# Quantifying evidence TOWARDS pathogenicity FOR rare phenotypeS: the case of succinate dehydrogenase genes SDHB and SDHD

A. Garrett1\*, C. Loveday1\*, L. King1\*, S. Butler2, R. Robinson3, C. Horton4, A. Yussuf4, S. Choi1, B. Torr1, M. Durkie5, G.J. Burghel6, J. Drummond7, I. Berry3, A. Wallace6, A. Callaway8, D. Eccles9, 10, M. Tischkowitz11, 12, K. Tatton-Brown13, 14, K. Snape13, 14, T. McVeigh15, L. Izatt16, E.R. Woodward17, 18, N. Burnichon19, 20, A.-P. Gimenez-Roqueplo19, 20, F. Mazzarotto21, 22, N. Whiffin21, 22, J. Ware23, 24, H. Hanson1, 14, T. Pesaran4, H. LaDuca4, A. Buffet19, 20, E.R. Maher8, 11 & C. Turnbull1, 15

1 Division of Genetics and Epidemiology, Institute of Cancer Research, Sutton, UK

2 Central and South Genomic Laboratory Hub, Birmingham Women’s and Children’s NHS Foundation Trust, Birmingham, UK

3 Yorkshire and North East Genomic Laboratory Hub, Leeds Teaching Hospitals NHS Trust, Leeds, UK

4 Ambry Genetics, Aliso Viejo, CA 92656, USA

5 Yorkshire and North East Genomic Laboratory Hub, Sheffield Children's NHS Foundation Trust, Sheffield, UK

6 Manchester Centre for Genomic Medicine and North West Laboratory Genetics Hub, Manchester University NHS Foundation Trust, Manchester, UK

7 East Genomic Laboratory Hub, Cambridge University Hospitals Genomic Laboratory, Cambridge University Hospitals, Cambridge, UK

8 Central and South Genomics Laboratory Hub, Wessex Regional Genetics Laboratory, Salisbury Hospital NHS Foundation Trust, Salisbury, UK

9 Cancer Sciences, Faculty of Medicine, University of Southampton, Southampton, UK

10 Human Genetics and Genomic Medicine, Faculty of Medicine, University of Southampton, Southampton, UK

11 Department of Medical Genetics, University of Cambridge and Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK

12 East Anglian Medical Genetics Unit, Cambridge University Hospitals NHS Trust, Cambridge, UK

13 St. George's University, London, UK

14 Department of Clinical Genetics, St. George's University Hospitals NHS Foundation Trust, London, UK

15 Cancer Genetics Unit, Royal Marsden NHS Foundation Trust, London, UK

16 Clinical Genetics, Guy’s and St Thomas’ NHS Foundation Trust, London, UK

17 Manchester Centre for Genomic Medicine, Manchester Academic Health Sciences Centre (MAHSC), Manchester University NHS Foundation Trust, Manchester, UK

18 Division of Evolution and Genomic Sciences, School of Biological Sciences, University of Manchester, MAHSC, Manchester, UK

19 Université de Paris, PARCC, INSERM, Equipe Labellisée par la Ligue contre le Cancer, F-75015 Paris, France

20 Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Service de Génétique, Paris, France

21The Wellcome Centre for Human Genetics, Oxford, UK

22 Centre for Personalised Medicine and St Anne's College, University of Oxford, Oxford, UK

23 National Heart and Lung Institute & MRC London Institute of Medical Sciences, Imperial College London, London, UK

24 Royal Brompton & Harefield Hospitals, London, UK

Correspondence to Dr. Clare Turnbull, Division of Genetics and Epidemiology, Institute of Cancer Research, Sutton SM2 5NG, UK; [clare.turnbull@icr.ac.uk](mailto:clare.turnbull@icr.ac.uk)

\* A.G., C.L. and LK contributed equally to this paper

## Abstract

### Purpose

The weight of evidence is uncertain to attach to observation of a novel rare missense variant in *SDHB* or *SDHD* in individuals with the rare neuroendocrine tumours phaeochromocytomas and paragangliomas (PCC/PGL).

### Methods

We compared the frequency of *SDHB*/*SDHD* very rare missense variants (VRMVs) in 6328/5847 cases of PCC/PGL to population controls to generate a Pan-Gene-Very-Rare-Missense-Variant-Likelihood-Ratio (PG-VRMV-LR). Via windowing analysis we measured regional enrichments of VRMVs to calculate the “Domain-Specific-Very-Rare-Missense-Variant-Likelihood-Ratio” (DS-VRMV-LR). We also calculated subphenotypic likelihood ratios (SP-LRs) for variant pathogenicity for various clinical, histological and molecular features.

