INVITED REVIEW

Investigating the role of somatic sequencing platforms for phaeochromocytoma and paraganglioma in a large UK cohort

Bettina Winzeler1,2,3 | Nicola Tufton4,5 | Eugenie S. Lim4,5 | Ben G. Challis6 | Soo‐Mi Park7 | Louise Izatt8 | Paul V. Carroll9 | Anand Velusamy9 | Tony Hulse10 | Benjamin C. Whitelaw11 | Ezequiel Martin3,12 | Fay Rodger3 | Melanie Maranian3 | Graeme R. Clark3 | Scott A. Akker4,5 | Eamonn R. Maher3 | Ruth T. Casey3,6

1Department of Endocrinology, Diabetology and Metabolism, University Hospital Basel, Basel, Switzerland
2Department of Clinical Research, University of Basel, Basel, Switzerland
3Department of Medical Genetics, and Cancer Research, UK Cambridge Centre, University of Cambridge, Cambridge Biomedical Campus, Cambridge, UK
4Department of Endocrinology, St. Bartholomew’s Hospital, Barts Health NHS Trust, London, UK
5Department of Endocrinology, William Harvey Research Institute, Queen Mary University of London, London,
6Department of Endocrinology, Cambridge University Hospital, NHS Foundation Trust, Cambridge, UK
7Department of Clinical Genetics, Cambridge University Hospital, NHS Foundation Trust, Cambridge, UK
8Department of Clinical Genetics, Guy’s and St. Thomas’ NHS Foundation Trust, London, UK
9Department of Endocrinology, Guy’s and St. Thomas’ NHS Foundation Trust, London, UK
10Department of Paediatric Endocrinology, Evelina London Children’s Hospital, Guy’s and St. Thomas’ NHS Foundation Trust, London, UK
11Department of Endocrinology, King’s College Hospital NHS Foundation Trust, London, UK
12Oncology Department, Cancer Molecular Diagnostics Laboratory, University of Cambridge, Cambridge, UK

Correspondence
Ruth T. Casey, Department of Medical Genetics, and Cancer Research, UK Cambridge Centre, University of Cambridge, Cambridge Biomedical Campus, Cambridge CB2 0QQ, UK.
Email: rc674@medschl.cam.ac.uk

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Abstract

Objectives: Phaeochromocytomas and paragangliomas (PPGL) are rare neuroendocrine tumours with malignant potential and a hereditary basis in almost 40% of patients. Germline genetic testing has transformed the management of PPGL enabling stratification of surveillance approaches, earlier diagnosis and predictive testing of at-risk family members. Recent studies have identified somatic mutations in a further subset of patients, indicating that molecular drivers at either a germline or tumour level can be identified in up to 80% of PPGL cases. The aim of this study was to investigate the clinical utility of somatic sequencing in a large cohort of patients with PPGL in the United Kingdom.

Design and Patients: Prospectively collected matched germline and tumour samples (development cohort) and retrospectively collected tumour samples (validation cohort) of patients with PPGL were investigated.

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1 | INTRODUCTION

Phaeochromocytomas and paragangliomas (PPGL) are rare neuroendocrine tumours that arise from chromaffin tissue from the adrenal medulla (phaeochromocytoma) or neural crest progenitors of extra-adrenal sympathetic or parasympathetic paraganglia (paraganglioma).12 The clinical signs and symptoms vary according to the localisation of the tumours and to their hormonal activity. Treatment options include surgery, peptide receptor radionuclide therapy, targeted therapies, chemotherapy and radiotherapy. Morbidity and mortality are high in patients with metastatic disease, which account for 10%–20% of PPGL.3,4

PPGL are considered to be the most heritable tumours and over the past two decades, the identification of more than a dozen PPGL susceptibility genes5 has transformed the management of PPGL patients. More recently, the significant proportion of germline negative PPGL has motivated interest in the role of somatic sequencing for PPGL in both research and clinical settings.6–8 Furthermore, tumour sequencing has become more amenable in the era of next-generation sequencing (NGS), which offers a faster, cheaper and higher throughput option to the conventional method of Sanger sequencing. Custom NGS panels for tumour have followed in the successful path generation sequencing strategy. A screen for variants within 'mutation hotspots' in 68 human cancer genes was performed.

Measurements: Clinical characteristics of patients were assessed and tumour and germline DNA was analysed using a next-generation sequencing strategy. A screen for variants within 'mutation hotspots' in 68 human cancer genes was performed.

