/1	1/1	1/1	31 (28-33)	51
Age: median (IQR)	53 (44-66)	51 (42-61)	48 (43-58)	57 (48-72)	18	27 (17-36)	30 (20-42)	56	34 (28-40)	45	55 (52-57)	26 (21-49)	40	31 (28-33)	40	31 (28-33)	51
Secretion: nolyes	3/7	0/5	0/5	0/3	0/3	0/11	7/14	7/14	2/0	2/0	1/0	0/3	0/1	0/2	0/1	0/1	0/1
Type of secretion*	A 6	A 4	NAd 3	NAd 3	NAd 3	NAd 9	NAd 13	NAd 13	-	-	-	NAd 3	A 1	NAd 1	A 1	NAd 1	A 1
N (%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)

Ger., age, secretion and type of secretion are only represented for the patients with these data available.
 *type of secretion is only expressed for the patients with fractionated catecholamine/metanephrine data available. A, adrenergic secretion (epinephrine/metanephrine); NAd, noradrenergic secretion (norepinephrine/normetanephrine). Ger., germline mutation; Som., somatic mutation.
 A-PGL, abdominal PGL; HN-PGL, head and neck PGL; N, number of cases; PCC, pheochromocytoma; PGL, paraganglioma; T-PGL, thoracic-PGL; TA-PGL, thoracic plus abdominal-PGL.

Importance of differentiating specific location

In addition to the predominant secretion, our study highlights the utility of differentiating tumour location to select not only the most appropriate DNA sample (germline or tumour), but also the genes to be studied. Our analysis enabled us to conclude that the study of germline DNA should be prioritised in HN-PGLs and T-PGLs, while the study of tumour DNA should be recommended in PCCs. Despite not finding statistically significant differences between the frequencies of somatic and germline mutations in A-PGLs, SDH genes were involved in 72% of mutated cases (being two somatic mutations). In addition, 20% (3/15) of *HRAS*-mutated cases in this series were A-PGLs, compared with the 4.2% (1/24) previously reported,^{11 24 26} highlighting the relevance of *HRAS* somatic testing in tumours located outside the adrenal glands. Finally, for A-PGLs, it seems appropriate to recommend a germline study (starting with the SDH genes) in cases with tumours negative for SDHB-IHC and somatic screening (excluding the SDH genes) in those with positive SDHB-IHC staining.

Metastatic behaviour

As expected, *SDHB* was the main gene involved in metastatic S-PPGLs, even in cases with PCC. It was the most commonly mutated gene among T-PGLs (38.5%). Somatic mutations in *VHL*, *RET*, *EPAS1*, *HRAS* and *SDHD* were detected in one case each. Thus, because knowing the driver mutation is especially important in the determination of the most appropriate therapeutic intervention,³ metastatic cases should not be excluded from comprehensive testing for somatic mutations.

Age at onset

Historically, paediatric age has been considered a predictor of the presence of germline mutations in PPGL-related genes. In fact, previous paediatric series have shown rates of germline mutation of around 80%.⁵⁰ The results from the present study show that at least a quarter of S-PPGLs can be explained by somatic mutations, even in paediatric cases. So, it is also important to study somatic mutations in young patients.

Although there is no agreement on the upper age limit to apply for genetic testing,^{1 13-15 18 20 51} 45 years resulted in a better identification of mutation-positive cases in the study of Erlic *et al*,¹³ where various clinical parameters were assessed using multiple logistic regression. If germline screening had not been performed in index cases older than 45 years in our series, we would have missed 11.1% hereditary cases of HN-PGLs, 25% of T-PGLs, 8% of A-PGLs and 3.2% of PCCs. Conversely, younger patients tend to be excluded from somatic studies. Thus, if the somatic screening had not been performed in index cases younger than 45 years, we would have missed the genetic diagnosis of 42.9% A-PGLs, and even more importantly, 53.3% of PCCs. Therefore, we recommend that a germline and somatic genetic diagnosis be carried out for all S-PPGLs, regardless of the age of diagnosis.

Moreover, paediatric cases with somatic mutations should be assessed with caution, as it is especially important to rule out the presence of a mosaic. Mosaicism has been demonstrated to occur in *EPAS1* patients.^{52 53} NGS is a useful diagnostic tool to accurately quantify the level of mosaicism through the study of different embryological lineage cells.⁴⁸ Thus, if a somatic mutation is found in a paediatric case, a search for the mutation in multiple tissues is encouraged to better evaluate the extension of the disease, as well as to improve the management and follow-up of the patient and their offspring.⁴⁸

