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# Update of the UMD-VHL database: classification of 164 challenging variants based on genotype–phenotype correlation among 605 entries

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## ABSTRACT

**Background** The von Hippel-Lindau (VHL) disease is a hereditary tumour syndrome caused by germline mutations in *VHL* tumour suppressor gene. The identification of *VHL* variants requires accurate classification which has an impact on patient management and genetic counselling.

**Methods** The TENGEN (French oncogenetics network of neuroendocrine tumours) and PREDIR (French National Cancer Institute network for Inherited predispositions to kidney cancer) networks have collected *VHL* genetic variants and clinical characteristics of all VHL-suspected patients analysed from 2003 to 2021 by one of the nine laboratories performing *VHL* genetic testing in France. Identified variants were registered in a locus-specific database, the Universal Mutation Database-VHL database (<http://www.umd.be/VHL/>).

**Results** Here we report the expert classification of 164 variants, including all missense variants (n=124), all difficult interpretation variants (n=40) and their associated phenotypes. After initial American College of Medical Genetics classification, first-round classification was performed by the VHL expert group followed by a second round for discordant and ambiguous cases. Overall, the VHL experts modified the classification of 87 variants including 30 variants of uncertain significance that were as (likely)pathogenic variants for 19, and as likely benign for 11.

**Conclusion** Consequently, this work has allowed the diagnosis and influenced the genetic counselling of 45 VHL-suspected families and can benefit to the worldwide VHL community, through this review.

## INTRODUCTION

The von Hippel-Lindau (VHL) disease is a hereditary tumour syndrome that predisposes to retinal and central nervous system (CNS), haemangioblastomas (HBs), pheochromocytomas and paragangliomas (PPGLs), clear cell renal cell carcinoma (ccRCC) and renal cysts, pancreatic cysts and neuroendocrine tumours (pNETs), endolymphatic sac tumour (ELST), epididymal and broad ligament cyst-adenomas. The VHL disease is caused by germline mutations in the *VHL* tumour suppressor

## WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The von Hippel-Lindau (VHL) disease is caused by germline *VHL* mutation that requires accurate classification.

## WHAT THIS STUDY ADDS

⇒ The TENGEN and PREDIR networks have collected and interpreted the data of all *VHL*-mutated patients in the French population during 20 years.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The VHL experts modified the classification of 87 variants, registered in Universal Mutation Database-VHL locus-specific database ([www.umd.be/VHL/](http://www.umd.be/VHL/)), impacting the clinical care and the genetic counselling of 45 VHL-suspected families.

gene with an autosomal dominant pattern. The estimated incidence is approximately 1:36.000 with a frequency of de novo mutation being nearly 20%.<sup>1</sup> The *VHL* gene (3p25) contains three exons that encode two transcripts containing the three exons (NM\_000551.3) or the exon 1 spliced with the exon 3 (NM\_198156.2). The canonical version containing the three exons (NM\_000551.3) is a 642-nucleotide transcript which can be translated in two protein isoforms differing by the presence or the absence of the 53 first amino acids (pVHL160 and pVHL213 that contain 160 and 213 amino acids, respectively). Indeed, an alternative initiation codon ATG is found at the 54th codon.<sup>2</sup> Recently, a cryptic exon has been identified deep in intron 1 and named exon E1'. It has been found mutated in patients with the VHL disease or polycythaemia, but its role is still unknown.<sup>3</sup> The N-terminal region of the human VHL protein (pVHL) is a disordered domain containing a repeat region of 8×5 amino acid tandem G-(PAVG)-E-E-(DAYSLE). This domain is poorly conserved during evolution and its function remains unclear.<sup>4</sup> pVHL is a component of a pVHL-elonginC-elonginB complex which acts as a ubiquitin-ligase E3 and normally leads to



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proteasome-dependent degradation of the hypoxia-inducible factor alpha subunit (HIF $\alpha$ ). The  $\beta$ -domain of pVHL contains the cytosolic chaperonin containing TCP-1 (CCT) complex binding region (codons 100–155) needed for protein folding<sup>5</sup> and the codons Trp88, Tyr98, His115 and Trp117 required to interact with the hydroxyproline of HIF $\alpha$ , establishing hydrogen and van der Waals bonds.<sup>6</sup> Moreover, the BC-box (codons 157–166) is a critical domain allowing the interaction between pVHL and elonginB/C and is necessary to the proteosomal degradation of pVHL.<sup>7</sup> Both protein isoforms (pVHL160 and 213) contain the domains involved in HIF regulation.