Results: Of 141 included patients, 45 (32%) had a germline mutation. In 37 (26%) patients one or more driver somatic variants were identified including 26 likely pathogenic or pathogenic variants and 19 variants of uncertain significance. Pathogenic somatic variants, observed in 25 (18%) patients, were most commonly identified in the VHL, NF1, HRAS and RET genes. Pathogenic somatic variants were almost exclusively identified in patients without a germline mutation (all but one), suggesting that somatic sequencing is likely to be most informative for those patients with negative germline genetic test results.

Conclusions: Somatic sequencing may further stratify surveillance approaches for patients without a germline genetic driver and may also inform targeted therapeutic strategies for patients with metastatic disease.

**KEYWORDS**
paraganglioma, phaeochromocytoma, somatic variant

of a somatic driver mutation in 32% and 37% of PPGL patients with NF1, HRAS, RET and VHL being the most frequently affected genes.7,9 Notable findings in previous studies were the association of somatic ATRX variants with aggressive tumour behaviour and the detection of mosaic mutations in SDHB and VHL.9,10 Mosaicism may be underestimated in patients with PPGL if germline DNA alone is tested.

On the basis of their underlying driver mutation at a germline or somatic level, PPGL can be divided into three main clusters.8 The first cluster includes tumours with mutations in citric acid cycle genes such as SDHx, FH, MDH2, as well as VHL gene mutations. The transcriptional signature of ‘cluster 1’ tumours is defined by abnormal stabilisation of HIF alpha transcription factors leading to pseudohypoxia.6 ‘Cluster 2’ tumours are characterised by an upregulation of kinase signalling pathways involving the mitogen-activated protein kinase pathway and the mechanistic target of rapamycin (mTOR) pathway and include mutations in genes such as RET, NF1, TMEM127 and MAX. Finally, the third cluster is defined by activation of the Wnt/beta-catenin pathway. Perturbations in this pathway have been exclusively described in sporadic PPGL with somatic variants in CSDE1 and MAML3 fusion genes.8

Despite significant advances in our understanding of PPGL tumourigenesis, a number of barriers to optimal clinical practice still exist. First, risk stratification and prediction of malignant potential have remained a major challenge.10,11 With the exception of germline SDHB mutations, no robust molecular marker for the aggressive disease is currently known.12 This poses a challenge for clinical surveillance practices as potential metastatic cases may have no germline genetic diagnosis.13,14 A lack of effective treatment options for PPGL is another significant unmet need in clinical practice.15,16 Precision therapeutics based on molecular tumour characteristics are a desirable and crucial next step to improving outcomes and quality of life for patients with these rare tumours.17
The primary aim of this study was to explore the prevalence and role of somatic driver variants in a large UK cohort of patients with PPGL using an NGS strategy to analyse ‘mutation hotspots’ in 68 human cancer genes.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

Two separate cohorts were recruited between 2018 and 2021. For the development cohort, patients from Cambridge University Hospitals, Guy’s and St. Thomas’ NHS Foundation Trust, London and from St. Bartholomew’s Hospital in London were included. For the validation cohort, tumour samples were recruited from different PPGL referral centres across Great Britain. For both cohorts, the diagnosis of PPGL was based on procedures provided by international clinical practice guidelines18,19 and was confirmed by histology in every case.

All patients provided written informed consent for sample and data collection as well as genetic testing (South Birmingham REC and East of England—Cambridge South REC, reference number: 5175 and East London and Cambridge East MREC 06/Q0104/133).

2.2 | Development cohort

Tumour and matched germline DNA samples were prospectively collected from patients with a new diagnosis of PPGL who underwent surgery or patients under ongoing clinical care for whom tumour tissue was available. Both sporadic and familial cases were included. Detailed clinical information (i.e., sex, age of onset, tumour localisation and extension, metastatic disease, secretion pattern and family history) was collected. In June 2021, follow-up information including recurrent disease (multiple tumours or metastatic disease) and survival was assessed for all patients.

2.3 | Validation cohort

Tumour DNA samples were retrospectively collected from patients with sporadic and hereditary PPGL tumours. Matched germline DNA was not available for these patients. Clinical information including sex, age of onset and primary localisation of the tumour was accessible, but other clinical characteristics and follow-up data were not available. Results of germline genetic testing were collected when possible.

2.4 | Targeted gene panel and sequencing technique

Tumour and matched germline DNA were sequenced and analysed using a custom-designed NGS panel based on the Ion AmpliSeq™ Cancer Panel covering ‘mutation hotspots’ in 68 human cancer genes and additional bespoke content to cover all exons and flanking sequences of 12 PPGL-related genes plus EPAS1- and VHL-targeted exons (Table S1).

