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Consensus Statement on next-generation-sequencing-based diagnostic testing of hereditary pheochromocytomas and paragangliomas

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EXPERT CONSENSUS DOCUMENT

Consensus Statement on next-generation-sequencing-based diagnostic testing of hereditary pheochromocytomas and paragangliomas

The NGS in PPGL (NGSnPPGL) Study Group, Rodrigo A. Toledo^{1,2}, Nelly Burnichon^{3,4}, Alberto Cascon⁵, Diana E. Benn⁶, Jean-Pierre Bayley⁷, Jenny Welander⁸, Carli M. Tops⁹, Helen Firth¹⁰, Trish Dwight⁶, Tonino Ercolino¹¹, Massimo Mannelli¹¹, Giuseppe Opocher¹², Roderick Clifton-Bligh⁶, Oliver Gimm¹³, Eamonn R. Maher¹⁰, Mercedes Robledo⁵, Anne-Paule Gimenez-Roqueplo^{3,4} and Patricia L. M. Dahia¹

Abstract | Pheochromocytomas and paragangliomas (PPGLs) are neural-crest-derived tumours of the sympathetic or parasympathetic nervous system that are often inherited and are genetically heterogeneous. Genetic testing is recommended for patients with these tumours and for family members of patients with hereditary forms of PPGLs. Due to the large number of susceptibility genes implicated in the diagnosis of inherited PPGLs, next-generation sequencing (NGS) technology is ideally suited for carrying out genetic screening of these individuals. This Consensus Statement, formulated by a study group comprised of experts in the field, proposes specific recommendations for the use of diagnostic NGS in hereditary PPGLs. In brief, the study group recommends target gene panels for screening of germ line DNA, technical adaptations to address different modes of disease transmission, orthogonal validation of NGS findings, standardized classification of variant pathogenicity and uniform reporting of the findings. The use of supplementary assays, to aid in the interpretation of the results, and sequencing of tumour DNA, for identification of somatic mutations, is encouraged. In addition, the study group launches an initiative to develop a gene-centric curated database of PPGL variants, with annual re-evaluation of variants of unknown significance by an expert group for purposes of reclassification and clinical guidance.

Phaeochromocytomas and paragangliomas (PPGLs) are catecholamine-secreting, neural-crest-derived tumours of the adrenal medulla and extra-adrenal sympathetic nervous system, respectively^{1,2}. Paragangliomas can also arise from the parasympathetic nervous system; these tumours are usually located in the head and neck and typically do not secrete catecholamines³. Approximately 50% of PPGLs are caused by a single driver germ line mutation, which means that these tumours are the most highly heritable tumours in humans¹. Due to this high heritability, genetic testing has been recommended in all patients with PPGLs independent of a clear family

history⁴. Another striking characteristic of PPGLs is their genetic heterogeneity. Over 15 different susceptibility genes have been implicated in familial cases; however, the susceptibility gene has not been identified in all cases, which indicates that this number will continue to grow in the near future^{1,2} (TABLE 1). As a result of this large number of driver genes, genetic diagnosis of PPGLs by traditional technologies, including PCR-based amplification followed by Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) for larger gene disruptions, is becoming impractical as they are laborious, costly and time consuming.

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Genetic testing algorithms based on clinical features (that is, tumour localization, malignancy and syndromic characteristics), biochemical profile (that is, types of catecholamines secreted by the tumour) or immunohistochemistry pattern have been developed to aid prioritizing genetic testing of a single or a few PPGLs susceptibility genes^{5–9}. Although this approach is helpful for patients in whom a pathogenic driver mutation is identified promptly, it can be cumbersome when this quick identification does not happen, as the analysis must be extended to the remaining susceptibility genes. Notably, when variants of unknown significance (VUS; variants for which the pathogenicity is not clear) are found in the initial test, expanded screening is required in an effort to identify a more plausible causative mutation¹⁰.

The technology that has become widely known as next-generation sequencing (NGS) was first introduced in 2005 (REF. 11). Using novel methods of sequencing by ligation or synthesis, NGS platforms enhanced the capability of genetic testing by many orders of magnitude. In the first decade of its use, NGS methodology was improved to increase throughput, accuracy and speed, while simultaneously reducing costs and experimental complexity. Currently available NGS platforms are powerful and flexible, and can be adapted easily to the analysis of a single gene region in thousands of samples, or for sequencing the entire genome of a single patient. The implementation of NGS has been a paradigm shift in genetics research and is now considered the gold standard for genetic diagnosis^{12,13}. NGS has also been widely embraced by the fields of cancer and hereditary diseases. Therefore, inherited neoplasia, a group to which PPGLs belong, represent a particularly relevant class of disorders where the use of NGS for diagnostic purposes deserves special focus.

Recognizing the need to develop standards for broad implementation of NGS as a methodology for clinical diagnosis of hereditary PPGL, a Study Group comprising international experts from the Pheochromocytoma

and paraganglioma REsearch Support Organization (PRESSOR, R.A.T., P.L.M.D., A.-P.G.-R., N.B., M.R., A.C., D.E.B., T.D., R. C.-B., J.P.B., C.M.T., J.W., O.G., H.F., E.M., M.M., T.E., G.O.) and the PPGL working group of the European Network for the Study of Adrenal Tumors (ENS@T, A.-P.G.-R., N.B., M.R., A.C., J.P.B., C.M.T., J.W., O.G., H.F., E.M., M.M., T.E., G.O.) was formed to spearhead discussions on the application of NGS for diagnostic genetic testing in PPGLs (NGSnPPGL).

Methods

The NGSnPPGL Study Group was comprised of 18 experts in PPGLs from ten separate institutions representing eight countries and included both clinicians who provide genetic counselling for their patients and basic researchers who design and perform the diagnostic tests. All participants have adopted, and reported on, NGS-based technologies in their research and/or clinical practice^{14–26}. Discussions took place via conference calls, e-mail communications and file exchanges and one plenary session (at the 14th ENS@T Scientific Meeting on November 20th, 2015, Munich, Germany). In these multiple exchanges, current practices and literature evidence were critically reviewed and a set of recommendations was developed to guide broad implementation of this methodology for the diagnosis of hereditary PPGLs. During these encounters, pertinent technical, ethical and reporting issues were addressed. This Consensus Statement summarizes the outcome of these discussions.

While many of the topics included in this Consensus Statement are common to other hereditary conditions and/or cancers, aspects unique to PPGLs were considered when making recommendations (BOX 1). As in many other fields, guidelines for NGS-based testing are continually evolving and the recommendations set out here will be subject to change as our knowledge advances. Therefore, the current guidelines are based on the evidence available in 2016.

