Metabolome-guided genomics to identify pathogenic variants in isocitrate dehydrogenase, fumarate hydratase, and succinate dehydrogenase genes in pheochromocytoma and paraganglioma

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Purpose: Metabolic aberrations have been described in neoplasms with pathogenic variants (PV) in the Krebs cycle genes encoding succinate dehydrogenase (SDH), fumarate hydratase (FH) and isocitrate dehydrogenase (IDH). In turn, accumulation of oncometabolites succinate, fumarate, and 2-hydroxyglutarate can be employed to identify tumors with those PV . Additionally, such metabolic readouts may aid in genetic variant interpretation and improve diagnostics.

Methods: Using liquid chromatography–mass spectrometry, 395 pheochromocytomas and paragangliomas (PPGLs) from 391 patients were screened for metabolites to indicate Krebs cycle aberrations. Multigene panel sequencing was applied to detect driver PV in cases with indicative metabolite profiles but undetermined genetic drivers.

Results: Aberrant Krebs cycle metabolomes identified rare cases of PPGLs with germline PV in *FH* and somatic PV in *IDHx* and *SDHx*, including the first case of a somatic *IDH2* PV in PPGL.

Metabolomics also reliably identified PPGLs with *SDHx* loss-of-function (LOF) PV. Therefore we utilized tumor metabolite profiles to further classify variants of unknown significance in *SDHx*, thereby enabling missense variants associated with *SDHx* LOF to be distinguished from benign variants.

Conclusion: We propose incorporation of metabolome data into the diagnostics algorithm in PPGLs to guide genetic testing and variant interpretation and to help identify rare cases with PV in *FH* and *IDHx*.

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INTRODUCTION

Pathogenic variants (PV) in Krebs cycle–related genes occur in different tumor entities, which hence share a common mechanism of tumorigenesis and might also respond to similar treatment strategies.[1,2] Currently, there are nine genes encoding several different Krebs cycle enzymes indicated as tumor drivers: fumarate hydratase (*FH*) PV are associated with renal cell carcinomas (RCC), leiomyomas, and pheochromocytomas and paragangliomas (PPGLs).[3,4] In isocitrate dehydrogenase genes 1 or 2 (*IDH1/2*) occur in gliomas, acute myeloid leukemia, cholangiocarcinomas, and rarely also in PPGL.[5–8] PV in any of the five succinate dehydrogenase subunits (*SDHx*) or accessory proteins can lead to gastrointestinal stromal tumors, RCC, PPGL, neuroblastoma, and

pituitary tumors.[9–11] Additionally, malate dehydrogenase 2 (*MDH2*) was found to be rarely mutated in PPGL.[12]

In PPGLs, catecholamine-producing tumors originating from adrenal or extra-adrenal chromaffin tissue, tumor development has been ascribed to PV in more than 20 susceptibility genes, with 30-40% of PPGLs having a hereditary background.[13] Identification of tumors due to *SDHx* and *FH* PV has implications for patient care and follow-up, since those patients are at higher risk for metastatic disease.[3,14] Moreover, distinguishing between sporadic tumors and cases with underlying germlinen PV is important for management of patients and their families.

SDHx-, *FH*-, and *IDH1/2*-mutated tumors are known to lead to abnormally high levels of one of several oncometabolites: succinate, fumarate, or 2-hydroxyglutarate (2HG). Increases in those result in the inhibition of α-ketoglutarate-dependent enzymes, including prolyl hydroxylases and DNA and histone demethylases, causing a pseudohypoxic and hypermethylator phenotype.[15] We and others have already established the succinate:fumarate ratio as a diagnostically highly accurate functional indicator of pathogenic *SDHx* variants.[16,17] Strong elevations of fumarate as a consequence of loss of function (LOF) PV in *FH* are well known from RCC and leiomyomas, and have also been described for PPGL.[3,18] Somatic hotspot PV of codons R132 and R172 within *IDH1* and *IDH2*, respectively, lead to a gain of function responsible for the production of the D-enantiomer of 2HG, D-2HG.[19] Measurement of metabolites can therefore provide a screening tool to identify tumors with underlying driver PV in those genes. On the other hand, metabolomics could also function as readout for classification of identified variants in metabolic enzymes.

Clinical practice guidelines recommend that genetic testing should be considered in all patients with PPGL, with testing and test interpretation carried out according to a clinical feature-driven diagnostic algorithm that establishes the likelihood of PV.[20] With the use of next-generation sequencing (NGS) now moving into routine clinical diagnostics, large numbers of genes can be tested simultaneously as part of one panel.[13,21–23] As a consequence, more variants are identified; however, for many the pathogenicity is unclear and caution is warranted for interpretation, in particular for germline variants with far-reaching implications for patients and their families.[21,22,24]

The importance of correct classification of gene variants is emphasized by the example of RET Y791F, a variant previously associated with the occurrence of medullary thyroid carcinoma. Recent findings, however, showed that this variant is likely benign and may have led to unnecessary prophylactic thyroidectomies.[25] The development of standards and guidelines for the correct interpretation of genetic variants and the need for new approaches to functionally assess variants of unknown significance (VUS) has become increasingly significant.[26] Apart from manipulated cell lines and in silico predictions, potential approaches to assess functionality include immunohistochemistry (IHC),[10]

expression profiling[27] as well as nuclear magnetic resonance,[28] and mass spectrometry metabolite profiling.[16,17]

The present study utilized mass spectrometry–based metabolomics of Krebs cycle intermediates in 395 PPGL samples to identify aberrant metabolite profiles, functionally relate them to variants in relevant genes, and evaluate their potential as a diagnostic tool.

MATERIALS AND METHODS

Tumor procurement

Primary tumor tissue was collected from 391 patients with PPGL from nine centers, specimens either snap frozen or formalin-fixed and paraffin-embedded (FFPE) after surgery (Supplementary information 1). Tumor procurement was approved under Intramural Review Board protocols at each center, where patients have signed informed consent. Samples with potential contaminants (e.g., those stored in RNA-preserving citrate-based buffers) were excluded from metabolic clustering analysis. We included 230 tumors from 229 patients from a previous study that analyzed succinate:fumarate in relation to *SDHx* PV[16] and 157 freshly frozen (FF) tumors from 154 newly diagnosed patients from seven centers (Bethesda/USA, Madrid/Spain, Florence/Italy, Nijmegen/The Netherlands, Dresden, Munich, Würzburg/Germany). Additionally, eight patients in whom only FFPE tissue was available were included.