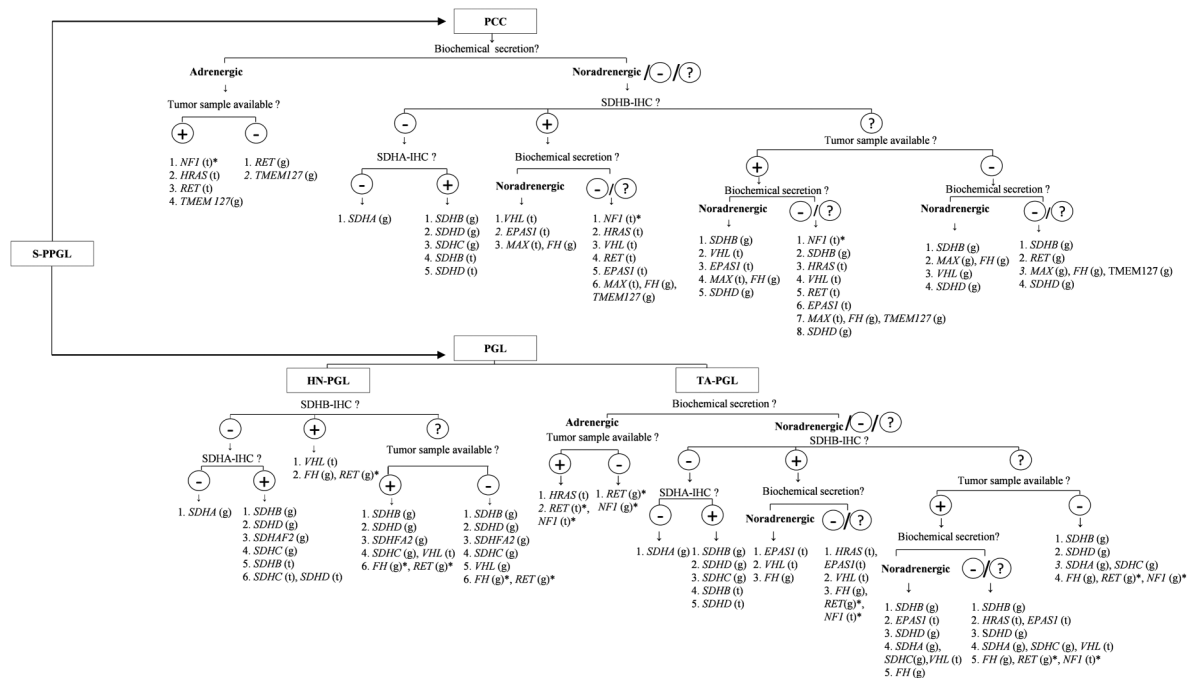


Figure 2 Proposed genetic testing algorithm for patients with sporadic-pheochromocytoma and paraganglioma (S-PPGL) based on SDHB-immunohistochemistry (IHC) in formalin-fixed paraffin-embedded tissue (if available) and biochemical phenotype. This algorithm has been elaborated considering previous reports' findings^{3 5 7 11 16 23 27–30 57} and the current series. (g), germline DNA; HN-PGL, head and neck-paraganglioma; PCC, pheochromocytoma; PGL, paraganglioma; (t), tumour DNA; TA-PGL, thoracic plus abdominal-paraganglioma. *Test if possible.

Paraffin-embedded tumour samples

The prevalence of mutations in the SDH genes in A-PGLs, metastatic cases, as well as paediatric S-PPGLs stresses the importance of using SDHB-IHC as a filter to optimise genetic screening, and therefore highlights the importance of having access to FFPE tumour material.^{3 32} Additionally, SDHA, MAX and S-(2-succinyl) cysteine IHC could also help to direct mutation testing of *SDHA*, *MAX* and *FH*, respectively.^{3 25 31 32 41} A good example of utility of performing IHC to guide the study was the case with a somatic mutation in *SDHD*. Somatic SDHs mutations are scarce and rarely analysed. When FFPE tumour material is unavailable, at a minimum *SDHB* germline mutations should be tested for, given the higher associated risk of developing metastases⁵⁴ and the presence of a founder effect, at least in the Spanish population.²⁰

Even where NGS can be carried out, the large number of variants identified by this platform means that having access to FFPE tumour samples to perform IHC studies can help to select the genetic variants that should be validated by Sanger sequencing, and to determine the pathogenicity of variants of unknown significance found in these genes.^{11 27 28}

Reasons to consider genetic screening in all cases of S-PPGLs

Genetic screening is expensive and time-consuming, especially if NGS is not available, but there are important implications of having a genetic diagnosis in S-PPGLs. According to the American Society of Clinical Oncology (ASCO)'s general recommendations for genetic screening, all patients with a risk of at least 10% of carrying a genetic mutation should be offered genetic testing, especially when the results would aid in diagnosis or influence the management of the patient or family members at hereditary risk of cancer.^{16 55}

The identification of a germline mutation in a patient with apparent S-PPGLs could lead to the early diagnosis of multiple tumours or additional syndromic neoplasias in the proband, as well as in relatives at risk.