Traditionally, two types of VHL disease are described:

- ▶ VHL type 1, which is characterised by a low risk of PPGL and associated with truncating pathogenic variant (PV) (nonsense, frameshift, splicing mutations or large deletions).
- ▶ VHL type 2 with a high risk of PPGL and associated with missense mutations.

The diagnostic criteria of clinical VHL disease are defined by the Massachusetts General Hospital criteria<sup>8</sup> as:

- ▶ One lesion of VHL and a positive family history.
- ▶ Two or more retinal or CNS HBs regardless of family history.
- ▶ One retinal or CNS HB and a visceral manifestation regardless of family history.

In addition, *VHL* mutations have been identified in patients with hereditary polycythaemia.<sup>9</sup> In these cases, the patients carry hypomorphic mutations on both alleles and they never develop tumours, except for two cases of HBs.<sup>10</sup>

Whereas truncating variants such as nonsense, frameshift and splice site variants ( $\pm 1$ ,  $\pm 2$ ) in *VHL* are typically pathogenic, the interpretation of missense, synonymous or deep intronic variants, in-frame deletions and variants upstream from the 54th codon is highly challenging.

However, the identification of *VHL* variants requires accurate classification. This step is essential for the diagnosis of VHL disease. It is also a prerequisite to propose *VHL* predictive genetic testing to relatives of affected patients and then a surveillance protocol to positive mutation carriers in order to foster a favourable clinical outcome.<sup>11</sup> Only PV and LPV allow those opportunities.

The UMD-VHL variant database was developed with the UMD (Universal Mutation Database) software and curated by the French expert group in oncogenetic neuroendocrine tumors (TENGEN) and the PREDIR (French National Cancer Institute network for Inherited predispositions to kidney cancer) networks from 2003, supported by the Institut National du Cancer. This database includes 1642 patients with 605 different variants and 162 protein variants identified by the nine French laboratories involved in genetic diagnosis of VHL disease (<http://www.umd.be/VHL/>). Here we report the classification of 124 missense variants and 40 variants with challenging interpretation carried out by the VHL expert TENGEN group.

## MATERIALS AND METHODS

### Data collection

*VHL* variants were shared by the nine French molecular biology laboratories performing *VHL* genotyping and belonging to the TENGEN network.

All missense variants, all variants located in upstream alternative ATG codon 54 and other challenging interpretation variants (deep intronic variants, in-frame deletions and synonymous variants) were selected for this study. All variants with an allele frequency (AF) up to 1% in the population Genome Aggregation Database (gnomAD V2.1.1) were excluded. In fact, it concerns

only two intronic variants: c.463+43A>G (AF: 1.19%) and c.340+5G>C (AF: 1.12%).

Clinical data of probands included type, number and localisation of tumours, age at onset, family history of VHL spectrum tumours, co-segregation data, metastatic status, recurrence and catecholamine secretion profile of PPGL.

Written informed consent from patients was obtained during one-on-one genetic counselling. An anonymised number was created for each patient.

### Sequencing data

Leucocyte DNA was locally extracted from peripheral blood. The full coding regions and exon–intron junctions were sequenced using Sanger sequencing and next-generation sequencing. The cryptic exon E1' was not consistently sequenced<sup>3</sup> and was investigated only in patients with polycythaemia in order to highlight a second hit if necessary.

All reported variants were annotated according to the NM\_000551.3 reference transcript on genome assembly GRCh37 as defined by the nomenclature of the Human Genome Variation Society.

### Initial American College of Medical Genetics classification

Following the publication of the American College of Medical Genetics (ACMG) recommendation,<sup>12</sup> for all missense variants, we consistently considered the PP2 criterion (missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease), since no missense variant with an AF >1% was reported in gnomAD.

The PS4 criteria (the prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls) were not considered due to its redundancy with PM2 criterion (absent from controls) for a rare disease such as VHL.