Krebs cycle metabolites

In a mass spectrometry–based screening assay eight organic acids of the Krebs cycle as well as pyruvate and lactate were measured in all 387 FF and 8 FFPE tissue samples. Additionally, four amino acids, glutamate, glutamine, aspartate, and asparagine, were analyzed in a sample subset. Metabolites were analyzed with a modification of a previously described method.[16] Briefly, metabolites were extracted from samples with methanol, dried, resuspended in mobile phase, and cleared with a 0.2-μm centrifugal filter. To improve separation, the elution gradient was changed as follows: 99% A (0.2% formic acid in water), 1% B (0.2% formic acid in acetonitrile) for 2.00 min, 100% B at 2.50 to 2.65 min, 1% B at 3.40 min, and equilibration with 1% B until 5.00 min. Multiple reaction monitoring with negative electrospray ionization was used for quantification according to transitions provided in Supplementary Table S1.

D-/L-Enantiomers of 2-hydroxyglutarate (2HG)

Samples were prepared as described previously.[29] Briefly, methanol extracts of tumor tissue were dried and derivatized using diacetyl-l-tartaric anhydride (DATAN) dissolved in dichloromethane and acetic acid (4:1). After 30 min incubation at 75 °C, samples were dried under a stream of nitrogen. After reconstitution in mobile phase (3.5% acetonitrile in water containing 2 mM ammonium formate, pH 3.6), samples were injected onto a Shimadzu UFLC LC-20ADXR-system coupled to a tandem mass spectrometer (Sciex QTRAP5500). Separation of D- and L-enantiomer derivatives of 2HG was

achieved using a Waters XBridge® BEH C18 column (100×3 mm, 2.5 μm) with an isocratic mobile phase flow at 0.44 ml/min.

Genetic testing

Patients were tested for germline PV in established susceptibility genes by centers of origin or by the Spanish National Cancer Research Centre (CNIO) institute in Madrid through a collaborative multicenter study (the Prospective Monoamine-producing Tumor Study, https://pmt-study.pressor.org/) using Sanger sequencing and/or NGS, and multiplex ligation-dependent probe amplification or custom array comparative genomic hybridization (CGH) for deletion detection.

Tumors with aberrant metabolite profiles and unknown genetic backgrounds were additionally analyzed by customized panel sequencing (Illumina, San Diego, CA) including coding exons and 20 bp of intronic flanking regions of known PPGL-associated genes *RET*, *VHL*, *NF1*, *MAX*, *TMEM127*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *MDH2*, *FH*, *EPAS1*, *HRAS*, *KIF1B*, *EGLN1*, *EGNL2*, *IDH1*, and *IDH2*. Library preparation was performed using Illuminas' TruSeq Nano DNA Library Prep Kit according to the manufacturer's instructions. One hundred fifty nt paired-end sequencing was carried out with a minimum median coverage of 1000-fold either on an Illumina MiSeq or NextSeq sequencer. Reads were aligned to the reference genome (GRCh37/hg19) and variant calling was performed using in-house workflows, as described previously.[22,30] Variants of interest were validated by Sanger sequencing.

Variant classification was performed in accordance with the standards and guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP),[26] considering loss of heterozygosity (LOH) in tumor samples as an additional supporting criterion for pathogenicity (PP6). For *SDHx* variants, immunohistochemical loss of the SDH complex as well as an elevated succinate:fumarate were interpreted as functional analyses (PS3) but were downgraded to moderate (PM7 and PM8) criteria. In contrast, positive immunohistochemistry of the SDH complex and normal succinate:fumarate were interpreted to support nonfunctional variants (BP8).

Immunohistochemistry

FFPE tumor tissue was sectioned and stained for SDHB or FH protein using rabbit polyclonal anti-SDHB (HPA002868, Sigma-Aldrich), anti-SDHA (ab14715, Abcam or 2E3GC12FB2AE2, Biorad), and anti-FH (HPA027341, Sigma-Aldrich).

Statistical analysis

Metabolome data were normalized to natural logarithms (LN) to account for large value discrepancies. Basic statistics and clustering analysis were performed using JMP statistics software (version Pro 12.1.0). Outlier analysis on LN-transformed values or ratios were used to identify candidates for panel sequencing of metabolic genes. For this purpose we focused on outliers above the 75th quartile $+ 1.5\times$ (interquartile range) marked by the top whisker in box and whisker plots. Significance between groups of patients was calculated using the nonparametric Wilcoxon test.

RESULTS

Metabolite profiling in PPGL

Clustering analysis of the full set of 395 PPGLs from 391 patients using ratios of Krebs cycle intermediates identified two clusters involving three main groups (Fig. 1a, Supplementary information 1): cluster I was characterized by high succinate and low levels of all other Krebs cycle metabolites and almost exclusively contained *SDHx*-mutated tumors. Additionally, lower levels of the amino acids aspartate and glutamate were present in *SDHx*-mutated PPGLs compared with PPGLs due to other causes (Supplementary Fig. S1). PPGLs of the first cluster, with the exception of three tumors, had succinate:fumarate ratios above a previously defined cut-off for *SDHx*-mutated PPGLs (Fig. 1b).[16]

Cluster II was divided into two main subclusters separated by different levels of citrate, isocitrate, and *cis*-aconitate (Fig. 1a). Subcluster IIa was characterized by low levels of the aforementioned metabolites and contained primarily tumors with driver PV ns in the hypoxia signaling axis (*VHL*, *EPAS1)*, as well as tumors with *FH* and *IDHx* PV (characterized below in detail). Subcluster IIb, characterized by higher citrate, isocitrate, and *cis*-aconitate levels, includes predominantly tumors with driver PV in the kinase signaling pathway (*RET*, *NF1*, *TMEM127*). This metabolic differentiation between groups with PV in common pathways reflects somewhat the clustering of PPGLs according to transcriptomics.[31]

By applying outlier analysis to our cohort, we identified outliers for 2HG (Fig. 1c), α-ketoglutarate (Supplementary Fig. S2A), the fumarate:malate ratio (Fig. 1d), fumarate (Supplementary Fig. S2B), and malate (Supplementary Fig. S2C). The following sections describe the identification of underlying PV in those samples.

2HG and α-ketoglutarate elevations and *IDHx* PV in PPGL

Twelve outliers were identified for 2HG. Of those, two were also α-ketoglutarate outliers (Fig. 1c, Supplementary Fig. S1A). Nine of the twelve 2HG outliers were available for further analysis. Patients 1 and 2 had the highest 2HG values of the set, with increases of up to 1800-fold (patient 2) and 100-fold (patient 1) over the median (Fig. 2a). The other seven tumors showed increases of 10- to 40-fold above the median and four of nine PPGLs also had increases in α-ketoglutarate of 9- to 48-fold over the median (Fig. 2b).