2.5 | Bioinformatics analysis

All samples were aligned to the hg38 version of the reference human genome using bwa 0.7.17 in alt contig aware mode as described by the authors.20 The generated SAM file was compressed into a BAM file and sorted by genomic position using samtools 1.9.21 The sorted BAM files were subject to Base Quality Score Recalibration and Indel Realignment as specified in the Genome-Analysis Toolkit (GATK)22 best practices.23,24 For somatic variant calling the following GATK’s MuTect25 was used. A panel of normals (PON) was generated using the germline samples with GATK’s (version 4.0.3.0) MuTect2 and CreateSomaticPanelOfNormals algorithms. Variants were called in all tumours using the PON and the matched germline sample with the GATK’s MuTect2 algorithm to generate a VCF file.25 Finally, the VCF files were filtered with GATK’s FilterMutectCalls algorithm. The resulting VCF file was annotated and prioritised using annovar.26

2.6 | Variant filtering

Synonymous variants and noncoding variants were removed. Variants were removed if the variant allele frequency was <10% or the minor allele frequency (MAF) greater than 0.1% in EVS6500 and/or 1000 Genomes. All variants with a read depth less than two standard deviations below the mean coverage (<500 reads) were filtered out. Variants in the intronic and intergenic regions, synonymous variants, variants which failed the ‘artefact-in-normal’ and ‘base quality’ (minimum base quality below 20) filters, were also discarded. Finally, variants that were classified as ‘benign’ or ‘likely benign’ on the Catalogue of Somatic Mutations in Cancer (COSMIC) (https://cancer.sanger.ac.uk/cosmic) or ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) were removed. For those tumour samples without a matched germline, further variant filtering was performed if a common germline variant or single nucleotide polymorphisms was identified (Figure 1).

2.7 | Variant classification

For the purpose of this study, a somatic variant was defined as a potential driver variant if the variant allele frequency was >10%. Sanger sequencing validation was performed on 10 samples with suspected somatic driver variants. Other validation methods including SDHB immunohistochemistry, ex-vivo tumour metabolomics using NMR spectroscopy and hybrid capture-based sequencing were performed on single tumour samples to validate specific somatic driver variants. Identified driver variants were classified as: pathogenic, likely pathogenic or a variant of uncertain significance (VUS).
based on evidence available from the Catalogue of Somatic Mutations in Cancer (COSMIC) (https://cancer.sanger.ac.uk/cosmic) or ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). ***<500 reads was less than two standard deviations below the mean coverage). ^Including multiple variants in the same tumour. Validation of suspected driver variants was performed using; (i) Sanger sequencing for 10 cases.

2.8 | Statistical analysis

Statistical tests were performed using the statistical software package R (R Foundation for Statistical Computing). Summary statistics include median and interquartile ranges (IQR) for continuous variables and frequency and percentage for categorical variables.

For detailed information about study methodology please see the Supporting Information Appendix.

3 | RESULTS

One hundred patients were analysed in the development cohort and 41 patients in the validation cohort.

3.1 | Baseline characteristics of the pooled cohort

In the pooled patient data set (development and validation cohort), 76 (54%) patients were male and the median (IQR) age at diagnosis was 47 (36, 62). Germline genetic testing results were available for all but one patient in the development cohort, and for 31 (76%) cases in the validation cohort. A third of tested patients (45/130, 35%) harboured a germline mutation, most frequently in the SDHx genes. The most frequent tumour location was adrenal in 89 (63%) patients, followed by an extra-adrenal abdominal location in 34 (24%) patients. Multiple tumours were noted in 10 (7%) patients and median [IQR] tumour size was 44.5 mm (31.5, 62.5).

In the development cohort, 52 patients (52%) had a noradrenaline-only secreting tumour, 16 patients (16%) had metastatic disease and 3 patients died from metastatic PPGL during the study period. Baseline characteristics of the pooled patient data set as well as the individual development and validation cohorts are shown in Table 1.

3.2 | Somatic and matched germline sequencing

Tumour DNA (all primary tumours) was extracted from paraffin-embedded tumour samples and fresh frozen tissue in 136 (96.5%) and five (3.5%) samples, respectively. Matched germline DNA was
extracted from blood in 97 patients and from adjacent normal tissue in two patients of the development cohort.

### 3.3 Quality assessment of sequencing assay

The mean coverage calculated across all sequencing runs was 2171.64 reads, median coverage was 2402.86 and the standard error was 91.62482 (SD 1044.68). The coverage ranged from 30.66 to 7071.21 reads (see Figure S1). A higher frequency of C>T variants consistent with DNA damage from formalin fixation was noted in the FFPE samples, however, this mutational signature was not significant at a higher allele frequency (>5%).