In silico analyses were performed including nucleotide conservation, amino acid conservation and Grantham distance score with the assistance of Alamut Visual software, Varsome, UMD-Predictor and Mobidetails (<https://mobidetails.iurc.montp.inserm.fr/MD/>),<sup>13</sup> a newly established French aggregator database. Each variant was checked in gnomAD V2.1.1 and in inherited disease databases ClinVar, LOVD, HGMDpro and VHLdb.<sup>14</sup>

For the missense variants, we used the Rare Exome Variant Ensemble Learner (REVEL) combined score with threshold at 0.2 (benign <0.2) and 0.7 (damaging >0.7). For splicing variants and synonymous variants, we used MaxEntScan with a cut-off at 15% and Splicing Prediction PiPeline (SPIP) following the interpretation of the software to predict the impact on the splicing mechanism.

Each laboratory classified *VHL* variants separately. Then, in accordance with the ACMG recommendations, all the variants found from 2003 were reclassified by a single curator in one of the five following classes: PV (class 5), LPV (class 4), variant of uncertain significance (VUS, class 3), likely benign variant (LBV, class 2) and benign variant (BV, class 1).

### Review and curation by experts

This initial ACMG classification was submitted to the experts of the TENGEN network and reviewed through several meetings between 2018 and 2021. After a first round of curation, a second round was conducted for the discordant cases and for variants which remained of unknown significance. This second round was carried out after a careful research of additional data: new clinical or family data, somatic genetic analysis, that is, examining for a loss of heterozygosity (LOH) status in the tumour or

a second event on the *VHL* gene or on other candidate genes and for carbonic anhydrase 9 (CA9) immunohistochemistry (IHC) results. A CA9 membranous staining is in favour of a loss of pVHL function in the tumour with a high sensitivity (96%) and specificity (89%) in PPGL (online supplemental figure 1).<sup>15</sup>

### Statistical analyses

Statistical analyses were performed using Prism V.9.3.1 (GraphPad Software, La Jolla, California, USA). The mean ages of patients with (L)PV and (L)BV were compared using a non-paired, non-parametric Mann-Whitney test. The comparison of the number of lesions between the  $\beta$ -domain and the BC-box was done using a  $\chi^2$  test of association. The threshold for statistical significance was  $p < 0.05$  (95% CI).

## RESULTS

### Variants from the UMD database included in this study

Overall, 164 unique variants (online supplemental table) were included from 301 index cases: 293 patients with VHL features, 7 patients with isolated polycythaemia and 1 with both. Ninety-eight variants were found in only a single patient. For 63 variants, the number of times they were detected was between 2 and 9 times, and only 3 variants were found 10 or more times.

These 164 unique variants included 124 (75%) missense variants and 40 (25%) variants with challenging interpretation. Among the last ones, 27 were intronic variants, 8 were synonymous variants, 1 was a nonsense variant localised at amino acid 52, 2 were in-frame deletions, 1 was a substitution in the translation initiation codon and 1 was a substitution in the last codon resulting in the extension of the coding sequence of 14 amino acids.

The variants were distributed all along the gene, in the coding and flanking regions (online supplemental figure 2). Eighty variants were localised within exon 1: in the 5' untranslated region (5'UTR), the coding part and its flanking regions or within exon E1', including 23 (13%) variants localised in or upstream the

alternative start codon at 54th codon. Among these 23 variants, 11 were missense variants. Respectively, 44 and 40 variants were localised within exons 2 and 3, and their flanking regions.

### Comparison between ACMG classification and VHL expert classification of the 164 variants

The 164 variants were initially classified according to the ACMG classification, as follows: 14 (9%) PVs, 75 (46%) LPVs, 63 (38%) VUSs, 10 (6%) LBVs and 2 (1%) BVs. Considering the great number of VUSs ( $n=63$ ) that deprive the patients of definitive diagnosis and relatives to predictive genetic testing, two rounds of curation by the VHL experts were done allowing to reclassify 87 variants. Thirty VUSs were reclassified as follows: 9 as PVs, 10 as LPVs and 11 as LBVs (figure 1B). Finally, among these 164 variants, 78 (47%) variants were classified as pathogenic, 29 (18%) as likely pathogenic, 34 (21%) as VUSs (33 remaining VUSs+1 LPV reclassified as VUS), 20 (12%) as likely benign and 3 (2%) as benign (figure 1A).

Interestingly, only one L(PV) (c.1-73delC) was localised before the alternative initiation codon 54. Among the 164 variants, 159 were found in patients with features of VHL, and only 5 in patients with isolated polycythaemia. The two cohorts of patients with VHL features or with polycythaemia were analysed separately in the following manuscript.

### Clinical characterisation of 205 patients of the VHL disease cohort bearing an (L)PV after VHL expert classification

Two-hundred five VHL-suspected index cases were found with a PV or an LPV. Clinical data were not available for 6 and incomplete for 10 of them (table 1).