To further characterize the 2HG elevations, D- and L-enantiomers of 2HG were measured. Seven PPGLs with moderate total 2HG levels served as controls with D-2HG to L-2HG ratios ranging from 0.2 to 1.6 (Fig. 2c). Tumors of patients 1 and 2 showed strongly increased D-2HG to L-2HG ratios of 127 and 901, respectively (Fig. 2d). Tumors from

patients 3 and 4 had L-2HG to D-2HG ratios of 13 with control tumors ranging from 0.4 to 4.3. The other five PPGLs were similar to controls.

Patient 1 has been described elsewhere with a somatic *IDH1* c.394C>T (R132C) PV,[6] explaining the strong increases in D-2HG now described here for the first time. In patient 2 we
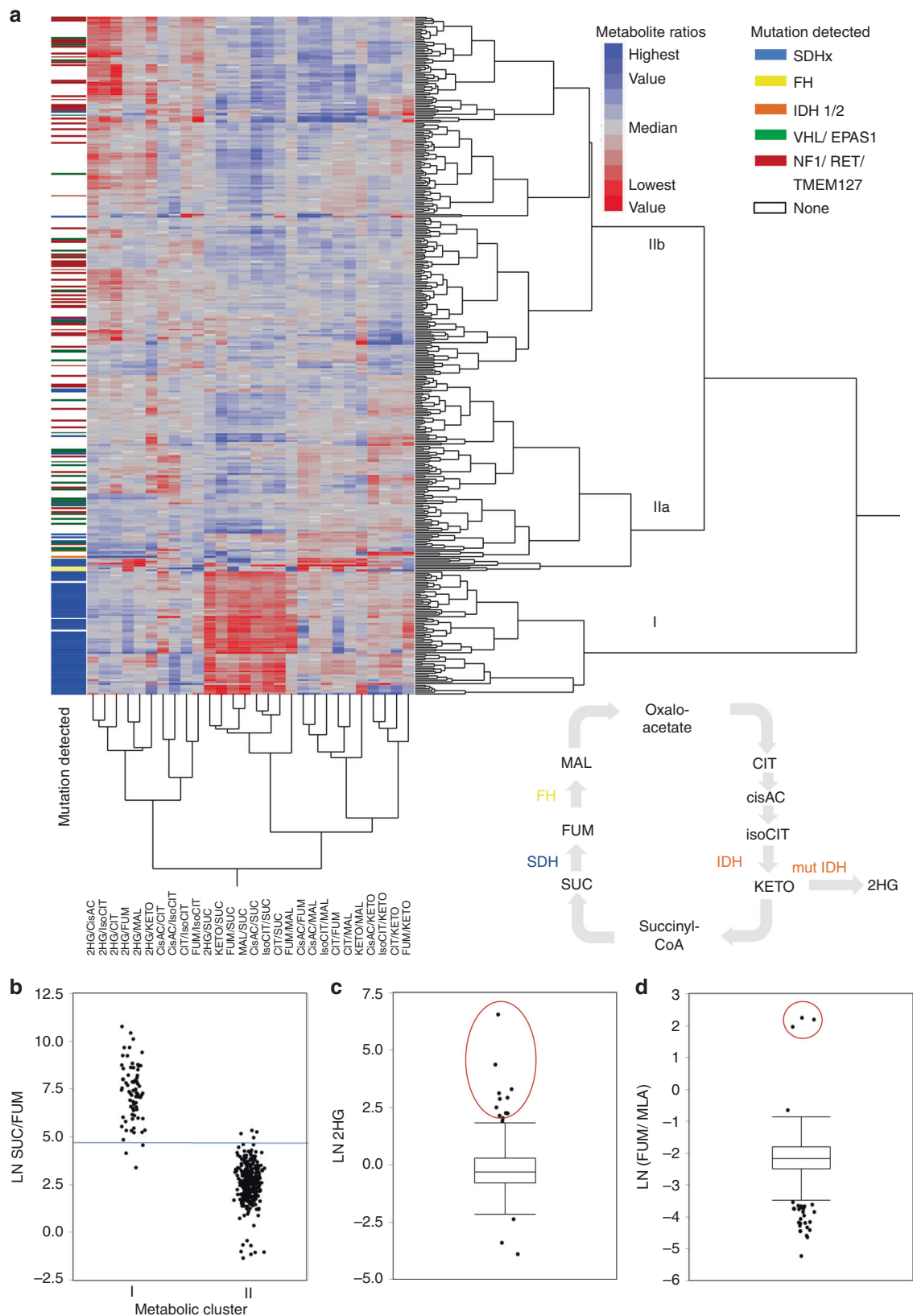
Fig. 1 Krebs cycle metabolite profiling of pheochromocytomas and paragangliomas (PPGLs). a Hierarchical cluster analysis (Ward) of 395 different PPGLs from 391 patients. All possible ratios between eight intermediates (citrate, isocitrate, *cis*-aconitate, α-ketoglutarate, 2-hydroxyglutarate, succinate, fumarate, malate) of the central carbon metabolism were generated and natural logarithm (LN) transformed. Mutational status (germline and somatic) is shown by color in the left column. Clusters are marked with roman numbers. The Krebs cycle with all measured metabolites is schematically shown; oxaloacetate and succinyl-CoA are not detectable with the used method. *2HG*-2 hydroxyglutarate, *CIT* citrate, *cisAC cis*-aconitate, *FUM* fumarate, *isoCIT* isocitrate, *KETO* α-ketoglutarate, *MAL* malate, *SUC* succinate. **b** The succinate:fumarate ratio as an indicator for PV in *SDHx* genes separates cluster I from II. Cut-off defined in ref. [16] marked in blue. **c** Box plots of 2HG levels with outliers marked as dots. Outliers above the top whisker (the 75th quartile + 1.5× [interquartile range]) were further investigated by next-generation sequencing analysis when the driver PV was unknown (red circle). **d** Outlier analysis for the fumarate:malate ratio

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identified a somatic *IDH2* c.514A>G (R172G) PV, to our knowledge the first *IDH2* described in PPGL to date (Fig. 2e). A detailed patient description is provided in Supplementary information 2. The other seven PPGLs with moderately elevated 2HG levels and normal D-2HG to L-2HG ratios did not have PV in any of the genes of our NGS panel; however, in five PPGLs we identified PV in other known susceptibility genes, including *NF1*, *HRAS*, *ATRX*, and *VHL* (Fig. 2f). In summary, *IDH1/2*-mutated PPGLs were characterized by elevations of total 2HG of at least 100-fold and of D-2HG to L-2HG ratios over 100-fold above control median values.