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Table 1 | Genes involved in PPGL pathogenesis

Gene	Frequency of mutations detected in PPGLs (mutation type)	Refs
ATRX	<5% (S)	96,98
BRAF	<2% (S)	15,45
CDKN2A	<2% (S)	96
EGLN1/PHD2	<1% (G or S)*	99,100
EPAS1	6–12% (M or S)	19,43,52,77
FGFR1	~1% (S)	14
FH	1–2% (G)	24,43,101
H3F3A	<2% (M)*	14
HRAS	7–8% (S)	43,102
IDH2	<0.5% (S)	103
KIF1B	<5% (G or S)	18,104,105
KMT2D	<2% (G or S)*	106
MAX	1–2% (G or S)	107
MDH2	<2% (G)*	108
MERTK	<2% (G)*	14
MET	<2% (G) or <2–10% (S)*	14,96
NF1	3% (G) or 20–25% (S)	109,110
RET	5–6% (G or S)	44,111
SDHA	<1% (G or S)	112
SDHAF2	<1%	113
SDHB	8–10% (G)	43,111
SDHC	1–2% (G)	43,47,111
SDHD	5–7% (G)	43,111
TMEM127	1–2% (G)	43,114
TP53	<5% (S)	96
VHL	7–10% (G or S)	43,111

The numbers shown here are in part based on data generated by the TCGA (The Cancer Genome Atlas) Research Network for Pheochromocytoma and Paraganglioma (unpublished data, publicly available through cBioPortal). G, germ line; S, somatic; M, mosaic; PPGLs, pheochromocytomas and/or paragangliomas. *Frequency based on one or two clinical cases.

General ethical considerations

Specific written informed consent must be obtained from all patients following standards for diagnostic genetic testing established by certified and accredited diagnostic laboratories of individual countries^{27,28}. Special consideration is required when whole-exome sequencing (WES), whole-genome sequencing (WGS) or extended gene panels (that is, not limited to PPGL susceptibility genes) are used. In these circumstances, patients should indicate if they wish to be informed of incidental findings. These findings represent significant genetic variants in a specific group of genes (unrelated to PPGLs) that are implicated in disorders that require medical action, such as those specified in recommendations by the American College of Medical Genetics and Genomics (ACMG)²⁹.

Samples

The sample requirements for NGS are similar to those currently adopted for clinical diagnosis of PPGLs using Sanger sequencing. For the analysis of germ line variants

in PPGLs susceptibility genes, laboratories should request blood (fresh (collected <7 days ago) or frozen) or a frozen leukocyte pellet. When a blood sample is not available, laboratories can accept buccal cells either as a cheek swab or in saliva obtained with specific collection kits containing preservatives³⁰. DNA extraction and quality assessment should follow standard procedures established for conventional genetic testing^{29,31,32}.

For the analysis of somatic variants in PPGLs susceptibility genes, laboratories should require fresh frozen tumour fragments (50 mg of frozen tissue provides a sufficient amount of high-molecular weight DNA for sequencing). Alternatively, formalin-fixed paraffin embedded (FFPE) sections or other fixed tumour material (for example, maintained in alcohol) might also be acceptable; however, the quality of DNA from these materials is variable and can be suboptimal^{33,34}. DNA from the tumour should be processed and assessed for quality according to standard protocols^{29,31,32}. Over the past few years, protocols for DNA, and even RNA, isolation from FFPE samples have considerably improved, and technical adaptations for handling potential artefacts generated from these materials have yielded increasingly reliable sequencing data, which has expanded the use of FFPE in clinical settings³⁵. Tumour tissue, when available, can provide valuable information that will aid interpretation of results from germ line samples. For example, identification of loss of heterozygosity (LOH) in a region where a potential pathogenic germ line mutation of a tumour suppressor gene is detected supports and reinforces the likelihood of pathogenicity. In patients with hereditary PPGLs, the tumour DNA is used exclusively for the purposes of supplementing the diagnostic value of germ line variants of unclear pathogenic status (see additional details in a subsequent section). To this end, the tumour sample could be analysed by either Sanger sequencing or specific targeted sequencing (single gene or exon); therefore, the tumour sample (frozen or FFPE) should be of sufficient amount and quality to provide reliable genotype results.

NGS-based platform and processing

After considering costs, turnaround time, autonomy of individual laboratories, assay flexibility, scalability, bioinformatics needs, data storage and data interpretation, a consensus was achieved by the Study Group that targeted NGS is currently the favoured method for genetic diagnosis of PPGLs. Specific recommendations for implementing this method are as follows.

Approach. Amplicon-based targeted sequencing was the approach preferred by the Study Group, as this approach has been adopted and successfully optimized by the majority of the group members in their own laboratories. However, no objections were raised regarding the use of the hybridization-captured NGS method.

Depth coverage. The minimum recommended sequence depth coverage was 100x for each sample from blood or saliva. Higher coverages (200x or higher) might be required for detection of mosaic variants in blood or saliva.

Box 1 | Features unique to PPGLs

Hereditary Mendelian diseases are caused by one driver mutation inherited in an autosomal dominant or recessive manner. This feature is relevant because the finding of a single unquestionably pathogenic mutation will define the proband's diagnosis and should trigger testing of the specific mutation in at-risk family members. Approximately 50% of pheochromocytomas and paragangliomas (PPGLs), a rate higher than any other human neoplasia, are caused by an autosomal dominantly inherited mutation detectable in the germ line^{1,2}.

Mosaic transmission, in addition to classic germ line transmission, of PPGLs can also occur. The *EPAS1* gene was found to be somatically mutated in PPGLs and in patients who had an association between these tumours and polycythaemia and/or, rarely, duodenal somatostatinomas^{19,75–77}. Further studies have demonstrated that these mutations can be mosaic, and are occasionally detected in non-tumorous tissue at a low frequency⁷⁸. Detection of these low-representation alleles requires the use of highly sensitive techniques such as NGS. Therefore, the Study Group suggests inclusion of *EPAS1* in the group of genes mutated at the germ line level.

The extent to which mosaicism occurs in PPGLs has not been systematically examined across all known susceptibility genes. Both *NF1* and *VHL*, which are established PPGL susceptibility genes, have been detected as mosaic, post-zygotic mutations in neurofibromatosis type 1 or von Hippel–Lindau syndrome; however, this finding has not been described in the specific setting of PPGLs^{79,80}. In 2015, mosaic mutations leading to a syndrome involving PPGL and giant cell tumours of bone were reported in association with the *H3F3A* gene¹⁴. Therefore, mosaic transmission might occur more frequently in PPGLs than hitherto appreciated.

In-laboratory validation. Sensitivity and specificity of the developed NGS assay should be established by individual groups based on data obtained from a set of samples carrying known mutations (identified by Sanger sequencing). This positive control group should include samples spanning a comprehensive set of mutations (point mutations and indels) and origins (germ line, mosaic and somatic). The Study Group also suggested that samples positive for rare mutations, which might not be available to every laboratory in their positive control set, could be shared among multiple laboratories to enable the development of more uniform and comprehensive ‘calibration sets’. Distribution of such DNA materials in an anonymized manner would be subject to sample availability, approval of the institutions’ ethics committees and material transfer agreement arrangements. Importantly, it is recommended that a set of normal reference samples of matching ethnic background is also sequenced using the same NGS platform to determine false positive rates of the assay and to establish the frequency of common and private or population-specific polymorphisms.