### 3.4 Detection of somatic variants of the pooled cohort

Somatic sequencing revealed the presence of one or more potential somatic driver variants in 37 (26%) patients of the pooled cohort including 26 pathogenic variants and 19 variants of uncertain significance (see Figure 1). Excluding patients with VUS, 25 (18%) of patients were found to have one (except V36 had two) pathogenic or likely pathogenic variant.

The most frequent affected genes (affected by both pathogenic variants and VUS) were \textit{NF1} (n = 7), \textit{VHL} (n = 5), \textit{HRAS} (n = 4), \textit{EPAS1} (n = 4) and \textit{RET} (n = 3). All but three somatic variants were detected in patients without a germline mutation (exceptions: D86 with germline and somatic \textit{SDHA} variant and a VUS in \textit{KRAS}, V11 with germline \textit{SDHD} variant and somatic VUS in \textit{FH}, V38 with germline \textit{SDHB} variant and two somatic VUS in \textit{SDHA}) (see Tables 2 and S3).

Pathogenic variants in ‘cluster 1’ genes (e.g., \textit{SDHx}, \textit{FH} and \textit{VHL} genes) were more frequent at the germline level, whereas ‘cluster 2’ genes (such as \textit{RET}, \textit{HRAS} and \textit{NF1}) were most frequently mutated at the somatic level (see Figure 2).

### 3.5 Genetic characterisation and clinical features of development cohort

In the development cohort 32 (32%) patients harboured a germline variant and 29 (29%) patients had one or more somatic variant (21 pathogenic, 9 uncertain). Genetic variants were exclusively at the germline or somatic level in all but one patient (D86).

The most common affected genes at the germline level were \textit{SDHx} (n = 19), \textit{TMEM127} (n = 3) and \textit{VHL} (n = 3). All germline variants were classified as pathogenic or likely pathogenic with the exception of a missense VUS in the \textit{TMEM127} gene (c.398A>G, p.His133Arg), (case D28, with bilateral phaeochromocytoma). Tumour sequencing from this case demonstrated loss of heterozygosity suggesting that the germline variant was likely pathogenic and causative in this case coupled with the phenotype of multifocal tumours at a young age.

| TABLE 1 Baseline characteristics of study patients |
|-----------------------------------------------|
| **Number of patients** | **Pooled cohorts (141)** | **Development cohort (100)** | **Validation cohort (41)** |
| Sex (male), n (%) | 76 (54) | 55 (55) | 21 (51) |
| Age at diagnosis (years), median [IQR] | 47 [36, 62] | 48 [37, 66] | 42 [35, 49] |
| Genotype, n (%) | No mutation 85 (61) | 67 (67) | 18 (44) |
| Mutation 45 (32) | 32 (32) | 13 (32) |
| No information 11 (8) | 1 (1) | 10 (24) |

| Genotype affected gene, n (%) | SDHB 19 (14) | 12 (12) | 7 (17) |
| SDHD 6 (4) | 2 (2) | 4 (10) |
| SDHA 6 (4) | 5 (5) | 1 (2) |
| SDHC 2 (1.4) | 2 (2) | 0 (0) |
| VHL 4 (3) | 3 (3) | 1 (2) |
| TMEM127 3 (2) | 3 (3) | 0 (0) |
| RET 2 (1.4) | 2 (2) | 0 (0) |
| NF1 1 (0.7) | 1 (1) | 0 (0) |
| MAX 1 (0.7) | 1 (1) | 0 (0) |
| FH 1 (0.7) | 1 (1) | 0 (0) |

| Tumour localisation, n (%) | Adrenal 89 (63) | 67 (67) | 22 (54) |
| Extra-adrenal abdomen 34 (24) | 24 (24) | 10 (24) |
| Extra-adrenal mediastinum 2 (1.4) | 2 (2) | 0 (0) |
| Head and neck 14 (10) | 5 (5) | 9 (22) |
| Bladder 2 (1.4) | 2 (2) | 0 (0) |

| Multiple tumours, n (%) | 10 (7) | 8 (8) | 2 (5) |
| Maximum tumour size (mm), median [IQR] | 44.5 [31.5, 62.5] | - |
| Metastatic disease, n (%) | 16 (16) | - |
| Death, n (%) | 3 (3) | - |
| Secretory pattern, n (%) | - | - |
| Nonfunctional | 9 (9) | - |
| Adrenaline | 4 (4) | - |
| Noradrenaline | 52 (52) | - |
| Mixed | 32 (32) | - |
| Family history, n (%) | 11 (11) | - |
Likely pathogenic and pathogenic somatic variants of the development cohort