Fumarate and malate elevations and *FH* PV in PPGL

Outlier analysis identified three samples (patients 10, 11, 12) with strong elevations in fumarate:malate ratios 60- to 80-fold above median values of controls (Fig. 1d). Five outliers for absolute fumarate levels were identified. Three of those also presented as outliers for malate and had normal fumarate: malate ratios (Figs. S2B–C); the other two (patients 10, 11) were also fumarate:malate outliers.

In all three patients with aberrant tumor fumarate:malate, NGS revealed heterozygous germline variants in *FH*: NM_000143.3:c.700A>G p.(Thr234Ala), c.908T>C p.(Leu303-Ser), and c.816_836del p.(Ala273_Val279del) (Figs. 3a–c). In all cases, the tumors showed loss of heterozygosity (LOH) for these variants. IHC confirmed the loss of FH protein in tumors 10 and 11, for tumor 12 no more tissue was available (Fig. 3d).

The *FH* variants in patients 10 and 11—p.(Thr234Ala) and p.(Leu303Ser)—are both missense variants predicted to be damaging by three different in silico tools (Polyphen2/ SIFT/MutationTaster). Both are listed three times in heterozygous states in the gnomAD database. *FH* p.(Thr234Ala) is rated with pathogenic/uncertain significance in ClinVar and dbSNP (rs372505976) and *FH* p.(Leu303Ser) as a VUS (rs201502246). The variant *FH* p.(Leu303Ser) was found in a girl together with a second compound heterozygous variant in *FH*, who suffered from autosomal-recessive fumarase deficiency (LOVD 0000116260).[32] The variant p.(Thr234Ala) has not, to the best of our knowledge, been reported in the literature. The third patient (patient 12) carried an in-frame deletion leading to loss of six amino acids in *FH* (p.[Ala273_Val279del]). This variant is rated pathogenic in ClinVar and dbSNP (rs863223985). It is not listed in gnomAD or described in the literature. With only this

information available, the variants would be interpreted as VUS according to ACMG-AMP criteria. The metabolomics-based aberrant tumor fumarate:malate ratios and elevated fumarate levels, which indicate loss of function of *FH*, combined with the IHC-confirmed loss of tumor FH protein in patients 10 and 11 and LOH, provides sufficient evidence to classify all three variants as likely pathogenic.

All three patients with germline *FH* PV (patients 10–12) had unilateral adrenal pheochromocytomas with a noradrenergic biochemical phenotype (see Supplementary information 2). None of the patients had metastatic disease at initial presentation (patient 10) or 6 (patient 11) to 18 years (patient 12) later. Family history included RCC in the father of patient 11 and thyroid cancer and melanoma in patient 10.

The three other fumarate outliers with normal fumarate: malate ratios, who also showed outlier profiles for malate (Supplementary Fig. S2C), did not show variants in *FH* but had *SDHx* variants classified as likely pathogenic or pathogenic (Table 1 and Supplementary material 3). Because those tumors did not show aberrant fumarate:succinate ratios, this might indicate that the outlier profiles for those samples are possibly related to sampling problems. In summary, fumarate:malate ratios above 7.0 with non-*FH* mutated PPGLs having values below 1.0 provide a useful guide to the presence of *FH* PV in PPGL. Increases in tumoral fumarate alone may not offer reliable guidance to the presence of *FH* PV.

Succinate:fumarate ratios, metabolic cluster 1, and *SDHx* variants in PPGL

In our cohort of 391 patients we identified 73 individuals having PPGLs with aberrant succinate:fumarate ratios.[16] All except five tumors belonged to the first metabolic cluster (Fig. 1a). Of those 73 patients, 34 had clear LOF PV (e.g., stop gain, frameshift, or exon-spanning deletions) in one of the *SDHx* genes in the germline. Furthermore, another 32 patients had missense *SDHx* variants in the germline that are well established as pathogenic or likely pathogenic ($n = 21$) or would be currently classified as VUS ($n = 10$) or likely benign (1), according to ACMG-AMP guidelines (see Tables 1 and 2 and Supplementary material 3). Additionally, three patients (57, 77, 92) had tumors of metabolic cluster I with confirmed pathogenic or likely pathogenic SDHx but with succinate: fumarate ratios below the cut-off.