Limitations. Special attention should be given to limitations of NGS methods for sequencing and detection of variants in specific regions of the genome, including homopolymer repeats, indels, AT-rich regions and GC-rich regions³⁶. Some NGS techniques, such as Ion Torrent (Life Technologies/ThermoFisher, Waltham, Massachusetts, USA), rely on single-nucleotide additions and can have a high error rate for indel detection (1%)³⁷. Illumina platforms have high sensitivity (0.1%); however, false-positive errors have also been reported^{37,38}. AT-rich regions and GC-rich regions are well known to be problematic in conventional PCR and Sanger sequencing³⁸. These areas can also be challenging for capture by target

and WES probes and, therefore, tend to be underrepresented by NGS. If regions of low coverage are noticed, complementary assays should be designed using a different method (for example, Sanger sequencing) to achieve the desired minimal coverage of the target region. Off-target sequencing (unwanted regions) might occur in genomic regions with low sequence complexity, which can be removed by filtering during sequencing analysis.

Confirmation. Given the reasons outlined in the previous section, the detection of a variation or mutation in a new sample should be confirmed using an orthogonal method, such as Sanger sequencing, real-time PCR genotyping or a distinct NGS-based assay. As in conventional genetic testing, whenever possible, confirmation of the NGS-identified variant in a separate aliquot of the patient’s DNA (ideally obtained from an independent blood or saliva sample) is highly recommended. However, the Study Group recognizes that this practice is not universally adopted by diagnostic laboratories.

Whole-exome sequencing. The Study Group chose WES as the preferred method for investigational genetic analysis for PPGLs, with the research purpose of discovering the primary mutation when none is found among the PPGLs susceptibility genes. WES coverage can vary greatly but a mean coverage of 50x or higher was recommended for identification of germ line variants. With decreasing costs of NGS methodology, the ability to sequence at progressively higher depth without added budgetary burden makes this coverage goal easily attainable.

Quality control. Efficient capture of exons and adjacent regions, quality of sequencing and error rates are influenced by the reagents and kits used in library preparation and exome capture, as well as by the chemicals and equipment used for sequencing³⁸. For laboratories that adopt commercial NGS services or institutional core facilities for processing their samples, it is critical to ensure that every step of the protocol is performed following strict quality control standards, using reliable reagents and sequencers with low error rates. The Study Group recommends establishing a bioinformatics pipeline in which at least two algorithms are used for sequence alignment with the goal of enhancing both the sensitivity and specificity of sequence calls¹².

Other considerations. Low-coverage WES is not suitable for clinical sequencing. WES should be considered for patients with PPGLs who have no germ line mutations in the genes analysed by targeted NGS, also referred to as a ‘negative PPGL’. However, before labelling a sample ‘negative’ it is imperative to establish a comprehensive analysis of all known PPGLs susceptibility genes. This analysis should not only include sequence evaluation of coding regions and exon–intron boundaries of target genes, but also large indels or gene rearrangements. These grosser defects, which have been reported in the *VHL*, *SDH* and *MAX* genes^{39–42}, might not be identifiable by WES performed at average depth of coverage. Instead, other

Table 2 | Modified ClinVar review status adapted for this Consensus Statement on PPGLs driver genes

Original ClinVar classification		Modified classification	
Number of gold stars	Description and review status	Evidence level	Specific applicability to PPGLs
None	No submitter provided an interpretation with assertion criteria (no assertion criteria provided), or no interpretation was provided (no assertion provided)	0	N/A
One	One submitter provided an interpretation with assertion criteria (criteria provided, single submitter) or multiple submitters provided assertion criteria but there are conflicting interpretations, in which case the independent values are enumerated for clinical significance (criteria provided, conflicting interpretations)	1	Single source (one published report)
Two	Two or more submitters providing assertion criteria provided the same interpretation (criteria provided, multiple submitters, no conflicts)	2	Two or more sources without functional validation
Three	Reviewed by expert panel	3	Two or more sources with some functional validation
Four	Practice guideline	4	Established evidence from: clinical, genetic, computational prediction, functional evidence and/or analysis of population frequency

N/A, not applicable; PPGLs, pheochromocytomas and/or paragangliomas.

methods that are well-established for detection of these genetic lesions, such as MLPA, quantitative multiplex PCR or other genome-wide copy number analysis assays, can be performed^{43,44}. Alternatively, targeted NGS panels can be designed to optimize detection of larger deletions, insertions or rearrangements, as reported in hereditary breast cancer diagnostic panels⁴⁵. Finally, the existence of epimutations, such as those detected in the promoter of the *SDHC* gene should also be considered in cases where no mutations are detected^{46,47}. Specific attention to the mode of inheritance and the existence of mosaicism are briefly discussed in BOX 1.

Targeted NGS PPGLs gene panels

With the important advances in our understanding of the genetics of PPGLs that have occurred in the past decade, a large number of genes have been implicated in susceptibility to PPGLs, which are also known as ‘driver’ genes (TABLE 1). Some of these driver genes are only mutated at the germ line level, while others can be mutated either at the germ line or somatic level. A third group of driver genes are only mutated somatically^{1,2,48}. The relative frequency of overall mutations and specific germ line and/or somatic events for each of these genes varies (TABLE 1). Although accumulated evidence regarding the role of some of these susceptibility genes is fairly extensive, as expected, the discoveries from the past few years have not yet been fully validated clinically, genetically or functionally.

An extensive discussion on the requirements for determining a *bona fide* ‘driver status’ of the PPGLs susceptibility genes is beyond the scope of this Consensus Statement. Therefore, to harmonize the current evidence available for each gene we have applied general concepts of tumour predisposition genes⁴⁹ and the ‘review status’ established by ClinVar, the public archive of reports of

the relationships among human variations and phenotypes curated by the [National Center for Biotechnology Information](#) (NCBI). ClinVar uses a five-level rank of evidence to establish variant pathogenicity that was suggested by the American College of Medical Genetics and Genomics^{50,51} (TABLE 2). In this Consensus Statement, we adopted a modified version of ClinVar’s ‘gold star’ scale to create three PPGLs panel types based on the current evidence of involvement of these genes in PPGLs susceptibility at the germ line (the basic premise for hereditary PPGLs screening) and somatic level. On the basis of the current literature, we propose the development of three sets of gene panels for the diagnosis of PPGLs (TABLE 3). TABLE 4 lists the genes that belong to each panel type, and summarizes the current level of evidence of their pathogenic driver status. Importantly, as our knowledge of the genetics of PPGLs evolves, re-evaluation of this list and reclassification of susceptibility genes will be warranted.

Basic panel. The basic panel includes genes with the highest level of evidence for their involvement in the pathogenesis of PPGLs and that are mutated at the germ line level. These genes have been extensively validated in the literature and are predominantly associated with familial disease or syndromic features.

Extended panel. The extended panel includes all ‘basic panel’ genes, along with other candidate susceptibility genes that are mutated at the germ line level and are found at a low frequency (<1% of hereditary PPGLs) but that have been proven to be functionally relevant. This panel also includes genes that can contain mutations with mosaic transmission and that might occasionally also be detected in non-tumour tissue, including blood or saliva (for example, *EPAS1*, also known as *HIF2A*).