Of the 21 patients with a pathogenic or likely pathogenic somatic driver variant 17 (81%) patients presented with an adrenal tumour, 3 (14%) with an extra-adrenal abdominal paraganglioma and one with a HNPGL (5%) (Figure 3). A pathogenic or likely pathogenic variant was identified in the NF1 gene in six cases, RET in two cases, VHL in four cases and HRAS in three cases. In the remaining cases somatic variants in EPAS1, SDHB, SDHD, IDH, FGFR3 and TP53 were identified (Table 2 and Figure 3).

The youngest patient in the cohort (case D22) was diagnosed with a phaeochromocytoma at age 9 years (age range in the cohort: 9–87 years). This patient did not have a germline pathogenic variant.

### Table 2: Molecular classification of detected driver somatic variants in the development cohort

| ID | Gene | Variant | rs ID | Variant type | Variant classification | Variant allele frequency (%) | Validated by Sanger sequencing* |
|----|------|---------|------|--------------|------------------------|-------------------------------|-------------------------------|
| D2 | HRAS | c.182A>G, p.Glu61Lys | rs121913233 | Nonsynonymous | Pathogenic | 38 | Yes |
| D5 | RET  | c.2753→C, p.Met918Thr | rs74799832 | Nonsynonymous | Pathogenic | 39 | No |
| D10 | RET  | c.2753→C, p.Met918Thr | rs74799832 | Nonsynonymous | Pathogenic | 39 | No |
| D15 | SDHD | c.14G>A, p.Try5Ter | rs104894310 | Stop gain | Pathogenic | 31 | No |
| D22 | VHL  | c.371C>T, p.Thr124Ile | rs193922610 | Nonsynonymous | Likely pathogenic | 36 | No |
| D23 | VHL  | c.250G>A, p.Val84Met | rs5030827 | Nonsynonymous | Pathogenic | 36 | No |
| D30 | RET  | c.1898T>G, p.L633R | - | Nonsynonymous | Uncertain | 30 | No |
| D32 | SDH4 | c.1679C>T, p.Val560Met | rs775350508 | Nonsynonymous | Likely pathogenic | 36 | No |
| D40 | EPAS1 | c.1589C>T, p.A530V | - | Nonsynonymous | Uncertain | 51 | No |
| D51 | BRAF | c.1801A>G, p.Glu61Met | rs121913364 | Nonsynonymous | Uncertain | 40 | No |
| D53 | NF1  | c.3338delT, p.L1113fs | - | Frameshift | Likely pathogenic | 45 | No |
| D54 | NF1  | c.2014G>T, p.Glu672X | - | Frameshift | Likely pathogenic | 60 | No |
| D56 | NF1  | c.3513delG, p.K1171fs | - | Frameshift | Likely pathogenic | 10 | No |
| D61 | KIF1B | c.1204C>T, p.L402F | rs764084679 | Nonsynonymous | Uncertain | 30 | No |
| D62 | EPAS1 | c.1681C>T, p.Q561X | - | Stop gain | Uncertain | 22 | No |
| D65 | FGFR3 | c.1125T>A, p.Y375X | - | Stop gain | Likely pathogenic | 45 | No |
| D67 | TP53 | c.527G>A, p.C176Y | rs786202962 | Nonsynonymous | Likely pathogenic | 13 | No |
| D68 | VHL  | c.366A>G, p.Leu122Pro | rs1559428119 | Nonsynonymous | Uncertain | 22 | No |
| D73 | HRAS | c.182C>T, p.Glu61Pro | rs121913233 | Nonsynonymous | Likely pathogenic | 29 | No |
| D77 | SDHB | c.423+1G>A | rs398122805 | Splice site | Likely pathogenic | 15 | Yes |
| D78 | VHL  | c.482C>T, p.Arg161Gln | rs730882035 | Nonsynonymous | Likely pathogenic | 33 | Yes |
| D79 | NF1  | c.2927_2933delCTGAAGG, p.Thr976fs | - | Frameshift | Likely pathogenic | 36 | Yes |
| D84 | IDH1 | c.394C>T, p.Arg132Cys | rs121913499 | Nonsynonymous | Likely pathogenic | 40 | Yes |
| D86 | KRAS | c.88G>A, p.Arg115Leu | - | Nonsynonymous | Uncertain | 13 | No |
| D86 | SDHA | c.1270G>T, p.Glu424X | - | Stop gain | Likely pathogenic | 27 | Yes |
| D87 | FBXW7 | p.Cys384fs | - | Frameshift | Uncertain | 70 | Yes |
| D88 | VHL  | c.245G>A, p.Arg82Leu | rs794726890 | Nonsynonymous | Likely pathogenic | 25 | Yes |
| D95 | HRAS | c.182A>C, p.Glu61Pro | rs121913233 | Nonsynonymous | Likely pathogenic | 41 | No |
| D97 | NF1  | c.792delC, p.Ser264fs | - | Frameshift | Likely pathogenic | 15 | Yes |
| D98 | NF1  | c.209delA, p.Thr700fs | - | Frameshift | Likely pathogenic | 40 | Yes |