In 7 of the 73 patients with elevated succinate:fumarate ratios, no *SDHx* variants were identified in the germline. In
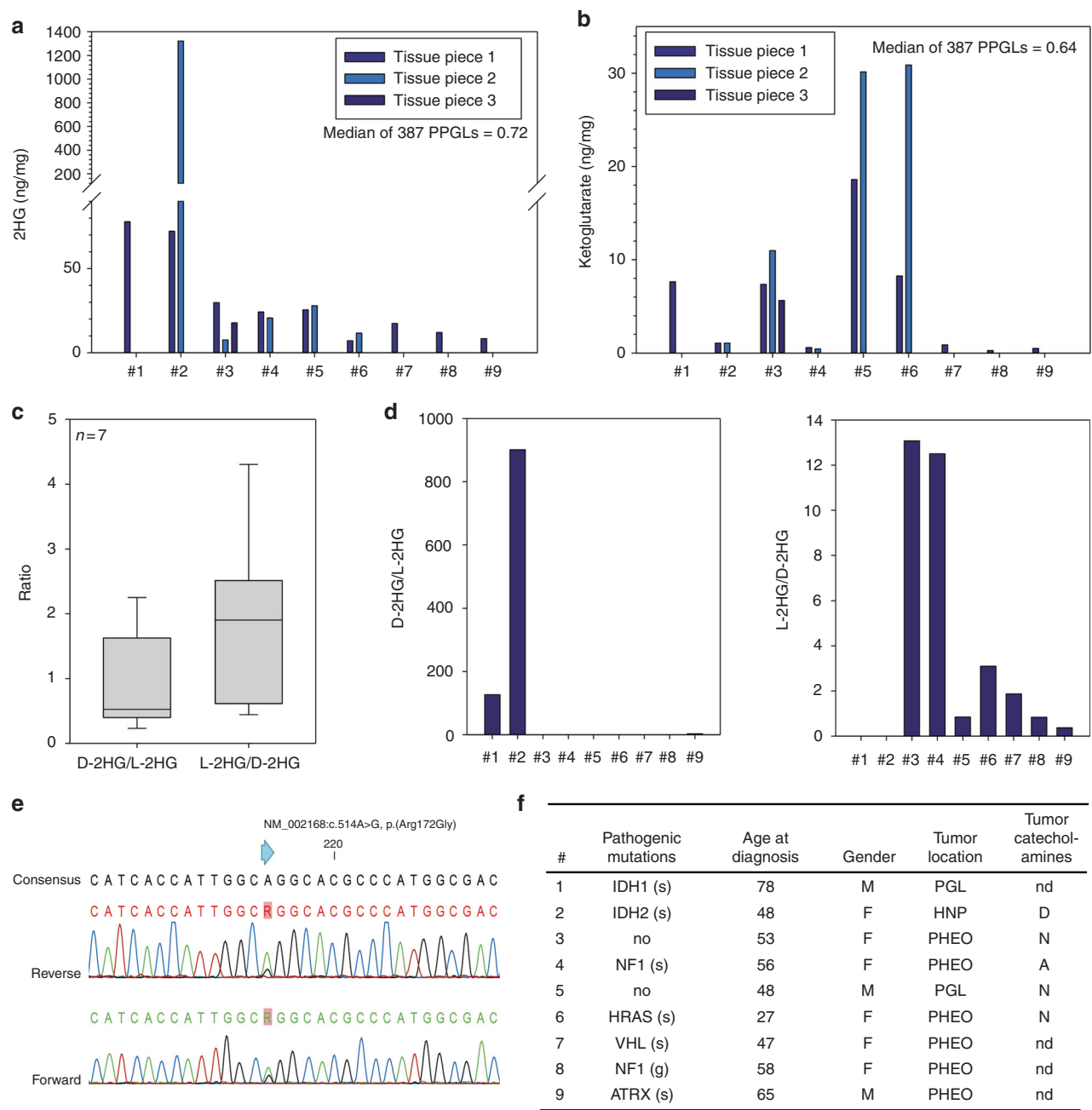
Fig. 2 Isocitrate dehydrogenase 1 and 2 gain-of-function PV in pheochromocytomas and paragangliomas (PPGLs). a Total 2-hydroxyglutarate (2HG) in nine tumors with the highest 2HG values of the set of fresh frozen tumors. **b** Total α-ketoglutarate in nine tumors with the highest 2HG values of the set. (**c**) Quantification of D- and L-enantiomers of 2HG in seven control PPGLs with moderate total 2HG. **d** Quantification of D-2HG and L-2HG in the nine tumors with highest total 2HG. **e** Sanger sequencing traces of PPGL 2 analyzed for a PV in IDH2. **f** Patient information of PPGLs with highest total 2HG. For further details refer to Supplementary information 1. *A* adrenergic, *D* dopaminergic, *g* germline, *HNP* head and neck, *N* noradrenergic, *nd* not determined, *PGL* extra-adrenal, *PHEO* adrenal, *s* somatic variant, # patient number

three of those patients we identified somatic *SDHx* variants: a stop PV (*SDHB* p.Tyr61*, 30); a startlossPV (*SDHD* c.3G>T, 70), and a missense PV (*SDHB* p.(Pro155Arg), 105) (Supplementary material 3). In a further patient we previously reported somatic *SDHC*-promoter methylation (107 [ref. [33]]) explaining the SDH-deficiency phenotype. Two cases (106, 108) currently remain unsolved. We excluded somatic *SDHx* PV and *SDHC*-promoter methylation in those cases. In 106

we reported germline variants in *OGDHL* and *PCK2*; however their significance is unclear.[6] The seventh case had a pathogenic germline *RET* c.1852T>G, p.(Cys618Gly) PV. Therefore, we interpreted the borderline succinate:fumarate elevation (109) as a false-positive result.

One patient (69) with an elevated tumoral succinate: fumarate was particularly interesting. He had a likely benign germline missense variant *SDHD* c.34G>A, p.(Gly12Ser),
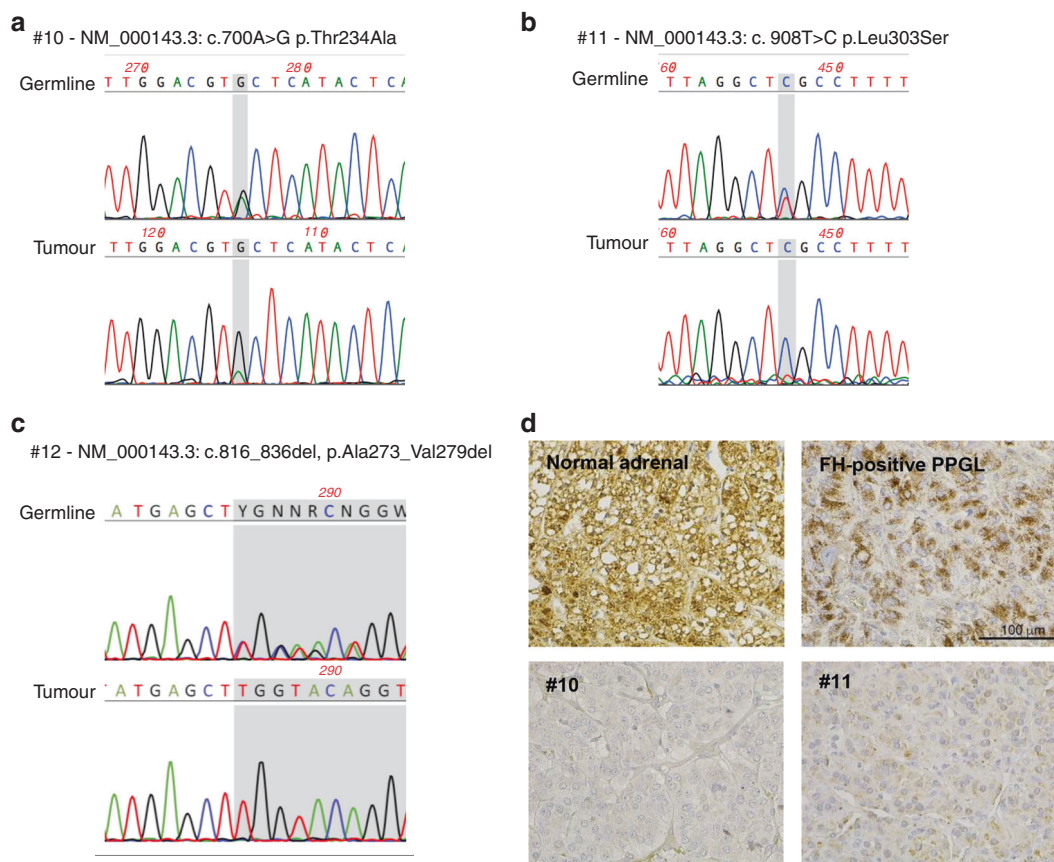
Fig. 3 Metabolite profiling identifies pathogenic fumarate hydratase (FH) PV previously classified as variants of unknown significance (VUS). Sanger sequencing traces of leukocyte (germline) and tumor DNA from **a** patient 10, **b** patient 11 and **c** patient 12. **d** Immunohistochemical staining for FH of normal adrenal, FH wild type, and FH mutated pheochromocytoma and paraganglioma (PPGL) tissue from patients 10 and 11; 400× magnification