Table 3 | Target panel versions based on ClinVar gold star variant evidence level

Panel type	Gene list	Sample type*
Basic	Evidence level 3 and 4 for germ line mutations	Blood
Extended	Evidence level 2 for germ line mutations (rare)	Blood
Comprehensive	All somatic mutations and all levels of evidence	Blood and tumour

*Buccal cells can be used as a source of germ line DNA when blood is not available.

Comprehensive panel. The comprehensive panel includes all ‘extended panel’ genes, genes found to be exclusively mutated at the somatic level and recently identified genes mutated at the germ line and/or somatic levels for which the evidence is still limited due to the low number of events. The comprehensive panel can be used for blood, saliva and tumour tissue analysis.

The target area

The suggested targeted panels should encompass coding exons and intron boundaries of the targeted genes. At this stage, the Study Group opted to exclude deep intronic, promoter and intergenic regions from the panel design, as the pathogenic relevance of variants detected in these genomic areas is of unclear diagnostic value. In addition, special caution should be taken when designing primers that target areas of homology to pseudogenes or other partially homologous sequences, which can confound variant interpretation. Furthermore, the inclusion of just the hotspot exons of two PPGLs oncogenes, *EPAS1* (exons 9 and 12) and *RET* (exons 8, 10, 11 and 13–16), instead of the entire coding region, was favoured for gene panels given the highly selective mutation distribution of these oncogenes^{2,15,43,52}. However, these settings might need to be re-evaluated as the field evolves⁵³.

Other sequencing methods

WGS is the most comprehensive method for mutation analysis, as it can be used to assess nearly all types of genetic disruptions (including large deletions and insertions) of the entire coding and noncoding regions of PPGLs susceptibility genes without amplification bias introduced by PCR. The main barriers for using WGS in clinical diagnostics are the high cost, the need for expert bioinformatics support to perform the analysis and the necessity for a multidisciplinary expert group to help with variant interpretation (see subsequent section on variant reporting). However, the technological advances in the past few years indicate that soon some of these issues will no longer be impediments. The sequencing of the entire human genome for less than US\$1,000 is finally possible with the launch of new sequencers focused on population-scale and production-scale genomics, such as Illumina X10 (REF. 54). In addition, automated bioinformatics analyses with cloud-based shared free software have been developed and continue to be implemented by many leading institutions in the field of advanced genomics and biocomputing⁵⁵. These shared spaces will enable the analysis of whole genomes without the requirement for individual institutional acquisition of super computers or rental of computer clusters.

Another NGS method that generates information on nucleotide variation is the sequencing of the entire collection of mRNA molecules (RNA-seq), which yields both expression profile and mutational status. Using RNA-seq could be considered when a fresh frozen tumour sample is available. A possible limitation of this technique is the difficulty in identifying mutations that lead to decreased or absent transcription or very unstable mRNA of the target gene. RNA-seq has been performed in only a small number of PPGLs^{14,26}. Although not tested for all known driver genes for PPGLs, at least in one report, truncating germ line mutations in *SDHB* and *SDHD* and a missense germ line mutation in *VHL* were promptly detected with evidence of LOH, and later validated by Sanger sequencing¹⁴, which supports the efficacy of RNA-based sequencing for screening of susceptibility mutations. RNA-seq might also enable detection of fusion transcripts that cannot be identified by WES²⁶. However, given the added technical and material source challenges, the Study Group recommends that DNA is the preferred source of material for mutation screening of PPGLs susceptibility genes in routine practice.

Somatic variants

Studies published over the past 5 years have demonstrated that somatic mutations frequently occur in PPGLs (TABLE 1). These mutations are only detected in the tumour DNA and not in germ line DNA, and, therefore, they do not have implications for heritability of the disease. Detection of a PPGL-related somatic mutation in the tumour of a germ line ‘negative PPGL’ suggests sporadic disease, and, consequently, averts the need for screening the patient’s relatives. Moreover, identification of a somatic mutation provides insights into tumour biology, and might guide targeted therapies, especially in patients with metastatic disease (when treatment options are limited)^{56–59}. The Study Group recommends that the analysis of somatic mutations in PPGLs be carried out whenever possible. Genes targeted at the somatic level are indicated in TABLE 1. These genes should be included in panels in which both germ line and tumour analyses are performed. The number of clinical trials available for patients with non-operable and/or metastatic PPGLs is currently small (ClinicalTrials.gov; search for active PPGL trials), so expanding our knowledge of potential targets can have a broad effect on therapeutic choices for these patients (see [Supplementary information S1](#) (table)).

Additional clinical trials based on therapies that target specific molecular findings are expected to be developed in the near future. Moreover, new discoveries

Table 4 | Gene panels of PPGLs based on current evidence

Gene	Review status*	Targeted panel†	Gene target area
<i>FH</i>	3	Basic	All coding exon–intron boundaries
<i>MAX</i>	4	Basic	All coding exon–intron boundaries
<i>NF1</i>	4	Basic	All coding exon–intron boundaries
<i>RET</i>	4	Basic	Exons 8, 10, 11, 13–16
<i>SDHA</i>	3	Basic	All coding exon–intron boundaries
<i>SDHB</i>	4	Basic	All coding exon–intron boundaries
<i>SDHC</i>	4	Basic	All coding exon–intron boundaries
<i>SDHD</i>	4	Basic	All coding exon–intron boundaries
<i>TMEM127</i>	4	Basic	All coding exon–intron boundaries
<i>VHL</i>	4	Basic	All coding exon–intron boundaries
<i>EGLN1/PHD2</i>	2	Extended	All coding exon–intron boundaries
<i>EPAS1</i>	4	Extended	Exons 9, 12
<i>KIF1B</i>	2	Extended	All coding exon–intron boundaries
<i>MET</i>	2	Extended	All coding exon–intron boundaries
<i>SDHAF2</i>	2	Extended	All coding exon–intron boundaries
<i>ATRX</i>	2	Comprehensive	All coding exon–intron boundaries
<i>BRAF</i>	1	Comprehensive	All coding exon–intron boundaries
<i>CDKN2A</i>	1	Comprehensive	All coding exon–intron boundaries
<i>EGLN2/PHD1</i>	1	Comprehensive	All coding exon–intron boundaries
<i>FGFR1</i>	2	Comprehensive	Exons 11, 13
<i>H3F3A</i>	1	Comprehensive	All coding exon–intron boundaries
<i>HRAS</i>	4	Comprehensive	All coding exon–intron boundaries
<i>IDH2</i>	1	Comprehensive	All coding exon–intron boundaries
<i>KMT2D</i>	1	Comprehensive	All coding exon–intron boundaries
<i>MDH2</i>	1	Comprehensive	All coding exon–intron boundaries
<i>MERTK</i>	1	Comprehensive	All coding exon–intron boundaries
<i>TP53</i>	2	Comprehensive	All coding exon–intron boundaries

PPGLs, pheochromocytomas and/or paragangliomas. *Based on ClinVar, related to TABLE 2. †Related to TABLE 3.

focused on personalized drug therapies for PPGLs are anticipated to guide the development of more research trials. In this context, the NGSnPPGL Study Group recommends the analysis of somatic mutations in all metastatic PPGLs, as mutations in ‘druggable’ genes might be identified, which could help guide therapeutic choices and/or select patients for genetics-based clinical trials.