### Results

We estimated the PG-VRMV-LR to be 76.2 (54.8-105.9) for *SDHB* and 14.8 (8.7-25.0) for *SDHD*. Clustering analysis revealed a region of *SDHB* (ɑɑ 177-260, p=001) for which DS-VRMV-LR was 127.2 (64.9-249.4) and for *SDHD* the enriched region (ɑɑ 70-114, p=0.000003) yielded a DS-VRMV-LR of 33.9 (14.8-77.8). SP-LRs exceeded 6 for invasive disease (SDHB), head-and-neck disease (SDHD), multiple tumours (SDHD), positive family history, loss of *SDHB* staining on immunohistochemistry and succinate:fumarate ratio >97 (SDHB, SDHD).

### Conclusions

Using methodology generaliseable to other gene-phenotype dyads, the likelihood ratios relating to rarity and phenotypic specificity for a single observation in PCC/PGL of a SDHB/SDHD VRMV can afford substantial evidence towards pathogenicity.

## Introduction

Clinical genomic analysis is typically undertaken with the aim of identifying an underlying monogenic cause in a patient with suggestive clinical features. For any genomic variant identified, a variety of evidence types are integrated to assess the likelihood of the variant being pathogenic. In 2015, the American College of Medical Genetics (ACMG) and Association of Molecular Pathology (AMP) published a framework prescribing how these disparate evidence elements should be combined by diagnostic laboratories for classification of a newly identified genomic variant1. They defined for the evidence elements four strengths, Supporting (P), Moderate (M), Strong (S) and Very Strong (VS), which could be combined towards classification categories of Pathogenic, Likely Pathogenic, Likely Benign and Benign. However, inclusion and strength of evidence elements often differ between diagnostic laboratories and produce discrepant classifications2.

Phaeochromocytomas and paragangliomas (PCC/PGL) are neuroendocrine tumours of the adrenal medulla and autonomic nervous system of estimated frequency 1/4000 and 1/16,000 respectively3-7. Head and neck paragangliomas (e.g. chemodectoma, glumus jugulare) (HNPGL) are derived from parasympathetic ganglia. Inherited predisposition to PCC/PGL is associated with constitutional pathogenic variants (PVs) in >15 genes including *SDHA,* *SDHAF2, SDHB, SDHC, SDHD, VHL, FH, MAX, TMEM127, RET, MEN1* and *NF1*5 8.Amongst the Mendelian PCC/PGL cases, the most sizeable contribution is from PVs in *SDHB*, followed by *SDHD*5 9. Associations with ‘subphenotypes’ of HNPGL, multiple, familial and/or young-onset disease and have been reported with underlying germline SDHx PVs, as well as metastatic disease for *SDHB* PVs10. The SDH proteins together form the succinate dehydrogenase enzymatic complex or mitochondrial complex II, disruption of which by mutation of any of the SDHx components may cause loss of *SDHB* expression in tumour material11. SDH succinate:faumarate ratio in the tumour has also been associated with underlying SDHx mutation12. Other tumours have been associated with PVs in SDHx but with much lower relative risks; these include wild-type gastrointestinal stromal tumours, SDH-deficient renal cell carcinoma, and pituitary adenomas11. In the case of *SDHD, MAX* and *SDHAF2*, disease is typically only manifest when PVs are transmitted paternally5 11. For the other SDHx genes, the pattern of disease transmission follows the normal autosomal dominant model of inheritance.

As per the classical Knudson two-hit model of loss-of-function, protein-truncating variants in *SDHB/SDHD* are typically pathogenic10. Interpretation and classification of missense variants is more challenging. On encountering a patient with PCC/PGL and a rare missense variant in *SDHx*, evidence towards pathogenicity could be inferred from (i) the very observation in an individual with the relevant rare PCC/PGL phenotype of a rare variant in an associated gene (PP2 in the ACMG/AMP framework) (ii) location of that variant within a sub-region of the gene particularly associated with pathogenicity (PM1) (iii) ‘subphenotypic’ features particularly associated with PVs in the *SDHx* gene, for example invasive disease or loss of *SDHB* staining on immunohistochemistry (IHC) (PP4). We demonstrate generalisable quantitative approaches and requisite datasets by which likelihood ratios can be calculated for each of these elements, using the genes *SDHB/SDHD*, the phenotype PCC/PGL and missense variation as our exemplar gene/phenotype/variant-class paradigm13.