which is listed 2035x heterozygously and 12x homozygously in the gnomAD database and has been described as a rare polymorphism.[34] The patient presented with sympathetic paraganglioma of the renal hilum at age 21 and had no family history of PPGL. Because germline testing was inconclusive in this case, we analyzed tumor tissue and identified a pathogenic somatic LOF variant in *SDHD* NM_003002.3: c.337_340del, p.(Asp113Metfs*21) in 41.47% of reads. Allele frequency of the germline variant c.34G>A was 69.7% in the tumor, indicating LOH of the wild-type *SDHD* allele and that the somatic and pathogenic *SDHD* variant p.(Asp113Metfs*21) occurred in the allele having the germline c.34G>A variant. We therefore assume that the somatic *SDHD* variant p.(Asp113Metfs*21) is responsible for SDH deficiency in this tumor and that the germline variant is likely benign and coincidentally observed together with the aberrant metabolite profile.

In ten patients with elevated succinate:fumarate ratios we identified eight different missense variants in *SDHx* (including one somatic) that would currently be classified as VUS. Most of the variants had previously been described in PPGL cases and integrating the metabolomics profiles together with all available information (see Supplementary information 3), we would now reclassify five of those variants as likely pathogenic (Table 2).

On the other hand, in 22 patients with succinate:fumarate ratios that were not suspicious, germline variants in *SDHx* were identified during routine diagnostics testing. Three are known benign or likely benign *SDHx* variants (two missense, one silent), in line with the normal succinate:fumarate ratios. However, nine patients had pathogenic or likely pathogenic germline PV, including one frameshift, one splice site, one gross deletion, and four different missense mutations already described in PPGL families. Three of those (57, 77, 92) had tumors belonging to metabolic cluster I despite their normal succinate:fumarate. Seven of the tumors furthermore were head and neck paragangliomas, known to contain high amounts of stroma. For none of the samples was histological estimation of tumor content performed. Testing of additional tumor specimens in these patients revealed highly elevated ratios in three cases, supporting that these were false negatives due to insufficient sampling of tumor cells.

The remaining ten cases with normal succinate:fumarate had variants that would be currently classified as VUS: seven missense, one in-frame deletion, one potential splice site, and one substitution in the 5'UTR. Metabolomics analysis for additional tumor samples in eight cases confirmed normal succinate:fumarate ratios. Further analyses revealed somatic pathogenic variants in *VHL* and *EPAS1* in two cases (14, 50)

Table 1 Pathogenic and likely pathogenic variants in *SDHx* genes identified in this study

Gene	Nucleotide change	Predicted protein change	Pathogenic Variant type	Germline status	S:F above cut-off	Phenotype	Reference SNP number	n patients (families)	AF in gnomAD in %	ClinVar	Known from literature/ disease databases
SDHB	c.72+1G>T	-	Splice	Germline	Yes	PGL	rs587782703	1	-	Pathogenic	Yes
	c.136C>T	p.(Arg46*)	Stop	Germline	Yes	3× xPGL	rs74315370	3	0.001805	Pathogenic	Yes
	c.166_170del	p.(Pro56Tyrfs*5)	fs	Germline	Yes	1× PGL	rs786202100	1	-	Pathogenic	Yes
	c.183T>G	p.(Tyr61*)	Stop	Somatic	Yes	1× PGL	-	1	-	-	yes
	c.268C>T	p.(Arg90*)	Stop	Germline	Yes	2× PGL, 1× HNP	rs74315366	3	0.00041	Pathogenic	Yes
	c.287-1G>C	-	Splice	Germline	Yes	1× PGL	rs397516833	1	-	-	Yes
	c.287-3G>C	-	Potential splice	Germline	Yes	2× PGL	rs397516834	2	-	-	Yes
	c.343C>T	p.(Arg115*)	Stop	Germline	Yes	1× PGL	rs751000085	1	0.00081	Pathogenic	Yes
	c.348_352del	p.(Ile117Hisfs*5)	fs	Germline	Yes	1× PHEO	-	1	-	-	-
	c.423+1G>A	-	Splice	Germline	Yes	3× PGL	rs398122805	3	0.0012	Pathogenic	Yes
	c.445_446delinsGGTATCT	p.(Gln149Glyfs*11)	Frameshift	Germline	Yes	1× PGL	-	1	-	-	Yes
	c.503dup	p.(Gln169Alafs*10)	Frameshift	Germline	Yes	2× PHEO	-	2(1)	-	-	-
	c.587G>A	p.(Cys196Tyr)	Missense	Germline	Yes	1× PHEO	-	1	-	Pathogenic	Yes
	c.649C>T	p.(Arg217Cys)	Missense	Germline	No[1]	2× HNP	rs200245469	2	-	Likely pathogenic	Yes
	c.689G>A	p.(Arg230His)	Missense	Germline	Yes	2× PGL	rs587782604	2	0.00041	Pathogenic/ likely pathogenic	Yes
	c.725G>A	p.(Arg242His)	Missense	Germline	Yes	2× HNP, 1× PGL	rs74315368	3	0.00081	Pathogenic	Yes
SDHC	c.43C>T	p.(Arg15*)	Stop	Germline	Yes	1× HNP	rs201286421	1	0.00082	Pathogenic	Yes
	c.126G>A	p.(Trp42*)	Stop	Germline	No	1× PGL	-	1	-	-	Yes
SDHD	c.3G>T		Startloss	Somatic	Yes	1× HNP	-	1	-	-	Yes
	c.14G>A	p.(Trp5*)	Stop	Germline	Yes	1× HNP	rs104894310	1	-	Pathogenic	Yes
	c.36_37del	p.(Ala13Profs*55)	Frameshift	Germline	Yes	1× PHEO	-	1	-	-	Yes
	c.49C>T	p.(Arg17*)	Stop	Germline	Yes	1× HNP	-	1	-	-	Yes
	c.53-2A>G	-	Splice	Germline	Yes	1× HNP	-	1	-	-	-
	c.53dup	p.(Leu19Serfs*50)	Frameshift	Germline	Yes	1× HNP	-	1	-	Pathogenic/ likely pathogenic	-
	c.64C>T	p.(Arg22*)	Stop	Germline	Yes	2× HNP	rs104894306	2(1)	-	Pathogenic	Yes
	c.169+1G>A	-	Splice	Germline	(Yes)[b]	1× HNP	-	1	-	-	Yes