Data reporting

A written report of the genetic test results has to be clear, concise, understandable by non-experts and in full compliance with general recommendations for reporting results of diagnostic genetic testing^{60,61}. The results report should include administrative information, such as name and full contact details of the laboratory performing the analysis, date, name and address of the referring physician and signature of the laboratory specialist who validated and interpreted the results.

Patient identification should include patient name (or unique identifier, as in the case of some referral diagnostic laboratories), date of birth, sex and, ideally, ethnicity. Sample details, such as tested material type, date of sample collection and arrival at the laboratory and unique sample identification number should also be part of the report.

Other sample information, including histological confirmation of PPGLs diagnosis, tumour location, tumour number, occurrence of metastasis, age at first diagnosis, hormonal phenotype, family pedigree with clinical information and personal and/or familial history of other diseases or clinical manifestations consistent with syndromic forms of PPGLs or any familial history of other associated tumours or diseases can add valuable information to the interpretation of the results. In addition, results from immunohistochemistry analysis of tumour sections, if available, can also be relevant.

A summary of technical information should be included, encompassing a list of targeted genes, the sequencing platform used, any appropriate kits (for example, library generation and capture), the mean sequence coverage achieved for the target genes (including areas of low coverage, if any) and the bioinformatics pipeline (alignment and annotation software used). The test results, their interpretation and technical limitations (that is, whether the test is capable, or not, of detecting specific variants such as mosaic mutations or copy number changes) must be specified. Importantly, test results and variant interpretation should be consistent across laboratories to avoid variability that could affect clinical decision-making.

The results report should mention whether the findings were confirmed by another method, and whether Sanger sequencing was used to fill gaps of poorly covered regions. These reports should also state whether MLPA or other methods have been performed to detect large rearrangements. Examples of effective NGS-based reports are included in the reference list^{60,61}.

Variant classification

The International Agency for Research on Cancer (IARC)⁶² has classified genetic variants into five categories (class 5: 'pathogenic'; class 4: 'likely pathogenic'; class 3: 'VUS'; class 2: 'likely not pathogenic'; and class 1: 'not pathogenic'), and this system has been adopted by most laboratories^{61,63}. The five-category system is the most comprehensive classification system for molecular geneticists and research experts; however, the Study Group recognizes that a simplified classification in three categories only ('pathogenic', 'VUS' and 'benign') can be considered for reports to physicians and for genetic counselling purposes, as this distinction is usually adequate for clinical decision-making.

The objective of gathering the information detailed in this section is to integrate all available evidence of pathogenicity or non-pathogenicity from the different criteria listed here to reach a conclusion on the status of the detected variants.

For variants identified in genes associated with PPGLs, the Study Group proposes a simplified framework (FIG. 1) that should provide an objective and reproducible classification for the majority of identified variants. Recognizing that some variants will have more complex requirements for classification, the Study Group recommends following the rules proposed by Richard and colleagues, which combine multiple criteria for classifying variants⁶³. Variant classification requires the combination of several criteria, which are described in the following paragraphs^{63,64}.

Criterion 1: the type of mutation. Whether or not it is likely to result in a null variant should be considered. For example, nonsense, frameshift or canonical splice sites (positions ± 1 or ± 2 bp) that result in frameshift of the coding sequence, mutations that affect the initiation codon, or those that lead to single exon or multi-exon deletion are generally considered null mutations, although this concept is proving to have some notable exceptions⁶⁵.

Criterion 2: the variant frequency in large population databases. These databases include [dbSNP](#), [Exome Aggregation Consortium \(ExAC\)](#), [Exome Variant Server](#) and [1000 Genomes](#). Of note, these databases do not include only healthy individuals and, therefore, can contain some pathogenic variants. This feature is particularly relevant in the case of low-penetrance alleles, such as *SDHB* mutations, and their association with malignant PPGLs⁶⁶. Thus, the simple presence of a VUS in any of these databases does not invalidate their potential pathogenic role in PPGLs. In addition, these databases are not independent datasets, so some redundancy exists. Local, population-specific reference datasets (such as the Spanish National database⁶⁷), if existent, can be informative as they help uncover population-based VUS frequency bias.

Criterion 3: the variant description in disease or gene-specific databases. These databases include [ClinVar](#), the [Human Gene Mutation Database](#), [Leiden Open source Variation Database](#) and in-house databases. These sources provide various levels of annotation of variants, including in many cases the clinical context in which they were described. Of note, not all variants from these databases have been systematically curated or are described with the most updated nomenclature. Therefore, pathogenicity of variants cannot be assumed solely by its presence in any of these databases (please see additional details later in the article).

Criterion 4: previous reporting of the variant in the literature. Variants reported as pathogenic or possibly pathogenic with limited evidence in a single patient, or a small number of patients, must be carefully evaluated before being considered a PPGL-predisposing mutation, as they could be rare or private polymorphisms.

Criterion 5: whether the variant was previously functionally evaluated. This analysis should determine whether the variant is pathogenic or not. Importantly, establishing the functional effect of a variation must involve rigorous standards. For example, certain variations in downstream effectors might not necessarily reflect pathogenicity and should not be used as the sole criterion to classify a variant^{68,69}.

Criterion 6: in silico predictions. These predictions focus on the pathogenicity of missense or splice site variants. The Study Group recommends using multiple prediction software packages (including SIFT, PolyPhen2 and MutationTaster for missense mutations or MaxEntScan, Splice Site Finder Like and NNSplice for splice prediction), all of which are freely available, or commercial software integrating multiple sources such as [Alamut Visual](#). The results generated by these prediction programmes can help interpretation, but should not be taken as the sole or final determinant of pathogenicity of a variant.