## Materials and methods

### Assembly of group clinical and laboratory experts for the gene-phenotype paradigm

Via our national UK multi-disciplinary network Cancer Variant Interpretation Group-UK (CanVIG-UK), we identified from the 23 UK genetics centres, the lead diagnostic laboratory scientists, clinical geneticists and endocrinologists for PCC/PGL to assemble the CanVIG-UK SDHx expert group, who guided sourcing of case data and focused survey of the literature14.

### Assembly of case variant data

For the case-control analyses, we were able to identify only one data series providing frequency for individual *SDHB*/*SDHD* variants, fully stratified by ethnicity, ascertained from full SDHx gene analysis in a PCC/PGL series unselected for subphenotypes: 179 cases of PCC/PGL recruited to ‘The Cancer Genome Atlas’ (TCGA dataset)15. We obtained summary-level per-variant frequencies for four additional PCC/PGL series from clinical testing: these series were all of predominantly Caucasian (Western European) ethnicity but detailed/individual-level ethnicity data were unavailable.

The ‘Birmingham’ and ‘Leeds’ datasets comprised per-variant summary results from unrelated probands with PCC/PGL, referred from UK clinical genetics and endocrinology centres to West Midlands Regional Genomic Laboratory Hub (2000-2020) and Yorkshire and North East Genomic Laboratory Hub (2015-2020), respectively comprising clinical testing (single gene/panel including dosage analysis) of *SDHB* and *SDHD* for 3044 and 2565 patients (Birmingham) and 215 and 215 patients (Leeds). The ‘Ambry’ dataset comprised per-variant summary results from clinical testing for SDHx undertaken at Ambry Genetics of 1338 PCC/PGL cases referred from US clinical genetics and endocrinology centres from 2012-2020. The ‘French’ dataset comprised per-variant summary results from single gene/panel testing for *SDHB* and *SDHD* of 1552 and 1550 French PCC/PGL cases accrued 2001-2010, as previously described by Buffet et al9. In total, 6328 and 5847 unrelated PCC/PGL probands were available for analysis for *SDHB* and *SDHD* respectively. For *SDHB*, we identified in total 308 PVs predicted to truncate the protein and 315 missense variants classified in ClinVar as (Likely) Pathogenic. For *SDHD*, there were 155 protein-truncating PVs and 139 (Likely) Pathogenic (of which 116 were c.242C>T p.Pro81Leu). These classifications are based for missense variants on ClinVar (≥1star, pathogenic/likely pathogenic) and for truncating variants on classification using ACMG criteria performed by a diagnostic clinical scientist.

For subphenotype analyses, we were able to access individual level *clinical* phenotype data for a subset of 709 of the Birmingham probands comprising (i) tumour location (head-and-neck/thoraco-abdominal), (ii) tumour behaviour (invasive/non-invasive), (iii) tumour number (multiple/single), (iv) family history (familial/isolated) and (v) age at diagnosis, as previously described by Maher and colleagues16 17. Data on the relevant molecular subphenotypes, namely *SDHB* IHC and SDH succinate:fumarate ratio (SSFR) were unavailable for any of our case series, so were instead derived from the literature. We identified suitable IHC data, stratified by SDHx variant type, generated by Van Nederveen et al, for 175 PCC/PGL cases of known germline SDHx status (retrospective series) and 45 PCC/PGL cases in whom SDHx germline testing was performed subsequently (prospective series). Two different commercial primary antibodies against *SDHB* (mouse monoclonal clone 21A11 and rabbit polyclonal HPA00286) were used to perform IHC18. We identified suitable SSFR data, stratified by SDHx variant type, generated by Richter et al for 210 PCC/PGL cases (69 with PVs, 14 with variants of uncertain significance and 127 with SDHx wild-type). Metabolites were measured using liquid chromatography-mass spectrometry and variant classification was conducted according to ACMG/AMP guidelines1 12.

### Assembly of control data-series

For the control comparison group, we made use of the publicly available gnomAD v2.1.1 (non-cancer) dataset: exome data from 118,479 individuals recruited via studies of common complex diseases, such as hypertension and type II diabetes (from which cancer-related series were excluded). The gnomAD v2.1.1 dataset comprised 51,377 non-Finnish European (NFE) individuals, 10,816 Finnish European, 17,130 Latino/Admixed American, 15,263 South Asian, 7,451 African/African-American, 8,846 East Asian, 4,786 Ashkenazi Jewish and 2,810 other)19. To extend representation of rarer ethnicities, we also utilised the 1000 Genomes Project (1000GP) Phase 3 data comprising 2,504 individuals from 26 subpopulations, recognising that some overlap between the 1000GP and gnomAD populations is reported20 21.