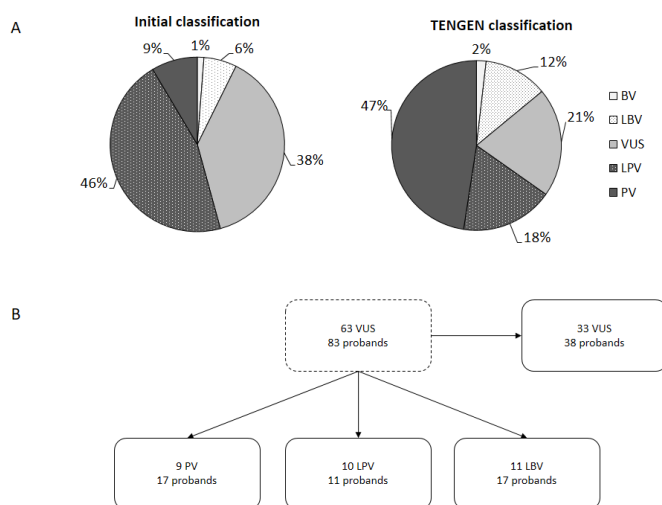
Only 105 index cases (53%) met the criteria of clinical VHL disease. Overall, 90 (48%) patients harboured only one lesion of the VHL spectrum, 33 (17%) had two lesions and 66 (35%) had three or more lesions. One hundred patients presented an HB, of whom 19 had retinal HB, 38 had CNS HB and 38 showed both localisations. The localisation was unknown at the time of genetic testing for five patients. One hundred and nineteen patients had PPGL, of whom 51 were bilateral or multiple and 7 were metastatic. Thirty-six patients presented a ccRCC, 23 had a pNET and 4 an ELST. The mean age of the first disease onset was 29 years (min: 3 years; max: 74 years).

These 205 patients carried 102 different (L)PVs including 92 missense variants and 10 other challenging variants. Interestingly, one patient had two (L)PVs. The first c.376G>A, p.(D126N) was classified pathogenic through clinical data, CA9 IHC and somatic LOH data available from another patient of the cohort. The second variant c.574C>T, p.(P192S) was classified as LPV and not seen in other patients of the cohort. This patient developed a PPGL as well as a polycythaemia. Unfortunately, the cis or trans status of both variants could not be clarified in the absence of segregation study.

### Correlation between the localisation of the 92 missense (L)PVs and the clinical characterisation of the patients

The 92 missense (L)PVs were found in 194 patients. Among those for which clinical data were available, 99 (53%) had a clinical VHL. All the 92 missense variants were localised after the alternative initiation codon 54 and showed similar distribution across the three exons of the gene (figure 2).

Interestingly, the 56 patients with variants within BC-box region developed significantly more lesions (>3 lesions in 52% of patients) than the 32 patients with variants within CCT binding region (>3 lesions in 37% of patients) ( $p=0.001$ , figure 3). The



**Figure 1** (A) Comparison of initial ACMG classification and classification by VHL experts of the 164 variants. (B) Reclassification of the 63 VUSs after reviewing by the VHL experts. Dotted line: initial classification, full line: after two rounds of review by Tengen work group. ACMG, American College of Medical Genetics; BV, benign variant; LBV, likely benign variant; LPV, likely pathogenic variant; PV, pathogenic variant; Tengen, French oncogenetics network of neuroendocrine tumors; VHL, von Hippel-Lindau; VUS, variant of unknown significance.

**Table 1** Clinical characteristics of the 205 patients bearing VHL (L)PV

	Variants n=102	Missense variants n=92	Other challenging variants n=10
Patients	205	194	11
Sex (F/M)	45/47	42/44	3/3
Mean age at first lesion (years) (min–max)	29 (3–74)	29 (3–74)	41 (12–67)
Clinical VHL* (%)†	105 (53)	99 (53)	6 (55)
Including VHL lesion family history (%)	46 (23)	43 (23)	3 (27)
Lesions of VHL spectrum‡			
HB (%)	100 (53)	93 (52)	7 (64)
PPGL (%)	119 (63)	113 (63)	6 (55)
ccRCC and renal cysts (%)	60 (32)	56 (31)	4 (36)
pNET and pancreatic cysts (%)	60 (32)	58 (33)	2 (18)
ELST (%)	4 (2)	4 (2)	0 (0)

\*According to Massachusetts General Hospital criteria.<sup>7</sup>  
 †From the 199 patients for whom the data were available.  
 ‡From the 199 patients for whom the VHL lesions were described.  
 ccRCC, clear cell renal cell carcinoma; ELST, endolymphatic sac tumour; F, female; HB, haemangioblastoma; (L)PV, likely pathogenic variant; M, male; pNET, pancreatic neuroendocrine tumour; PPGL, pheochromocytoma/paraganglioma; VHL, von Hippel-Lindau.