Criterion 7: co-occurrence of pathogenic variants. The co-occurrence can either be in the same gene or in another susceptibility gene. On the basis of the general premise

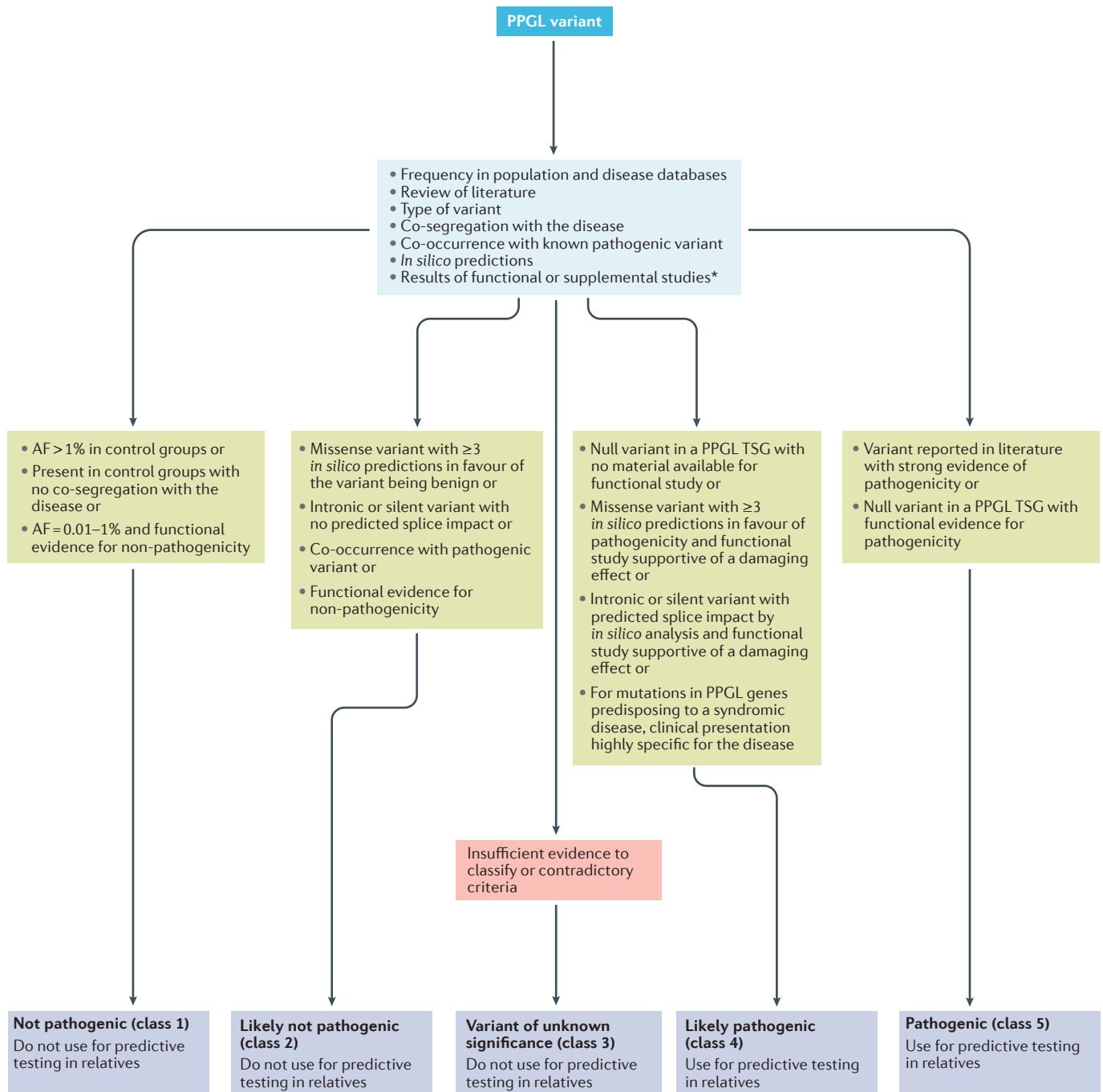


Figure 1 | **Framework for variant interpretation of pheochromocytomas and/or paragangliomas (PPGLs) susceptibility genes into five classes based on the likelihood of pathogenicity.** The classification relies on multiple criteria listed in each box and further detailed in the text. AF, allele frequency; TSG, tumour suppressor gene. *See TABLE 5 for additional details.

of mutual exclusivity of driver mutations in PPGLs¹, the detection, in the same individual, of a known pathogenic variant either in the same gene or in other PPGL genes essentially excludes pathogenicity of the unknown variant.

Criterion 8: analysis of co-segregation of the disease in families. The presence of the same variant in other

affected relatives supports pathogenicity of the variant. However, more importantly, the lack of co-segregation (for example, at least one affected relative without the variant) excludes its role as the susceptibility mutation in that family. Absence of the candidate variant in unaffected relatives can also be helpful to ascribe a role for a candidate variant in families.

Box 2 | PPGLs-associated syndromes, other associations and shared susceptibility

Pheochromocytomas and paragangliomas (PPGLs) have been reported as part of well-established hereditary syndromes, including multiple endocrine neoplasia type 2A and 2B (*RET*), von Hippel–Lindau syndrome (*VHL*), neurofibromatosis type 1 (*NF1*) and familial paraganglioma syndromes, type 1 (*SDHD*), 2 (*SDHAF2*), 3 (*SDHC*), 4 (*SDHB*) and 5 (*SDHA*)^{1,2,48}.

Gastrointestinal stromal tumours can associate with paragangliomas due to germ line *SDH* mutations (Stratakis–Carney dyad)^{81,82}. *EPAS1* mosaic mutations have been detected in patients with multiple paragangliomas, duodenal somatostatinomas and polycythaemia^{80,83}. Pituitary adenomas were reported in patients with PPGLs in familial settings in which an *SDH* mutation was detected^{84,85}. Germ line *FH* mutations have been described in patients with pheochromocytomas and uterine leiomyomas^{14,20,24}, and, in the past year histone gene mutations (*H3F3A*) were found in a new syndrome of multiple paragangliomas and giant cell tumour of bone, which implicates chromatin remodelling defects in PPGL tumorigenesis and susceptibility¹⁴.

The growing link between susceptibility to renal carcinomas and PPGLs is worth highlighting. An increasing number of genes can be responsible for development of both tumour types individually or in association, which suggests a closer connection than previously appreciated^{86–89}. In addition to *VHL*, *SDH*^{90,91}, *FH*⁹² and *TMEM127* (REFS 93,94), genes can also be mutated in renal carcinomas either with or without co-occurrence of PPGL. The *MET* gene, previously known to cause hereditary papillary renal cancer⁹⁵, was also found to be mutated in PPGLs^{14,96}. Finally, somatic mutations in chromatin remodelling genes are recurrently detected in renal carcinomas and PPGLs^{14,96–98}.

Further associations between PPGLs and other conditions might be detected in the future. Their rare occurrence makes it challenging to establish a causative link, but those infrequent associations can offer invaluable insights into the biology of these tumours and possible paths to their development.

Criterion 9: concordance with phenotype. Concordance with phenotype is especially relevant in susceptibility genes for established syndromes, such as neurofibromatosis type 1, von Hippel–Lindau disease or multiple endocrine neoplasia type 2, for which other clinical manifestations generally coexist with PPGL (BOX 2). Given the diversity of PPGL susceptibility genes and associated subphenotypes, the Study Group recommends that the referring physicians provide detailed phenotypic information to assist the laboratory in analysing and interpreting the results of testing. This information could help to prioritize variants for further consideration⁶¹. An example of this situation is the *RET* gene, in which the identification of an unquestionable pathogenic mutation has clear clinical implications (for example, thyroidectomy to prevent the development of the associated, highly penetrant medullary carcinoma of the thyroid). By contrast, detection of *RET* VUS should not trigger indiscriminate screening of relatives⁶⁹.