### Calculation of predicted maximum tolerated allele frequency (MTAFpred)

We calculated a predicted maximum tolerated allele frequency (MTAFpred) for pathogenicity for *a newly identified* missense variant in PCC/PGL for each of *SDHB* and *SDHD* based on the methods described by Whiffin et al, namely MTAFpred = disease prevalence\*maximum allelic contribution\*1/penetrance, where maximum allelic contribution = genetic heterogeneity\*allelic heterogeneity22. MTAFpred represents the estimated allele frequency in the population above which a *newly identified* very rare missense variant (VRMV) is not plausibly pathogenic. We sought guidance from the CanVIG-UK SDH expert group to ensure best estimation of the constituent parameters underpinning MTAFpred estimation22.

Disease Prevalence: As PCC/PGL is typically a time-limited condition resolved via surgery, for this analysis we took lifetime risk to approximate to disease prevalence. Estimates of the frequency in the population of PCC/PGL vary widely3 4 23 24. For example, estimated PCC/PGL incidence in the Netherlands was 0.04-0.21 per 100,000 person-years (equating to an approximate lifetime risk of ~1:6,000 to 1:31,000), whilst estimated PCC/PGL incidence in the USA was 500 to 1,600 cases per year (equating to an approximate lifetime risk of ~1:2,500 to 1:8,000)23 24. We utilised a widely-cited cancer-registry-derived estimate of lifetime risk for PCC from Pacak et al. of 1:4,500 with a frequency of paraganglioma estimated 4-fold less common (1:18,000), totalling a combined lifetime incidence of 1:3,6003.

Penetrance: We utilised estimates of penetrance from Andrews et al, which comprised prospective follow up of 371 and 67 unaffected *SDHB* and *SDHD* PV-positive non-probands respectively, ascertained on account of an affected index case in the family, the largest series we could identify. Penetrance to age 60 and 80 for non-probands was estimated for *SDHB* to be 22% and 39%. For *SDHD*, penetrance in non-probands for paternally-inherited *SDHD* PVs was estimated to be 50% to age 6017. Applying to *SDHD* the proportionate age-related penetrance of *SDHB*, we would thus predict a penetrance to age 80 for paternally-inherited PVs of 88.6%. At population-level, assuming absence of sex-selection in transmission of pathogenic *SDHD* alleles, we would thus predict an overall penetrance for *SDHD* to age 80 of ~44%.

Genetic Heterogeneity: We used data from our amalgamated series to estimate genetic heterogeneity: the frequency in our PCC/PGL cases of missense (Likely pathogenic/Pathogenic Variants was 315/6328 (4.9%) for SDHB and 139/5847 (2.3%) for SDHD.

Allelic Heterogeneity: As these are well-characterised genes for which extensive clinical testing has been performed, it is likely that majorly recurrent variants have been identified and thus we estimated conservatively that any *newly identified* variant of standard penetrance is unlikely to constitute >10% to the totality of missense PVs22.

Using parameter estimates for disease frequency (1/3600), penetrance (*SDHB*: 0.39, *SDHD*: 0.44), genetic heterogeneity for missense variants (0.049 for *SDHB*, 0.023 for *SDHD*) and allelic heterogeneity (0.1) we estimated the MTAFpred to be 1.7 x 10-6 for *SDHB* and 7.3 x10-7 for *SDHD*22. Assuming a Poisson distribution, adequate coverage and estimates based on the lower 95th confidence interval, the MTAFpred (VRMV threshold) for each of *SDHB* and *SDHD* is compatible with observation of a maximum of 1 allele in gnomAD v2.1.1(non-cancer)NFE (102,754 alleles or 51,377 individuals), and maximum of 0 alleles in any of the other gnomAD v2.1.1(non-cancer) subpopulations or 1000GPall (largest being gnomAD Latino/Admixed Americans at 34,260 alleles or 17,130 individuals).

As would be anticipated, some of the more common recurrent/founder pathogenic mutations occur at a frequency in controls too high for inclusion as a ‘VRMV’, namely *SDHB* c.286G>A p.Gly96Ser (frequency=2 in v2.1.1(non-cancer)NFE), *SDHB* c.688C>T p.Arg230Cys (frequency=2 in v2.1.1(non-cancer)NFE), *SDHB* c.725G>A p.Arg242His (frequency=3 in v2.1.1(non-cancer)NFE) and *SDHD* c.242C>T p.Pro81Leu (frequency=4 in v2.1.1(non-cancer)NFE).