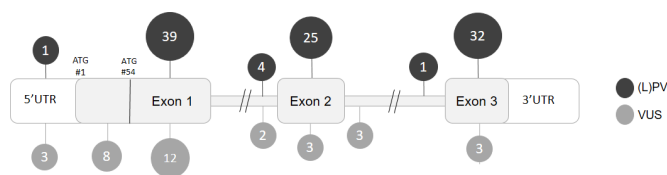
age of occurrence of each lesion was not available for further analysis, but the age at the diagnosis of the first lesion was similar between the two groups (28.6 vs 26.4 years). The type of VHL lesion was similar in the two groups (online supplemental figure 3).

**Localisation of the 10 challenging (L)PVs and their clinical characterisation**

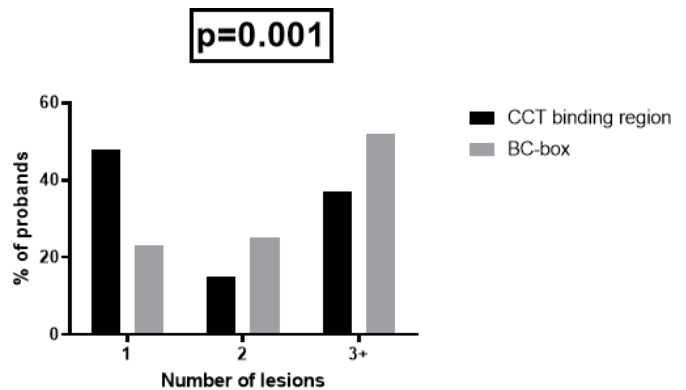
These variants concerned 11 patients and included two synonymous variants, five splicing variants, one in-frame deletion, one extension with the loss of the native terminal codon and a 5'UTR variant. Interestingly, the latter was the only LPV/PV localised before the alternative start codon. This variant, c.1-73delC, was found in two probands:

- ▶ One presented a ccRCC in early 40s with multiple CNS HBs and bilateral ccRCC in his family. The genetic testing showed a genotype–phenotype co-segregation (four related out of five, see online supplemental figure 4).
- ▶ The other presented bilateral ccRCC and his father, brother and nephew had a ccRCC (online supplemental figure 4).

The lesions in these 11 patients were similar to those seen in patients with missense (L)PVs and no significant difference was present between the two groups (table 1).



**Figure 2** Localisation of the 102 likely pathogenic variant ((L)PV in black) and the 34 variants of unknown significance (VUSs in grey) on *VHL* gene after classification by VHL expert group. VHL, von Hippel-Lindau.



**Figure 3** Comparison of the number of VHL lesions in the 56 patients with a missense (L)PV in the CCT binding region (codons 100–155) with the 32 patients with a missense (L)PV in the BC-box domain (codons 157–166). The data are expressed as percentage of the total number of patients having a missense (L)PV in the CCT binding region or in the BC-box domain. The statistical analysis was performed with a X<sup>2</sup> test of association (p=0.001). L(PV), likely pathogenic variant; VHL, von Hippel-Lindau.

**Clinical description of 38 patients of the VHL disease cohort carrying a VUS after VHL expert classification**

Thirty-eight probands carried 34 different VUSs after two rounds of curation. None of these 38 patients presented with a clinical VHL and all harboured only one lesion of the VHL spectrum, with the exception of one who had a paraganglioma and renal cysts, without any family history.

Most of the variants (23 of 34, 68%) were localised in the 5' region of the gene including the 5'UTR and the coding part of exon 1. Eleven (33%) were localised before the alternative initiation codon 54 and 12 (35%) after it (figure 2). Additionally, we found two VUSs in cis: c.(290C>T; 347T>C), p.((Pro97Leu; Leu116Pro)) in a patient presenting with a pheochromocytoma, multiple thoracic–abdominal paragangliomas and a familial pheochromocytoma. These two variants were not found in any other patient of the cohort in heterozygous state.