Variant reporting

Reports should provide a list of variants with clinical interest only. Gene name, zygosity status, cDNA nomenclature and protein nomenclature must be clearly defined and follow Human Genome Organization (HUGO) criteria⁷⁰ (see later).

The Study Group recommends that variants classified as not pathogenic (class 1) or likely not pathogenic (class 2) should not be reported, as the report of a common SNP or silent variant can generate anxiety for patients and relatives. In addition, reports should clearly distinguish known pathogenic (class 5) or likely pathogenic (class 4) variants from VUS (class 3).

Whether and how to report VUS can be laboratory-dependent but physicians should be aware of the policies about reporting VUS^{61,62}. When multiple variants that might be clinically important are identified (for example,

class 4 variants), they should be prioritized according to their relevance to the patient's phenotype. For example, in a patient with a syndromic clinical presentation suggesting von Hippel–Lindau disease, if NGS identifies a VUS in the *VHL* gene and a second VUS in another gene associated with PPGL, the *VHL* variant is more likely than the second variant to be disease-causing and, therefore, should be emphasized. A comprehensive review of interpretation of VUS of the *RET* gene has discussed this challenge⁶⁹.

Nomenclature

The gene names used in reports should adhere to the approved [HUGO Gene Nomenclature](#)⁶¹. The reference nucleotide and protein sequence accession number (and version number) should be indicated.

The [Human Genome Variation Society](#) (HGVS) nomenclature is currently the standard worldwide and is recommended for variant reporting⁷¹. Unambiguous naming of the variants is critical for the patient's medical records as well as for the pre-symptomatic genetic testing that could be offered to the patient's relatives. Indeed, screening for the mutation in a family generally comprises Sanger sequencing that exclusively targets the mutation identified in the proband. Misleading mutation nomenclature could lead to the amplification and sequencing of a different gene region and return a false negative result in relatives. Correct nomenclature is also critical for unambiguous registration of the data in human variation databases and for accurate searches for a previous description of any identified variant.

Interpretation

The interpretation section of the report should clearly state whether any identified variant is likely to be responsible for the patient's PPGL development and should also include the evidence that supports the

Table 5 | Supplementary and/or functional tests available for PPGL genes

Type of material required	Test description	Expected results	Target gene	Target mutation type	Evidence level of test*	Refs
Tumour or leukocyte RNA	cDNA sequencing	Identification of aberrant RNA transcript	All PPGLs susceptibility genes	Splice mutations	B	108,114
Tumour DNA	Sequencing, MLPA, SNP array or microsatellite markers study (for copy number analysis)	Identification of LOH (by deletion or somatic mutation of the contralateral allele)	PPGLs tumour suppressor genes	All	B	105,107, 110,111
	Methylation analysis	Identification of methylation of the contralateral allele	PPGLs tumour suppressor genes	All	B	21
Frozen tumour tissue	SDH enzymatic activity measurement	Loss of SDH enzymatic activity	<i>SDH</i>	All	A	115
	Fumarase enzymatic activity measurement	Loss of fumarase enzymatic activity	<i>FH</i>	All	A	20
	Succinate concentration	Accumulation of succinate	<i>SDH</i>	All	B	20
	Fumarate concentration	Accumulation of fumarate	<i>FH</i>	All	B	20
	Metabolome profiling by HRMAS NMR spectroscopy	Accumulation of succinate	<i>SDH</i>	All	C	124
FFPE tumour tissue section	SDHB/SDHD IHC	SDHB negative or SDHD positive IHC	<i>SDH</i>	All	A	5,116–118
	SDHA IHC	SDHA negative IHC	<i>SDHA</i>	All	A	112,117,119
	MAX IHC	MAX negative IHC	<i>MAX</i>	Truncating mutations	B	22
	2-SC IHC	2-SC positive IHC	<i>FH</i>	All	B	101
<i>In vitro</i> assay	PC12 rat pheochromocytoma cell-based luciferase reporter assay (mutagenesis)	Alteration of MAX regulatory effects on MYC	<i>MAX</i>	Missense	C	120
	Endomembrane localization or subcellular distribution of expressed mutant constructs by confocal microscopy	Intracellular distribution of TMEM127 mutant	<i>TMEM127</i>	Missense	C	94,114,121
	SDH cellular distribution	Loss of SDH mitochondrial localization	<i>SDH</i>	All	C	122
<i>In vivo</i> assay	<i>In vivo</i> succinate detection by magnetic resonance spectroscopy	Accumulation of succinate <i>in vivo</i>	<i>SDH</i>	All	C	123

FFPE, formalin-fixed and paraffin-embedded; IHC, immunohistochemistry; LOH, loss of heterozygosity; MLPA, multiplex ligation-dependent probe amplification; PPGLs, pheochromocytomas and/or paragangliomas; RT-PCR, reverse-transcriptase PCR; SDH, succinate dehydrogenase; 2-SC, succinyl-cysteine, SNP, single nucleotide polymorphism. *Evidence level of test: A, widely used, with strong functional accuracy, and independently validated by multiple laboratories; B,

variant classification. The report should list additional studies, if available, that could be performed to assist in further clarifying the variant classification. Supplemental material (such as the frozen tumour sample, FFPE tumour block or slides and/or RNA samples) required for these additional studies should be requested from the referring physician. Similarly, the participation of family members for segregation analysis should be requested, if appropriate. Finally,

the report should mention whether a pre-symptomatic genetic test can be offered to first-degree relatives or not.

VUS and tools for classification

The interpretation of VUS is challenging and in general these variants should not be used for clinical management of patients and families. VUS can be classified based on multiple parameters (described in a previous section)⁶⁹.

Box 3 | Available PPGL-related variant databases

- NF1; https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php?select_db=NF1
- VHL; <http://www.umd.be/VHL/>
- RET; http://www.arup.utah.edu/database/men2/men2_welcome.php
- SDHA; <http://databases.lovd.nl/shared/genes/SDHA>
- SDHB; <http://databases.lovd.nl/shared/genes/SDHB>
- SDHC; <http://databases.lovd.nl/shared/genes/SDHC>
- SDHD; <http://databases.lovd.nl/shared/genes/SDHD>
- SDHAF2; <http://databases.lovd.nl/shared/genes/SDHAF2>
- FH; <http://databases.lovd.nl/shared/genes/FH>
- MAX; https://grenada.lumc.nl/LOVD2/shared1/home.php?select_db=MAX
- TMEM127; <http://databases.lovd.nl/shared/genes/TMEM127>
- EPAS1; https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php?select_db=EPAS1
- EGLN1; https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php?select_db=EGLN1

PPGL, pheochromocytoma and paraganglioma.