### Sensitivity Analysis

Sensitivity analysis was undertaken testing of a range of plausible parameter estimates for disease frequency (1/2000, 1/3500, 1/5000, 1/10,000), penetrance (10%-50%) and allelic\*genetic heterogeneity (0.001-0.006), examining the impact on MTAFpred and maximum allele count in the different-sized population datasets (Supplementary Table 1).

### Generation of Likelihood Ratios

We generated positive likelihood ratios and confidence intervals, based on the rate of the entity under study in positives (true positive rate) compared to the rate of entity under study in negatives (false positive rate), (a/a+c)/(b/b+d), where a=true positive, b=false positive, c=false negative, d=true negative25. We generated a negative likelihood ratio based on the rate of absence of the entity under study in negatives (true negative rate) compared to the rate of absence of the entity under study in positives (false negative rate), (d/b+d)/(c/a+c). Where one or more cells contained zero counts, we universally applied to those analyses a Haldane correction (adding 0.5 to each cell): this correction dampens signal of association towards the null and thus is inherently conservative.

### Calculation of “Pan-Gene-Very-Rare-Missense-Variant-Likelihood-Ratio” (PG-VRMV-LR)

The PG-VRMV-LR was generated as the positive likelihood ratio for *SDHB/SDHD* for frequency of VRMVs in PCC/PGL cases compared to population controls. To estimate the frequency of VRMVs in *SDHB*/*SDHD* in the general population, we made comparison to the largest available single-ethnicity control population, the v2.1.1(non-cancer)NFE series. We also performed a modified PG-VRMV-LR estimation excluding established pathogenic VRMVs observed recurrently in the case series. We defined these as variants classified in ClinVar as Pathogenic/Likely Pathogenic *and* observed in more than 8 probands in our series. This rather conservative threshold, derived from visual inspection of the frequency distribution, equates to a variant present in >1/800 probands or constituting in our series >5% of all VRMVs).

### Calculation of “Domain-Specific-Very-Rare-Missense-Variant-Likelihood-Ratio” (DS-VRMV-LR)

Using the windowing method described by Walsh et al, we performed a clustering algorithm to examine agnostically regional enrichment of VRMVs reported in cases versus VRMVs reported in controls26.

### Calculation of subphenotype likelihood ratios (SP-LR)

Clinical subphenotypic data captured at ascertainment for (i) tumour location (head-and-neck/thoraco-abdominal), (ii) tumour behaviour (invasive/non-invasive), (iii) tumour number (multiple/single) and (iv) family history (familial/isolated) were used to generate positive and negative likelihood ratios. All cases with a variant of uncertain significance (VUS) in any SDHx gene were excluded from the ‘SDHx wildtype group’. Age was excluded from the multivariable analysis as on visual inspection, there was a complex relationship between PV status and age, not well captured by categorical groupings. We quantified and adjusted for co-linearity among subphenotypic variables using univariable and multivariable logistic regression. For the univariable analysis, all individuals with data for that parameter were included. For the multivariable analysis, only those with complete data on all clinical subphenotypes were included.

Using the combined van Nederveen series, positive and negative likelihood ratios for PVs against SDHx wildtype/untested were calculated for loss on IHC. Using the Richter et al series, positive and negative likelihood ratios for PVs against SDHx wildtype were calculated for SSFR >97 versus SSFR ≤97. For all subphenotypes, data are presented for missense PVs and all PVs.

## Results

The proportion of individuals in whom a VRMV was identified was 366/6,328 in PCC/PGL probands and 39/51,377 in controls (*SDHB*) and 37/5,847 in PCC/PGL probands and 22/51,377 in controls (*SDHD*). We calculated the Pan-Gene-Very-Rare-Missense-Variant-Likelihood-Ratio (PG-VRMV-LR) to be 76.2 (54.8-105.9) for *SDHB* and 14.8 (8.7-25.0) for *SDHD*. PG-VRMV-LRs were broadly consistent when analysed for the five case series individually. These frequencies do not include recurrent founder mutations observed in controls at a frequency exceeding the MTAFpred threshold, namely *SDHB* c.286G>A p.Gly96Ser (frequency 17 in cases/2 in v2.1.1(non-cancer)NFE), *SDHB* c.688C>T p.Arg230Cys (10/2), *SDHB* c.725G>A p.Arg242His (19/3), *SDHD* c.242C>T p.Pro81Leu (116/4).