**Clinical characteristics of patients with (L)BV after VHL expert classification**

We classified as (L)BV 23 variants that were present in 54 patients (table 2); 11 of 23 were localised in the 5'UTR and in the coding region of exon 1. The mean age at diagnosis was 42 years, which was significantly older (p<0.0001) than patients with (L)PV. Most patients presented with an isolated lesion including 23 isolated PPGLs, 17 isolated ccRCCs, 6 isolated HBs and 1 isolated pNET. However, three patients with an isolated (L)BV showed clinical VHL:

- ▶ Two carried a variant in intron 1, c.341-37G>A, p.(?) found in gnomAD at a frequency of 0.6% and in homozygous state in 57 cases. The first patient had multiple cerebellar HBs and a family history of VHL: a spinal HB and a pheochromocytoma in his first sister, pancreatic cysts in his second sister and a pheochromocytoma in his son. The second patient had a cerebellar HB and a pancreatic lesion.
- ▶ One patient had a variant in intron 2, c.463+8C>T, p.(?) found in gnomAD at a frequency of 0.05% and in the homozygous state in one case. This variant was reported 11 times in ClinVar (9 of 11 as (L)BV). No splicing alteration was described by in silico analysis software. This patient presented with multiple cerebellar HBs.

**Table 2** The 23 (likely)benign variants and their in silico analysis

Variant	c.	p.	Position	Classification	Number of occurrences	Revel score	gnomAD frequency (%)
#1	c.1-77C>T	p.?	5'UTR	Benign	2	NA	0
#2	c.-61_-51dup	p.?	5'UTR	Likely benign	3	NA	0.009
#3	c.1-12A>T*	p.?	5'UTR	Likely benign	2	NA	0.0007
#4	c.5C>T*	p.(Pro2Leu)	Exon 1	Likely benign	3	0.266	0.008
#5	c.74C>T	p.(Pro25Leu)	Exon 1	Benign	3	0.155	0.298
#6	c.91_92DelinsTT*	p.(Glu31Leu)	Exon 1	Likely benign	2	NA	0
#7	c.114C>T	p.(Ser38Ser)	Exon 1	Likely benign	2	NA	0.003
#8	c.119C>T	p.(Pro40Leu)	Exon 1	Likely benign	3	0.2879	0.023
#9	c.150C>G	p.(Ala50Ala)	Exon 1	Likely benign	3	NA	0.050
#10	c.154G>A*	p.(Glu52Lys)	Exon 1	Likely benign	2	0.559	0.008
#11	c.183C>G	p.(Pro61Pro)	Exon 1	Likely benign	4	NA	0.096
#12	c.340+15C>T	p.?	Intron 1	Likely benign	1	NA	0
#13	c.340+20G>A	p.?	Intron 1	Likely benign	1	NA	0.003
#14	c.340+109G>T	p.?	Intron 1	Likely benign	1	NA	0
#15	c.341-98C>T	p.?	Intron 1	Likely benign	1	NA	0
#16	c.341-88G>C	p.?	Intron 1	Likely benign	2	NA	0
#17	c.341-37G>A	p.?	Intron 1	Benign	8	NA	0.690
#18	c.341-22C>T*	p.?	Intron 1	Likely benign	1	NA	0.001
#19	c.449A>G	p.(Asn150Ser)	Exon 2	Likely benign	1	0.8889	0.0003
#20	c.463+8C>T	p.?	Intron 2	Likely benign	5	NA	0.050
#21	c.463+37_463+39del	p.?	Intron 2	Likely benign	1	NA	0.036
#22	c.612G>A	p.(Glu204Glu)	Exon 3	Likely benign	2	NA	0.003
#23	c.639T>C	p.(Asp213Asp)	Exon 3	Likely benign	1	NA	0.004

\*No LOH and/or no CA9 membranous staining in tumour.

CA9, carbonic anhydrase 9; gnomAD, Genome Aggregation Database; LOH, loss of heterozygosity; NA, not available; 5'UTR, 5' untranslated region.

### Clinical and genomic features of the patients with isolated polycythaemia

Seven patients of the cohort presented with an isolated polycythaemia (table 3). Two were homozygous and three were compound heterozygous, in one case with a variant in exon E1' (patient #5). For the remaining two probands (#3 and #4) with only one identified variant, the exon E1' sequencing was not performed. Among the nine variants found in these seven probands, five were exclusively found in this cohort of patients with isolated polycythaemia, the other four were also found in VHL cases at a heterozygous state.