Note: Clinical and genetic characteristics of the validation cohort are shown in Table S3.

*YES means that Sanger sequencing was performed and the variant confirmed. NO means that Sanger sequencing was not performed.
but a likely pathogenic somatic variant in VHL (c.371C>T, p.Thr124Ile, variant allele frequency [VAF] 28%), with no evidence of this variant in germline DNA on the analysis performed with greater than 200× coverage.

A likely pathogenic somatic variant in SDHB was identified in case D77. This patient presented aged 19 years with a metastatic para-aortic paraganglioma with local lymph node involvement and underwent curative surgery. Immunohistochemistry of this tumour demonstrated loss of expression of the SDHB protein indicating an SDH deficient tumour. Germline genetic analysis revealed no pathogenic variant. The likely pathogenic SDHB variant (c.423+1G>A) identified in this tumour (validated by Sanger sequencing) had a variant allele frequency of only 15% suggesting that this variant alone is not sufficient to explain the SDH deficiency. Promoter methylation analysis of the SDHC promoter in this tumour did not reveal hypermethylation and further extended genetic analysis of tumour and germline DNA is planned for this case.

A somatic driver variant was identified in one further case with an extra-adrenal paraganglioma (case D84). This patient presented at 50 years of age with an extra-adrenal abdominal paraganglioma. A likely pathogenic variant in IDH1 (c.394C>T, p.Arg132Cys) was identified and validated by Sanger sequencing and ex vivo tumour metabolomics confirming pathological accumulation of 2-hydroxyglutarate in the tumour tissue.

3.7 | Variants of unknown significance of the development cohort

A somatic VUS in a candidate driver gene was identified in nine cases in the development cohort including one case (case D86) with a metastatic paraganglioma in whom a pathogenic germline and an additional somatic driver variant in SDHA, as well as a VUS in KRAS, was found (Table 2 and Figure 3).

A missense variant in EPAS1 (c.1589C>T, p.A530V, VAF 51%) was identified in a patient with abdominal PGL (case D40). This variant lies in a mutation ‘hot-spot’ in the vicinity of prolyl hydroxylase residues and has been reported in patients with Pacak–Zhuang syndrome. To date this case has not developed any other tumours or manifestations suggestive of Pacak–Zhuang syndrome. Neither germline nor salivary DNA was available for further analysis for this patient at the time of manuscript preparation but the patient remains under close follow up.

A novel truncating variant was identified in EPAS1 in case D62 but the pathogenicity of this variant is not clear. The majority of reported disease-causing variants in EPAS1 have been missense variants affecting specific ‘hotspots’ between amino acids 529 and 539 of the protein. This case presented with a noradrenergic phaeochromocytoma at age 50 years and to date has not developed any further tumours (Figure 3).

3.8 | Metastatic disease in the development cohort

In this cohort, 16 patients had metastatic disease of whom seven were carriers of a pathogenic germline mutation (four in SDHB, one each in FH, SDHA and TMEM127). Ten patients with metastatic disease had an extra-adrenal abdominal tumour and six harboured a phaeochromocytoma only. Three cases of metastatic PPGL were found to harbour a potential driver somatic variant. This included case D86 with a somatic SDHA variant and a coexisting pathogenic germline SDHA variant. The remaining two cases included case D77 discussed above with a driver somatic variant in SDHB and case D87. This patient developed widespread...
metastatic disease within 5 years of her initial presentation and died from progressive metastatic phaeochromocytoma. A truncating somatic VUS was identified in the \textit{FBXW7} gene in her tumour. This variant was confirmed by capture-based sequencing.