However, whilst observed at sufficiently low frequency in controls to constitute a VRMV, a number of variants were observed in multiple independent probands and are well-documented in ClinVar as (Likely) Pathogenic. On removal of these ‘recurrent-pathogenic-VRMVs’, the frequencies reduced to 156/6,118 probands in *SDHB* and 37/5847 in *SDHD*, thus down-adjusting the PG-VRMV-LR to 34.6 (24.3-49.2) for *SDHB* and 14.8 (8.7-25.0) for *SDHD* (Table 1, Supplementary Table 2, 3).

From the clustering analysis, we identified a region comprising 30% of the coding region of *SDHB* (ɑɑ 177-260) enriched for VRMVs in cases compared to controls (p=0.001). This generated “Domain-Specific-Very-Rare-Missense-Variant-Likelihood-Ratios” of DS-VRMV-LR= 127.2 (64.9-249.4) for variants within the region and DS-VRMV-LR=60.9 (41.6-89.0) for those outside. For *SDHD,* there was also a cluster region (ɑɑ 70-114, 28% of coding region, p=0.000003), such that DS-VRMV-LR= 33.9 (14.8-77.8) inside and DS-VRMV-LR= 5.9 (2.6-13.0) outside of that region. Excluding the ‘recurrent-pathogenic-VRMVs’ reduced the hot-DS-VRMV-LR to 59.7 (28.5-125.2) and the cold-DS-VRMV-LR to 28.2 (18.8-42.4) for SDHB; for SDHD, DS-VRMV-LRs were unchanged (Figure 1).

Based on PV-positive versus SDHx wild-type case-only adjusted comparisons, invasive disease was predictive for *SDHB* missense PV status compared to SDHx wild-type status (SP-LR= 6.5 (3.9-10.7). Both head-and-neck disease (SP-LR= 10.6 (8.8-12.7)) and multiple tumours (SP-LR= 9.5 (5.3-17.1) were predictive for *SDHD* missense PV status compared to SDH wild-type status. Family history of at least one affected first degree relative was highly predictive for missense PV in *SDHB* (SP-LR= 18.7 (8.7-40.0)) and *SDHD* (SP-LR= 54.4 (25.6-115.5)) compared to wild-type status (Table 2, Supplementary Tables 4, 5). In univariable analysis, loss of *SDHB* staining on immunohistochemistry was strongly predictive of PV in both *SDHB* (SP-LR= 17.9 (14.7-21.8) and *SDHD* (SP-LR= 18.1 (16.6-19.8) compared to wildtype SDHx status (Table 2, Supplementary Table 6). Succinate Fumarate ratio >97 was also strongly predictive of PV in *SDHB* (SP-LR= 108.9 (92.9-127.6)) and *SDHD* (SP-LR= 93.1 (78.3-110.8)) compared to wildtype SDHx (Table 2, Supplementary Table 7). In Supplementary Table 8, some hypothetical variant scenarios are presented to illustrate combination of these LRs under the points-based Bayesian adaptation of the ACMG variant classification framework.

**Discussion**

Prior to evolution of the ACMG/AMP framework, assignment of a variant as pathogenic was frequently based primarily on observation thereof in an individual with the “correct” phenotype (along with absence on sequencing of a few hundred control chromosomes). However, this adage led to erroneous classifications of many innocuous variants as pathogenic on account of (i) insufficient size of population/control data series for confirmation of requisite rarity and/or (ii) application in the context of non-specific phenotypes such as “familial breast cancer”.

The notion of phenotypic specificity is not simple. For a given gene/phenotype/variant-class scenario, phenotypic specificity encompasses (i) rarity of the clinical phenotype in the general population, (ii) how much of the phenotype is attributable to that gene (iii) the level of enrichment of gene variants of a particular class in that phenotype (i.e. penetrance) (iv) how “noisy” the gene is for innocuous variants of that variant-class. There may in addition be (v) regional variation for pathogenic compared to innocuous variants of that variant-class, (vi) gene-specific subphenotypic features that are particularly associated with pathogenicity.

Using *SDHB*/*SDHD*, PCC/PGL and missense variants as our gene/phenotype/variant-class exemplar, we have demonstrated quantitation of these likelihood ratios, namely encompassing (i) identification of a rare missense variant in an individual with the correct rare phenotype in a gene variably constrained for those variants (ACMG/AMP criterion: PP2), (ii) enrichment for rare variants in cases compared to controls within specific gene regions (PM1) and (iii) presence of macroscopic or molecular subphenotypic features particularly associated with germline PVs in a specific gene (PP4).