### DISCUSSION

We present here a collaborative work collecting a total of 605 VHL variants from the TENGEN and the PREDIR networks, from which we focused on 124 missense and 40 other challenging variants from 301 probands.

Overall, the review of variants by VHL experts resulted in the reclassification of 87 variants, of which 30 were initially classified as VUS. Fifty-four variants out of the 164 were not reported in either the HGMDpro or VHLdb databases, and 25 of them were not found in ClinVar database. This reclassification by VHL experts allowed an improvement of the management of 45 probands with VHL lesions and for their families to benefit from genetic counselling (figure 1A).

**Table 3** Seven patients with isolated polycythaemia

Patient	Sex	Family history	c.	p.	Position	Status	Classification	Variant found in other patients of the cohort with VHL features
#1	Unk	No	c.162G>C	p.(Met54Ile)	Exon 1	Homozygous	PV	No
#2	M	No	c.241C>G	p.(Pro81Ala)	Exon 2	Compound	(L)PV	No
			c.430G>A	p.(Gly144Arg)	Exon 2	heterozygous	PV	H&N PGL at 21
#3	Unk	No	c.538A>G	p.(Ile180Val)	Exon 3	Heterozygous	(L)PV	No
#4	M	No	c.550C>T	p.(Leu184Phe)	Exon 3	Heterozygous	(L)PV	HB at 62 and ccRCC; pheo at 48 and pancreatic cyst
#5	F	Twin sister and brother asymptomatic without E1' variant	c.575C>G	p.(Pro192Arg)	Exon 3	Compound	(L)PV	No
			c.340+770T>C	p.(?)	E1'	heterozygous	PV	No
#6	M	No	c.598C>T	p.(Arg200Trp)	Exon 3	Compound	PV	Clinical VHL; isolated ccRCC at 47
			c.376G>A	p.(Asp126Asn)	Exon 2	heterozygous	PV	Pheo; PGL
#7	M	Polycythaemia	c.598C>T	p.(Arg200Trp)	Exon 3	Homozygous	PV	Clinical VHL; isolated ccRCC at 47

ccRCC, clear cell renal cell carcinoma; E1', cryptic exon; F, female; HB, haemangioblastoma; H&N, head and neck; (L)PV, likely pathogenic variant; M, male; PGL, paraganglioma; pheo, pheochromocytoma; PV, pathogenic variant; Unk, unknown; VHL, von Hippel-Lindau.

The *VHL* variants that were classified in this study were distributed along the entire coding region of the gene (online supplemental figure 1). Nevertheless, (L)PVs were more frequent in exons 2 and 3 coding sequences (25 and 32 (L)PVs for a length of the coding sequence of 123 and 179 bp, respectively) than in exon 1 (39 (L)PVs for 340 bp of coding sequence), and only one (L)PV was identified in the 5'UTR. In fact, VUS and (L)BV were mainly localised in the 5' region of the gene, up to the end of exon 1. The concentration of VUS and LBV in the N-terminal region of pVHL supports the fact that the function of this disordered region remains unknown and has been poorly conserved during evolution, despite the fact that no polymorphism was present in the *VHL* coding region including this 5' region. The most frequent variant p.(P25L) found in gnomAD has an AF of 0.299%.

The classification of variants located upstream the 54th codon is the most challenging, since these variants do not affect translation of the p160 isoform of pVHL. Among the 23 variants located upstream the 54th codon, we classified only one as (L)PV (c.1-73delC), p.(?), based on the clinical and familial phenotype (online supplemental figure 3). This nucleotide is localised in the putative binding sequence for the AP4 transcription factor, six nucleotides before the canonical transcription initiation site.<sup>16 17</sup>

In the context of the rare *VHL* disease, we have considered that all truncating *VHL* variants, except those localised upstream the 54th codon, can be easily classified as pathogenic. Indeed, according to the ACMG criteria, the PVS1 argument (null variant in a gene where loss of function is a known mechanism of disease) and the low frequency of this type of variation described in the population gnomAD (PM2) are sufficient to classify the variant as pathogenic. Indeed, in gnomAD (V2.1.1, 2022), only 10 loss of function variants were observed, 5 upstream and 5 downstream the 54th alternative start codon. Among the five located upstream the 54th codon, one showed four occurrences (AF: 1.79<sup>e-5</sup>) and one had seven occurrences (AF: 4.09<sup>e-5</sup>). In contrast, all five variants located downstream the 54th codon have only shown one occurrence.