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Table 1 continued

Gene	Nucleotide change	Predicted protein change	Pathogenic Variant type	Germline status	S:F above cut-off	Phenotype	Reference SNP number	n patients (families)	AF in gnomAD in %	ClinVar	Known from literature/ disease databases
	c.170-1G>T		Splice	Germline	Yes	1× PHEO	-	1	0.00041	-	Yes
	c.242C>T	p.(Pro81Leu)	Missense	Germline	Yes (5/7)	7× HNP	rs80338844	7	0.0018	Pathogenic	Yes
	c.274G>T	p.(Asp92Tyr)	Missense	Germline	Yes	2× HNP	rs80338845	2	-	Pathogenic	Yes
	c.284T>C	p.(Leu95Pro)	Missense	Germline	Yes	1× PHEO, 1× PGL, 1× HNP	rs80338846	3(2)	-	Pathogenic	Yes
	c.298_301del	p.(Thr100Phefs*34)	Frameshift	Germline	Yes	1× HNP	rs786203067	1	-	Pathogenic	Yes
	c.325C>T	p.(Gln109*)	Stop	Germline	Yes	1× PHEO, 1× PGL	-	2(1)	-	Pathogenic	yes
	c.337_340del	p.(Asp113fs)	Frameshift	**Somatic**	Yes	1× PGL	-	1	-	Pathogenic	Yes
	c.341A>G	p.(Tyr114Cys)	Missense	Germline	Yes	2× HNP	rs104894304	2	-	Pathogenic	Yes
	c.416T>C	p.(Leu139Pro)	Missense	Germline	**No**	1× PHEO	rs80338847	1	-	Pathogenic	Yes
SDHA	c.1753C>T	p.(Arg585Trp)	Missense	Germline	Yes	1× PHEO	rs200397144	1	0.0022	Inconclusive	Yes
SDHAF2	c.232G>A	p.(Gly78Arg)	Missense	Germline	Yes	3× HNP	rs113560320	3(2)	-	Pathogenic	Yes

AF allele frequency, *gnomAD* the Genome Aggregation Database, *HNP* head and neck paraganglioma, *S:F* succinate:fumarate, *PGL*, extra-adrenal pheochromocytoma, *PHEO* adrenal pheochromocytoma, *SNP* single-nucleotide polymorphism

[a]Metabolic cluster 1 tumor despite normal S:F ratio indicating aberrant SDH (likely normal tissue contamination)

[b]Normal succinate:fumarate ratios were found in the first tumor tissue, but measurement of a second piece confirmed highly elevated succinate:fumarate ratios

Table 2 Variants in *SDHx* genes identified in this study that would be currently classified as VUS according to ACMG with potential reclassification based on succinate:fumarate ratios

Gene	Nucleotide change	Predicted protein change	Pathogenic variant type	Germline status	S:F above cut-off	Phenotype	Reference SNP number	n patients (families)	AF in gnomAD in %	ClinVar	Known from literature/disease databases	Further information	Final classification
SDHB	c.32G>A	p.(Arg11His)	Missense	Germline	**No**	1x PHEO	rs111430410	1	0.051	Conflicting	Yes	Reported in SDHB positive tumor (IHC)	**Likely benign**
	c.49A>G	p.(Thr17Ala)	Missense	Germline	**No**	1x PHEO	-	1	-	Uncertain significance	Yes	Somatic likely pathogenic VHL, no LOH	**Likely benign**
	c.119A>C	p.(Lys40Thr)	Missense	Germline	**Yes**	1x HNP	-	1	-	-	-		VUS
	c.325A>C	p.(Asn109His)	Missense	Germline	**Yes**	1x PGL	-	1	0.00041	-	-		VUS
	c.380T>G	p.(Ile127Ser)	Missense	Germline	**Yes**	1x PGL/1x HNP	rs786201095	2	0.0012	Pathogenic/likely pathogenic	Yes		**Likely pathogenic**
	c.418G>T	p.(Val140Phe)	Missense	Germline	**Yes**	2x PGL	rs267607032	2	0.0012	Pathogenic/likely pathogenic	Yes		**Likely pathogenic**
	c.464C>G	p.(Pro155Arg)	Missense	Somatic	**Yes**	1x PGL	-	1	-	-	Yes		**Likely pathogenic**
	c.572G>A	p.(Cys191Tyr)	Missense	Germline	**Yes**	1x HNP	-	1	-	-	Yes	SDHB negative	**Likely pathogenic**
	c.574T>C	p.(Cys192Arg)	Missense	Germline	**Yes**	1x PHEO	rs786202732	1	-	Pathogenic	Yes		**Likely pathogenic**
SDHC	c.179G>T	p.(Ser60Ile)	Missense	Germline	**Yes**	1x HNP	-	1	-	-	-	SDHB IHC positive	VUS
	c.200T>C	p.(Met67Thr)	Missense	Germline	**No**	1x PGL	-	1	0.00041	-	-	No LOH	VUS
	c.256_257insTTT	p.(Gly86delinsValCys)	In-frame del	Germline	**No**	1x HNP	-	1	-	-	Yes		VUS
	c.379C>T	p.(His127Tyr)	Missense	Germline	**Yes**	1x PHEO	-	1	-	-	Yes	No LOH, SDH loss in IHC described in literature	**Likely pathogenic**
SDHD	c.169+5G>T		Potential splice	Germline	**(Yes)**[a]	1x HNP	-	1	-	-	Yes	IHC SDHB positive; LOH; skipping exon 2 cDNA in literature	**Likely pathogenic**
SDHA	c.239T>C	p.(Leu80Pro)	Missense	Germline	**No**	1x PHEO	-	1	-	-	-		VUS
	c.-7A>C		5'UTR substitution	Unknown	**No**	1x PHEO	rs751633537	1	0.067	Inconclusive	-		VUS
	c.1421C>T	p.(Ser474Leu)	Missense	Germline	**No**	1x PHEO	-	1	-	-	-	Somatic EPAS1 c.1592C>G	**Likely benign**
SDHAF2	c.1772C>T	p.(Ala591Val)	Missense	Germline	**No**	1x HNP	rs367621815	1	0.0018	-	-		VUS
	c.320G>A	p.(Arg107His)	Missense	Germline	**No**	1x PGL	rs535627239	1	0.018	-	-	No LOH	VUS