These analyses demonstrate a substantial Pan-Gene-Very-Rare-Missense-Variant-Likelihood-Ratio” (PG-VRMV-LR) for *SDHB* in particular, which is quite striking even after removal of the recurrently-reported pathogenic VRMVs. It is plausible that for other gene/phenotype/variant-class scenarios in which the gene is constrained and/or the phenotype is rare, the PG-VRMV-LR may be equivalently substantial and we may currently be ‘underscoring’ evidence afforded by single observation of a very rare variant in the relevant phenotype. Wide variation in aetiologic fraction, a similar metric, has been demonstrated for genes associated with cardiomyopathies27.

These analyses also exemplify potential clinical utility for formal quantitation of likelihood ratios for subphenotypic features, in this case abnormality on IHC, high SSFR, head-and-neck disease, invasive disease, multiplex tumours and familial disease. Although we were only able to undertake multivariable regression to adjust for collinearity between clinical subphenotypic features, collinearity between the clinical and molecular features would not a priori be predicted Stringent technical validation would be a prerequisite for inclusion of locally-generated laboratory data: whilst methodology and quality assurance for IHC is well explored, there is no international reference method for SSFR and assay thresholds may be influenced by tumour input material. The corresponding negative likelihood ratio should be applied where absence of the subphenotypic feature has high specificity for wild-type status (for example, absence of head-and-neck disease for *SDHD*). Currently the ACMG framework lacks formal designation of a ‘negative-phenotype’ evidence item.

As demonstrated by Tavtigian et al, ACMG/AMP categorical evidences strengths can be converted to likelihood ratios (Supporting (P): likelihood ratio=2.08, Moderate (M) likelihood ratio=4.33, Strong (S) likelihood ratio=18.8 and Very Strong (VS) likelihood ratio=350)28. Thus, the three types of evidence items we have described can be combined together with other relevant LRs in a Bayesian framework where the Posterior Probability is a function of the Prior Probability and the product of relevant likelihood ratios ([Likelihood Ratio]a x [Likelihood Ratio]b x [Likelihood Ratio]c. x [Likelihood Ratio]d x [Likelihood Ratio]e….).

Application to *SDHB*/*SDHD*-PCC/PGL illustrates a number of challenges and limitations in the methods presented. Firstly, critical to the Pan-Gene-Very-Rare-Missense-Variant-Likelihood-Ratio (PG-VRMV-LR) is conversion of the maximum tolerated allele frequency (MTAFpred) to a filtering allele count for a given control dataset. Even the largest subpopulation of the cancer-free gnomAD series (NFE) provides poor precision at low values of MTAFpred. Observation of 1/51,377 in the NFE group is consistent with underlying frequency of 4.93x10-7 - 0.0001084 (95% confidence interval of binomial distribution). Accordingly we may be over-estimating the frequency of VRMVs in controls (and thus underestimating the PG-VRMV-LR). As illustrated in our sensitivity analyses (Supplementary Table 1), access to larger control series for our VRMV case-control comparison will improve the precision by which we filter for MTAFpred29.

Secondly, limited size of non-NFE control series precludes accurate filtering of variants in non-NFE populations. Variants with a true frequency above the MTAFpred may still be sufficiently rare to be absent in these modest-sized control series. Although case-series used were predominantly Western European, in the non-Caucasian minority we may be erroneously including as VRMVs (i) variants common in ethnic groups entirely unrepresented in gnomAD or 1000GP or (ii) variants absent in the respective partition of gnomAD/1000GP but of frequency exceeding the MTAFpred for the relevant ethnicity group . Erroneous inclusion of such variants as VRMVs in the case series may result in overestimation of PG-VRMV-LR. Furthermore, due to limited control and case data, we have had to develop parameters and apply them to the same dataset: access to additional independent datasets would allow ‘testing’ of reproducibility.

Thirdly, for most rare phenotypes, parameter estimates for population frequency, lifetime penetrance and genetic heterogeneity vary widely and may be subject to substantial ascertainment bias. For pleomorphic syndromic phenotypes, it is only feasible to estimate MTAFpred by ‘pulling out’ a specific component of the syndrome and estimating frequency, penetrance, and genetic heterogeneity for this component (e.g. type II renal papillary cancer for the FH gene (Hereditary Leiomyomatosis and Renal Cell carcinoma-HLRCC) or medullary thyroid cancer for the RET gene (Multiple Endocrine Neoplasia Type II))30.