3.9 Genetic characterisation and clinical features of validation cohort

In the validation cohort, a germline variant was identified in 13 (32%) patients. One or more somatic variants were identified in eight (20%) patients (five pathogenic or likely pathogenic and 10 variants of uncertain significance). No pathogenic somatic variant was identified in patients with germline variants in the validation cohort but two patients with germline variants harboured a somatic VUS (Case V11 and case V38, Table S2). Two patients with abdominal PGL (case V7 and V16) were found to harbour an \textit{EPAS1} VUS (c.1589C>T, p.A530V) with VAFs of 67% and 36% respectively. This \textit{EPAS1} variant was the same as the one detected in case D40 of the development cohort and lies as discussed in a mutation ‘hot-spot’ in the vicinity of prolyl hydroxylase residues. A likely pathogenic variant in \textit{HRAS} (c.182A>G, p.Q61R, VAF 65%) and a likely pathogenic \textit{ERBB4} variant...
(c.2828C>T, p.P943L, VAF 22%) were each found in a patient with a pheochromocytoma (cases V15 and V37). Detailed molecular information for the validation cohort is provided in Table S3.

4 | DISCUSSION

In this large UK cohort of 141 patients, a pathogenic germline variant was recorded for 45 (32%) cases while a pathogenic somatic variant was identified in 25 (18%) patients, taking the overall number of patients with a somatic and or germline genetic driver to 69 (49%).

The frequency of somatic variants noted in this study was less than others (see Table S4)7–9 but this may be explained by the higher proportion of patients with germline genetic mutations that were included in this study, the sequencing method used (panel vs. WES) and the gene panel selection, which was missing some key genes (e.g., ATRX).

Somatic driver variants in NF1, VHL and HRAS were among the most commonly identified in this study and this correlates with published reports from large somatic sequencing studies in PPGL and the COSMIC somatic genetic variant data for PPGL.7–9 Somatic variants in NF1, HRAS, KRAS and BRAF affect the RAS/RAF/ERK pathway. Therefore, therapeutic targeting with agents such as MEK, RAF or ERK1/2 inhibitors may be an option for patients with malignant PPGL and driver variants in these genes in the future.29

A likely pathogenic somatic variant in genes involved in hypoxia signalling was identified in 14 cases (10%) in this study including five patients with a variant in VHL, four cases with a pathogenic variant in one of the SDHx genes and one patient with an IDH1 mutation.

Further four patients had a VUS in EPAS1, three of them had a variant affecting a mutation ‘hot-spot’. This is noteworthy as Belzutifan (PT2977, MK-6482), a highly selective small molecule that inhibits the function of the HIF-2α transcription factor, is currently under investigation in phase-2 studies for patients with advanced solid tumours and may prove beneficial for patients with genetic alterations affecting the oxygen-sensing pathway. Furthermore, tumours with citric acid cycle gene mutations at risk of metabolic vulnerability and accumulation of oncometabolites such as succinate and 2-hydroxyglutarate (e.g., case D84), may also be more susceptible to synthetic lethal targeting with poly(ADP)-ribose polymerase inhibitors.30

Finally, a single case with metastatic PPGL and a truncating somatic variant in FBXW7 was identified in this study (D87). Mutations in the FBXW7 gene have been implicated in renal neoplasia31–33 and studies have suggested that inactivation of FBXW7 may predict clinical response to mTOR inhibitors.34 Unfortunately, this patient died from progressive disease before experimental therapies could be considered.

In addition to informing potential therapies for patients with metastatic PPGL, somatic profiling may allow a more personalised follow-up strategy for patients with apparently sporadic PPGL. In 3 (2.2%) cases of this cohort, a somatic variant in EPAS1 (c.1589C>T, p.A530V), which lies within a mutation ‘hot-spot’, was identified. Although these cases have not developed features suggestive of Pacak–Zhuang syndrome, mutations in EPAS1 are considered to be exclusively somatic or mosaic predisposing to the development of other tumours including multiple paragangliomas, neuroendocrine tumours and polycythaemia.27 Mutations in EPAS1 are best identified

| ABL1 | FGFR1 | PIK3CA |
| AKT1 | FGFR2 | PRKACA |
| ALK | FGFR3 | PTEN |
| APC | FH | RB1 |
| ATM | FLT3 | RET |
| ATRX | GNAS | SDHA |
| BRAF | G0T2 | SDHB |
| BAP1 | H3F3A | SDHC |
| CACNA1D | HNF1A | SDHD |
| CDKN1B | HRAS | SDHAF2 |
| CDKN1C | IDH1 | SDT2 |
| CDKN2A | IDH2 | SLC25A11 |
| CDKN2C | KDR | SMAD4 |
| CTNNB1 | KDM2B | SMARC1 |
| DAXX | KIF1B | SMO |
| DLST | KIT | TMEM127 |
| DNMT3A | KRAS | TPS3 |
| EGFR | MAX | TET1 |
| EGLN1 | MDH2 | TET2 |
| EGLN3 | MEN1 | VHL |
| EPAS1 | MET | |
| ERBB2 | NF1 | |
| ERBB4 | NSD3 | |
| EZH2 | NRAS | |
| FBXW7 | PDGFRA | |