ACMG American College of Medical Genetics and Genomics, *AF* allele frequency, *cDNA* complementary DNA, *gnomAD*, the Genome Aggregation Database, *HNP* head and neck paraganglioma, *IHC* immunohistochemistry, *LOH* loss of heterozygosity, *PGL* extra-adrenal paraganglioma, *PHEO* adrenal pheochromocytoma, *S:F* succinate:fumarate, *SNP* single-nucleotide polymorphism, *UTR* untranslated region, *VUS* variant of unknown significance

[a]Normal succinate:fumarate ratios were found in the first tumor tissue, but measurement of a second piece confirmed highly elevated succinate:fumarate ratios

and we would reclassify the *SDHx* variants as likely benign (Table 2). Another variant, p.(Arg11His, patient 23), has been described in an SDHB-positive tumor and together with metabolite data we would classify it as likely benign.[35] In a patient (78) with a potential splice-site variant in *SDHD* (c.169+5G>T) metabolomics of a second tumor specimen revealed highly elevated succinate:fumarate ratios and we would altogether reclassify this variant as likely pathogenic. For the remaining six variants, despite normal succinate: fumarate, there is currently insufficient evidence for or against pathogenicity to reclassify these variants (see Table 2 and Supplementary information 3).

Among the total of 19 VUS in *SDHx* identified in our cohort, subsequent to comprehensive analysis of the metabolome and the tumor genome, we would reclassify seven (37%) as likely pathogenic and three (16%) as likely benign (Table 2). The overall diagnostic performance was comparable with previously published results and is summarized in Supplementary information 4 (ref.[16]).

DISCUSSION

Stratification of PPGL patients according to underlying PV is a crucial step of personalized patient management. PV in Krebs cycle–related genes are established drivers of tumor development, acting through the inhibition of α-ketoglutarate-dependent enzymes. Identifying such driver PV has important implications for the surveillance of patients at risk of recurrence or metastasis or of carriers of germline variants who are at risk of tumor development.

Here we show that quantification of metabolites in tumor tissue can guide identification of underlying driver germline or somatic PV in patients with unresolved results of genetic testing. More specifically, characteristic metabolite profiles involving high succinate:fumarate or fumarate:malate ratios or high levels of 2HG in combination with high D-2HG/ L-2HG ratios can respectively be used to guide detection of *SDHx*, *FH*, and *IDH1/2* PV. The described mass spectrometry–based assay is simple, cheap, and uses the same instrumentation required for measurements of metanephrines, a standard test in the workup of patients with suspected PPGL.[36] Metabolite data together with the catecholamine biochemical phenotype and clinical presentation can be used to narrow down the list of genes for Sanger sequencing.[13] Moreover, isolated PPGL cases with rare somatic PV in *IDHx* or *SDHx* can be identified, and thereby rule out a hereditary disease in those patients.

A particular advantage of metabolome-guided genomics is that tumors with similar phenotypic presentations in terms of metabolite accumulation or depletion can be characterized, leading to identification of mechanisms of silencing known PPGL genes or even of novel susceptibility genes. Recent examples are the identification of *SDHC*-promoter methylation and a *GOT2*-activating PV in PPGL with a similar clinical presentation as in *SDHx*-mutated PPGLs.[6,33] In our cohort, we identified five cases with somatic *SDHx* alterations, including one *SDHC*-promoter methylation, all currently considered extremely rare events.[6,33] We also for the first time report a somatic *IDH2* PVmuta in a PPGL.

The metabolome-guided identification of three patients with germline *FH* PV provides further information to clarify the clinical presentation of *FH*-mutated PPGLs. Consistent with the adrenal locations of tumors in our three patients, 9 of 11 previously published cases also involved adrenal tumors.[3,37–39] All three pheochromocytomas of the current study had the expected noradrenergic phenotype, but such phenotypic information has only been available for one previously described patient also with a noradrenergic tumor. Two patients under surveillance had not developed metastasis 6 or 18 years after initial diagnosis. Follow-up remains strictly recommended also due to the increased risk for RCC and leiomyomas.[4]

In addition to guiding genetic testing, metabolome analysis is also useful for guiding data analysis and interpretation of gene variants. The latter is particularly valuable with multigene panels that are associated with increased identification of coincidental, nonpathogenic variants. Interpretation of test results is often difficult, because many variants are uncharacterized and their disease-related impact remains unknown, leading to uncertainty for both patients and health-care providers. Provision of false-positive results to patients can have significant negative consequences for patients and their families. Guidelines published by the ACMG and AMP give clear instructions on the interpretation of germline variants.[26] Here we show that characterization of tumor tissue through mass spectrometry–based analysis of Krebs cycle metabolites can be employed to guide variant interpretation. When aberrant metabolomics profiling is in concordance with a VUS in a matching Krebs cycle gene, we propose that this can be used as supporting criteria for pathogenicity; if tumor metabolomics does not support a deficiency in the corresponding gene, this can be a supporting criterion against pathogenicity. Therefore, if germline genetic testing is inconspicuous or inconclusive (VUS), genetic testing of tumor tissue accompanied by metabolomics can be employed along with immunohistochemistry where appropriate. Importantly, in cases with a clear pathogenic metabolite tumor profile but without a clear pathogenic PV in the corresponding genes, alternative methods to analyze the same genes, such as promoter methylation, should be considered.

Unremarkable metabolite profiles should be interpreted in context and do not exclude a Krebs cycle gene PV ; false-negative results can be due to high amounts of normal or stromal tissue within the sample. In line with a previous study, we found that false-negative results mainly occur in head and neck paragangliomas[16] (Supplementary information 4), which may be explained by subforms with low amounts of tumor cells relative to tumor volume.[40] If possible, several tissue specimens from different regions of the tumor should be investigated. Importantly, tissue specimens should be scored for tumor content by a pathologist for both metabolomics and genetic testing.

In summary, metabolomics is a useful tool for guiding identification of *SDHx*, *FH*, and *IDH1/2*PV s and in variant interpretation with implications not only in PPGL, but also in other tumor diseases caused by Krebs cycle gene PV. Based on our experiences, we propose a cumulative approach for comprehensive PPGL diagnostics, including sequencing of germline and tumor tissue, as well as metabolic analyses in patients with inconclusive results of genetic testing; particularly in cases with VUS in Krebs cycle–associated genes and unsolved cases to guide diagnostic decision making.[13] Our study underlines that genetic diagnostics in PPGLs is a multidisciplinary approach combining the expertise of geneticists, endocrinologists, clinical chemists, and pathologists.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (https://doi.org/10.1038/s41436-018-0106-5) contains supplementary material, which is available to authorized users.

DISCLOSURE

The authors declare that they have no conflicts of interest.

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ARTICLE

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