Fourthly, a specific ‘case definition’ (ascertainment framework) is required to which the VRMV-LR is applicable. In practice, eligibility for clinical gene testing likely varies in space and time, rendering it challenging to pinpoint too precisely the framework for ascertainment and case inclusion.

Fifthly, the VRMV-LR metrics are based on and applicable only to observed variants which themselves are very rare (i.e. are observed in the control population at frequency below the MTAFpred). Variants which are disease-associated but of lower penetrance will likely occur in the population but at frequencies above the MTAFpred. Such variants will not have been included in the VRMV case control analyses and the VRMV-LR metric will not be applicable to them.

Thus, whilst our the parameter estimates were deliberately conservative and the limited size of NFE control data may have caused under-estimation of PG-VRMV-LR, failure to guarantee full exclusion of VRMVs in non-NFE cases would support a conservative translation of our estimates of PG-VRMV-LR to evidence strengths for clinical variant classification13.

Although we used *SDHB*/*SDHD*, PCC/PGL and missense variation as our exemplar gene/phenotype/variant-class paradigm, the principles, requisite datasets and methodologies illustrated here are applicable universally to any other gene/phenotype/variant-class scenario. We propose that adoption for other Mendelian rare cancer syndromes of the methodologies illustrated would improve consistency and accuracy of quantitative estimation of the rare variant/rare phenotype phenomenon (PG-VRMV-LR for PP2), of variant location in the right hot-spot (DS-VRMV-LR for PM1) and of quantitative evaluation of subphenotypes (SP-LR for PP4).

## Legends

**Figure 1. Variant position schematic.** Lolliplot showing the position of *SDHB* and *SDHD* variants in 51,377 controls and 6328 and 5847 cases of pheochromocytomas and paragangliomas respectively. Variants identified in cases represented by red circles and those in controls by yellow circles, with proportional representation for variants identified in both. Exon-exon boundaries are shown with a dashed line. Protein domains are represented by coloured blocks. Variant cluster regions, as defined using a custom clustering algorithm (see methods), are shown as black rectangles below each protein (*P* < 0.004). Fer\_2\_3, 2Fe-2S iron-sulfur cluster binding domain (red); Fer4\_17, 4Fe-4S dicluster domain (purple); CybS, succinate dehydrogenase cytochrome B small subunit (blue). Escore inter-species conservation is presented.

**Table1: Pan-Gene-Very-Rare-Missense-Variant-Likelihood-Ratios for *SDHB* and *SDHD*.** Frequency in cases of pheochromocytomas and paragangliomas and population controls (gnomAD v2.1.1 (non-cancer)NFE) of VRMVs in *SDHB* and *SDHD* (i) all VRMVs (ii) VRMVs excluding recurrent ‘founder’ pathogenic variants.

**Table 2: Case-only subphenotype analyses .** Analysis of clinical subphenotypic features in 206 *SDHB* PV-positive, 66 *SDHD* PV-positive and 427 SDH-wildtype cases of PCC/PGL. Analysis of *SDHB* immunohistochemistry (IHC) staining in 21 *SDHB* PV-positive, 53 *SDHD* PV-positive and 118 SDH-wildtype/untested cases of PCC/PGL. Analysis of SDH succinate:fumarate ratio (SSFR) in 14 *SDHB* PV-positive, 15 *SDHD* PV-positive and 127 SDH-wildtype cases of PCC/PGL.

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## Author Information

Conceptualization: C.T., C.L., A.G., M.D., A.C., G.J.B., R.R., J.D., I.B., A.W., H.L.D, T.P., H.H.; Data curation: L.K., S.C., S.B., R.R, C.H., A.Y., H.L.D, T.P., E.M., N.B., A.P.-G.R., A.B., K.S., K.T-B.; Formal Analysis: C.L., A.G., C.T.; Funding acquisition: C.T., M.T., D.E.; Methodology: C.T., C.L., E.M., J.W., N.W., T.McV, L.I., E.R.W., F.M.; Project administration: L.K., B.T.; Visualization: C.L., C.T.; Writing – original draft: C.T., A.G., C.L., E.M.; Writing – review & editing: all authors

## Ethics Declaration

The human variant data used was all de-identified. The data used for frequency analyses were provided in summary form only; all these data were wholly deidentified and thus IRB approval is not required. Collection and analysis of individual-level subphenotypic data was approved by the South Birmingham Research Ethics Committee as per the previous publications describing these data10 16.

## Data Availability

The publicly available data analysed are available as per references/URLs provided. Any materials and data developed during this study will be made available upon request from the corresponding author.

## Disclosure

The authors declare no conflict of interest.

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