FIGURE 4 Gene wish list for a targeted PPGL (pheochromocytomas and paragangliomas) gene panel. Gene wish list was selected based on published literature7,40–43 and the top 20 mutated genes in PPGL on COSMIC (Catalogue of Somatic Mutations in Cancer)
through tumour sequencing as variant allele frequency can be below the threshold of detection in blood using conventional sequencing methods such as Sanger sequencing. It should be noted that mosaic variants in other genes including VHL and SDHB\textsuperscript{35,36} have been reported in patients with PPGL. Indeed, two young cases (including one paediatric case) in this cohort, aged 9 and 21 years, were identified with a somatic driver variant in VHL (D22, D68) and although the variants were not identified in the germline DNA (analysed using NGS with 200× coverage) from either case, the patients remain under close follow up. Therefore, the identification of a mosaic variant detected through tumour and germline sequencing (and or other normal tissue, e.g., saliva), should prompt lifelong surveillance analogous to the surveillance that would be considered for a germline carrier of the identified gene.

It is also noteworthy that no somatic driver variants in HRAS were identified in cases of hereditary PPGL in this study, reiterating the observation that variants in HRAS and known hereditary PPGL genes are mutually exclusive drivers of tumourigenesis.\textsuperscript{37} Increased utility of somatic sequencing in clinical practice will allow further validation of this observation and may facilitate stratification of long-term management and reassurance regarding a potential missed germline genetic driver in patients with an identified somatic HRAS variant. The observation that somatic driver variants are more frequent in sporadic versus hereditary PPGL in this study and others\textsuperscript{7,8} would also suggest that somatic molecular profiling may be best utilised as a potential biomarker in sporadic PPGL.

The translation of tumour sequencing into routine clinical practice requires consideration of both clinical utility for the specific disease as well as key practical implications. In the past reliance on fresh frozen tumour samples to facilitate tumour sequencing has proven prohibitive in a clinical setting, however in recent years protocols for DNA isolation from paraffin-embedded tumour samples and protocols for bioinformatics analysis have advanced,\textsuperscript{38,39} thus facilitating good quality somatic sequencing from paraffin-embedded tumour samples, which are more readily available in clinical practice. Targeted gene panels have a number of associated benefits including cost-effectiveness, low DNA concentration requirements and high sequencing depth, making them a popular choice in a clinical setting for tumour sequencing. Gene panels can be bespoke and modified from centre to centre but the panel adopted should aim to include the most commonly implicated genes and to balance the potential for translational research by including novel research genes versus the risk of identifying frequent variants of uncertain significance (see Figure 4).

In conclusion, a pathogenic or likely pathogenic driver somatic variant was identified in 25 (18%) patients with PPGL in this large UK cohort, including 3/16 (18%) patients with metastatic disease. This study has highlighted clinical applications of PPGL tumour sequencing including the potential for specific somatic variants to inform long-term surveillance strategies and the potential to select more personalised treatment options. The implementation of somatic sequencing for PPGL into routine clinical practice may further advance personalised treatment and surveillance strategies for patients with PPGL.

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**CONFLICT OF INTERESTS**

The authors declare that there are no conflict of interests.

**DATA AVAILABILITY STATEMENT**

We may share deidentified, individual participant-level data that underlie the results reported in this article and related documents. Data will be available with the publication of the manuscript on receipt of a request detailing the study hypothesis and statistical analysis plan. All requests should be sent to the corresponding author. The steering committee of this study will discuss all requests and decide on the basis of the scientific rigour of the proposal whether data sharing is appropriate. All applicants are asked to sign a data access agreement.

**ORCID**

Bettina Winzeler https://orcid.org/0000-0001-8305-2700
Nicola Tufton https://orcid.org/0000-0003-2382-9711
Ben G. Challis https://orcid.org/0000-0002-1130-2851
Louise Izatt https://orcid.org/0000-0003-1258-4843
Scott A. Akker https://orcid.org/0000-0003-3893-3116
Ruth T. Casey https://orcid.org/0000-0003-4058-3135

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SUPPORTING INFORMATION
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