Additional tests and functional studies, listed in TABLE 5, can be performed to assess the pathogenicity of variants. These tests require different types of biological material and have distinct degrees of complexity and accuracy. When a VUS is identified in a PPGL tumour suppressor gene, the demonstration of tumour LOH, either by deletion or additional somatic mutation, is a strong argument supporting its pathogenicity, according to the Knudson ‘two-hit’ hypothesis⁷². However, it is important to note that the accuracy of the LOH analysis might be dependent on the marker or primer that was used. In addition, the frequency of somatic deletion of the region of interest can vary considerably (that is, chromosome 1p LOH, which spans the *SDHB* gene locus, is frequent in PPGLs but chromosome 5p LOH, which comprises the *SDHA* locus, is not). Thus, results of LOH analyses have to be interpreted with caution.

Molecular geneticists and researchers of the Study Group agreed on sharing data and protocols of functional assays previously developed in their respective laboratories or institutions, as well as on providing specialized technical assistance for newly identified variations that can aid in their classification. As distinct assays have been developed and optimized by individual laboratories, sharing of these protocols and controls will occur on a case-by-case basis, according to the specific variant and gene involved.

An international PPGL variant database

Public gene-specific databases already exist for various genes that predispose to PPGLs (BOX 3). The Study Group emphasizes the importance of multi-institutional, internationally shared efforts to compile resources of genomic and clinical data, as well as publicly accessible deposition of novel variants (for example, *ClinVar* and *Decipher*). In the interest of economy of scale and technology development, the Study Group recommends adopting the existing gene-centred database integrated in the framework of

the Leiden Open-source Variation Database (LOVD) system⁷³ rather than creating a disease-specific database. Accurate classification of variants requires databases specifically curated by a panel of PPGLs experts who span the range of expertise for each gene and associated functional studies. As recommended by the IARC, a consensus opinion on variant pathogenicity validated by a panel of experts should be established before making the report available^{62,74}.

To that end, the Study Group launched an initiative to establish gene-oriented groups of experts (including both basic researchers and clinicians) from multiple institutions worldwide to submit and review variants. During the 14th ENS@T Scientific Meeting on November 20th, 2015, Munich, Germany, the Study Group launched the first pilot of the PPGL Database Project, which focused on the *SDHB* gene. The objectives of this project are, firstly, to collect standardized genomic and clinical data for each submitted *SDHB* variant. Secondly, to review manually each variant and combine multiple lines of evidence for classification. Requests for additional information or supplementary functional analyses will be made when necessary. Thirdly, to develop standardized, transparent and consensus criteria for variant classification. Fourthly, to conclusively assign each variant to one of the existing classes (1–5; FIG. 1). Fifthly, to update and re-evaluate the variant list annually (see subsequent section).

Re-evaluation of VUS

This re-evaluation process will take place during ENS@T or PRESSOR face-to-face meetings or conference calls. These meetings will also address other details pertinent to the structure and configuration of the LOVD databases. A summary of the proposed database algorithm is shown in FIG. 1.

During the annual update of the PPGL Database Project, undefined variants (also known as class 2 or class 3 variants, which are not usable for predictive testing in relatives) could be assigned to the pathogenic or disease-causing mutation group (usable for predictive testing), on the basis of new evidence from the literature or functional assays. In that situation, information about variant re-classification will be disseminated to all Study Group participants. These professionals, in turn, should advise the referring physicians of the new classification status, when applicable. A summary of a suggested flow-chart for genetic testing is depicted in FIG. 2. More rarely, change in status of class 1 or class 5 mutations will also trigger a similar process.

Conclusions

Advances in sequencing technologies within the past few years led a majority of genetics laboratories to adopt NGS as the new gold standard for routine diagnosis. NGS is especially pertinent for diseases with broad genetic heterogeneity, as is the case with hereditary PPGLs. Considering the technological challenges inherent to NGS methodology, PPGL experts emphasize the

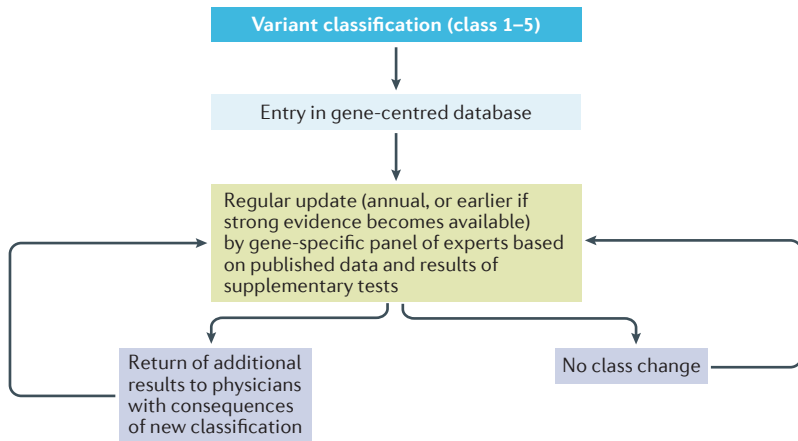


Figure 2 | Proposed workflow for re-evaluation of genetic variants detected in pheochromocytomas and/or paragangliomas (PPGLs). An annual review of variants might lead to re-classification based on new research and/or clinical evidence, with an effect on clinical follow up and screening of at-risk family members. Class 1 (not pathogenic), class 2 (likely not pathogenic), class 3 (variant of unknown significance), class 4 (likely pathogenic), class 5 (pathogenic).

need for specific recommendations. The main objective of this Consensus Statement is to support worldwide good laboratory practices and quality standards for

clinical application of NGS for PPGLs diagnosis, taking into account technical, interpretational and reporting aspects of genetic variants.

In brief, the Study Group recommends using a validated targeted gene panel for clinical genetic diagnosis of hereditary PPGLs. At present, WES or WGS should be adopted preferentially for research purposes, although these strategies will probably be incorporated as diagnostic tools in the future once they become more affordable. Blood or buccal (saliva) DNA is an adequate biological material for germ line diagnosis, but analysis of tumour DNA can aid interpretation of germ line variants and is also of interest to detect somatic mutations that could be targeted therapeutically. The Study Group acknowledges technical limitations of NGS and the need for orthogonal validation of findings. In addition, the Study Group highlights the importance of fostering collaborations to achieve consensus on VUS classification, the development and application of functional assays to aid in interpretation of findings, and, finally, implementation of curated variant databases. As NGS technologies are still evolving, these guidelines are subject to change and will be updated when necessary. Re-evaluation of these guidelines will require ongoing communication among the experts in the PPGL field.

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Author contributions

R.A.T., N.B., A.-P.G.R. and P.L.M.D. researched data for the article, made substantial contributions to discussion of the content, wrote the article and reviewed and/or edited the manuscript before submission. A.C., D.E.B., J.-P. B., J.W. C.M.T., H.F., T.D., T.E., M.M., G.O., R.C.-B., O.G., E.R.M. and M.R. contributed to discussion of the content and reviewed and/or edited the manuscript before submission. A.-P.G.R. and P.L.M.D. are senior authors with equal contribution and R.A.T and N.B. are first authors with equal contribution.

Competing interests statement

The authors declare no competing interests.



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Exome Aggregation Consortium (ExAC): <http://exac.broadinstitute.org/>
Exome Variant Server: <http://evs.gs.washington.edu/EVS/>
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