This study highlights the crucial role played by the *VHL* database, curated by *VHL* experts, in *VHL* diagnosis, since of the 205 patients with an (L)PV in our series, 100 (47%) did not fulfil the Massachusetts *VHL* criteria and were diagnosed through identification of a *VHL* mutation. Conversely, we found three patients with clinical *VHL* and bearing only an (L)BV. *VHL* mosaicism could be strongly suspected in those patients bearing multiple *VHL* lesions and without family history of *VHL* disease. The frequency of *VHL* mosaicism is largely unknown and is probably under-reported, being from 5% to 8.5% according to the literature.<sup>18-20</sup> Identification of patients with *VHL* disease without a *VHL* gene defect in the sequenced region raises the question of the existence of a *VHL* abnormality in other regions, such as the deep intronic region. An example is the identification of mutations in exon E1' located in intron 1 in a large family presenting with a typical *VHL* disease over three generations and stayed for a long time undiagnosed because no mutations in the *VHL* canonical exons were identified.<sup>3</sup> More recently, Ricketts *et al*<sup>21</sup> reported a patient with *VHL* bearing a germline balanced translocation between chromosomes 1 and 3, t(1;3)(p36.3;p25), with a breakpoint in the second *VHL* intron, creating a pathogenic alteration in *VHL*, undetectable by standard sequencing. This translocation mechanism has already been highlighted in some cases of hereditary ccRCC without other clinical features of *VHL*, most frequently between chromosomes 3 and 6.<sup>22-26</sup>

Finally, we found seven patients with isolated polycythaemia. Five of whom presented two hits in the *VHL* gene. For the two

patients with only one identified *VHL* gene defect, the exon E1' region was not sequenced. This highlights the need to sequence the cryptic exon E1' region in such cases. As expected, the variation p.(R200W) associated with Chuvash polycythaemia was identified in two of seven cases. However, the p.(R200W) variation, not usually associated with *VHL* features, was also found in two patients of the cohort without polycythaemia, one with a ccRCC and one with clinical *VHL*. In both cases, the p.(R200W) mutation was the only abnormality detected in the gene, raising the issue of the role of this variant in *VHL* lesions. Unfortunately, for these two patients, the *VHL* lesions were not available to allow exploration of the putative relationship between this variant and the lesion, and consequently to be able to provide adapted genetic counselling. Of note, p.(R200W) has been associated with isolated CNS HB in the literature,<sup>10</sup> but no recommendation exists in such situation.

The literature data reported a *VHL* hypomorphous variant with a low penetrance and an important clinical variability. This variant was a substitution of a proline by a serine on the codon 81. From our cohort, two probands had the p.(P81S): one with a metastatic pheochromocytoma and one with a ccRCC. Another proband with polycythaemia had a variant at the same codon, p.(P81A), with a compound heterozygous status with another *VHL* PV. The p.(P81S) variant is described numerous times in the literature. Rana *et al* reported a case of an isolated HB at age 40 years, with three relatives bearing the variant without any clinical manifestation at ages 36, 38 and 72 years.<sup>27</sup> Yoshida *et al* reported a patient with a CNS HB and a ccRCC.<sup>28</sup> Zbar *et al* reported two families with *VHL* without pheochromocytoma,<sup>29</sup> and Hes *et al* described a case with an isolated HB and four clinically unaffected first and second-degree relatives aged between 17 and 77 years.<sup>30</sup> Furthermore, Chesnel *et al* demonstrated that the p.(P81S) variant affects the function of pVHL213 and provides invasiveness to the 786-O kidney cancer cell line.<sup>31</sup> We have classified this variant as LPV, taking into consideration its frequency among the probands in the mutation databases. The difficulty in these cases involving hypomorphous variants is to provide an appropriate follow-up for probands and their relatives, but no published recommendation exists, as far as we know.

In conclusion, we report here the classification of 164 missense and challenging interpretation variants. This expert-curated *VHL* variant database is freely available to the scientific community in the UMD-*VHL* database (<http://www.umd.be/VHL/>), with regular updates every 6 months, and is crucial to provide guidance for both the diagnosis of *VHL* and the subsequent follow-up.

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