On the other hand, the identification of a somatic mutation benefits: (1) family screening, as it frees relatives from the need for genetic screening and clinical follow-up (more caution has to be taken in the case of 'somatic mutations' in paediatric cases since the possibility of a germline mosaicism cannot be excluded); (2) diagnosis, making unjustified the exhaustive follow-up required for patients harbouring germline mutations associated with a high risk of developing different cancer types; (3) prognosis, as it is known that mutations in some genes have a well-known high risk of metastatic behaviour and (4) therapeutic opportunities, since the identification of the somatic mutation opens up the possibility of new therapeutic approaches if surgery is not curative. Mutations involving cluster 1 genes could be targeted using an antiangiogenic approach, mutations in cluster 2 genes could be treated by targeting the mTOR and the RAS-RAF pathway and, specifically, for *FH*-related and *SDH*-related malignant PPGLs, drugs targeting epigenetic pathways could be an option. Finally, the identification of a somatic mutation avoids additional germline genetic screening as new susceptibility genes are discovered, which is associated with considerable anxiety and psychological ill health, especially in relatives of paediatric and metastatic cases.^{2 3 27 54 56}

In conclusion, the results of this study should bring to an end years of controversy and debate, as it brings new evidence that highlights the need to recommend genetic testing for all patients with an apparently S-PPGL, regardless of the age at first PPGL diagnosis.

Finally, based on the present and previous findings,^{3 5 7 11 16 23 27–30 57} we propose a genetic testing algorithm

based on tumour location for sporadic single PPGL (figure 2), for laboratories where NGS is not available or not optimised. Where NGS can be used, the sample type that should be tested is tumour DNA for PCCs and germline DNA for HN-PGLs and T-PGLs. For A-PGLs, it seems crucial to have a FFPE tumour sample available in order to perform SDHB-IHC, the findings from which can be used to determine the ideal source of DNA sample to study.

Author affiliations

- ¹Hereditary Endocrine Cancer Group, Spanish National Cancer Research Centre, Madrid, Spain
²Centro de Investigación Biomédica en Red de Enfermedades Raras, Madrid, Spain
³Department of Endocrinology, Hospital General Universitario de Ciudad Real, Ciudad Real, Spain
⁴Department of Endocrinology, Hospital Universitario Severo Ochoa, Madrid, Spain
⁵Department of Endocrinology, Hospital Universitario Puerta de Hierro, Madrid, Spain
⁶Department of Endocrinology, Hospital Universitario La Paz, Madrid, Spain
⁷Department of Endocrinology, Hospital General Universitario Gregorio Marañón, Madrid, Spain
⁸Department of Endocrinology, Hospital Infanta Sofía, San Sebastián de los Reyes, Spain
⁹Department of Endocrinology, Hospital Universitario 12 de Octubre, Madrid, Spain
¹⁰Department of Endocrinology, Hospital Clínico San Carlos, Madrid, Spain
¹¹Department of Endocrinology, Hospital El Bierzo, León, Spain
¹²Department of Endocrinology, Complejo Hospitalario Universitario de Albacete, Albacete, Spain
¹³Department of Endocrinology, Hospital Universitario de La Princesa, Instituto Princesa, Madrid, Spain
¹⁴Oncologic Pathology Group, Institut de Recerca Biomèdica de Lleida, Lleida, Spain
¹⁵Department of Endocrinology, Hospital General Universitario Reina Sofía, Murcia, Spain
¹⁶Department of Pediatrics and Clinical Genetics Unit, Clínica Universidad de Navarra, Navarra, Spain
¹⁷Department of Genetics, Hospital Universitario Virgen de las Nieves, Granada, Spain
¹⁸Department of Endocrinology, Hospital Universitario de Móstoles, Madrid, Spain
¹⁹Department of Pediatric Oncology and Hematology, Hospital Universitari Vall d'Hebron, Barcelona, Spain
²⁰Department of Genetics, Hospital Universitario Donostia, Gipuzkoa, Spain
²¹Department of Endocrinology, Complejo Hospitalario de Navarra, Navarra, Spain
²²Department of General Surgery and Digestive Tract, Complejo Asistencial de León, León, Spain
²³Department of Endocrinology, Hospital Virgen de la Salud—Complejo Hospitalario de Toledo, Toledo, Spain
²⁴Department of Otorhinolaryngology, Hospital General Universitario Gregorio Marañón, Madrid, Spain
²⁵Genetic Counselling Cancer Unit, Hospital Universitari i Politècnic La Fe, Valencia, Spain
²⁶Department of Endocrinology, Hospital General Universitario de Alicante, Alicante, Spain
²⁷Department of Endocrinology, Hospital Universitario de Fuenlabrada, Madrid, Spain
²⁸Erasmus Universitair Medisch Centrum Rotterdam, Rotterdam, The Netherlands

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