PVS1 / PM4 case studies

James Drummond

Presented to CanVIG 13/11/20

PALB2 c.1240C>T p.(Arg414Ter)

Variant classification:

Based on Garrett et al 2020 (https://jmg.bmj.com/content/jmedgenet/suppl/2020/03/15/jmedgenet-2019-106759.DC1/jmedgenet-2019-106759supp001_data_supplement.pdf).

Appendix 1: CanVIG-UK Consensus Specification for Cancer Susceptibility Genes of ACGS Best Practice Guidelines for Variant Classification (v1.2 03/03/20)

Guidance notes:

- For the following evidence items, CanVIG-UK have no additional specification for CSGs to add beyond that provided in ACGS Best Practice Guidelines for Variant Classification 2020[1]: PVS1, PS1, PS2, PM4, PM5, PM6, PP1, BS4, BP3, BP7 **(shaded white).**
- For the remaining evidence items, whilst remaining consistent with ACGS Best Practice Guidelines for Variant Classification 2020[1], there are more specific recommendations pertaining to CSGs contained within the CanVIG-UK Consensus Specification (**shaded grey).**
- A number of disease-specific expert panels have been established by the USA ClinGen Sequence Variant Interpretation (SVI) Working Group, generating disease/gene specific variant interpretation guidelines. Following evaluation within the CanVIG-UK group, in subsequent updates to the CanVIG-UK specification, we shall include specific recommendations regarding adoption and implementation of these disease/gene specific-guidance.

PVS1	_VSTR	_STR	_MOD	_SUP
Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where LOF is a known mechanism of disease				
See LOF decision tree and criteria (Tayoun *et al* 2018)[2]				

PALB2 c.1240C>T p.(Arg414Ter)

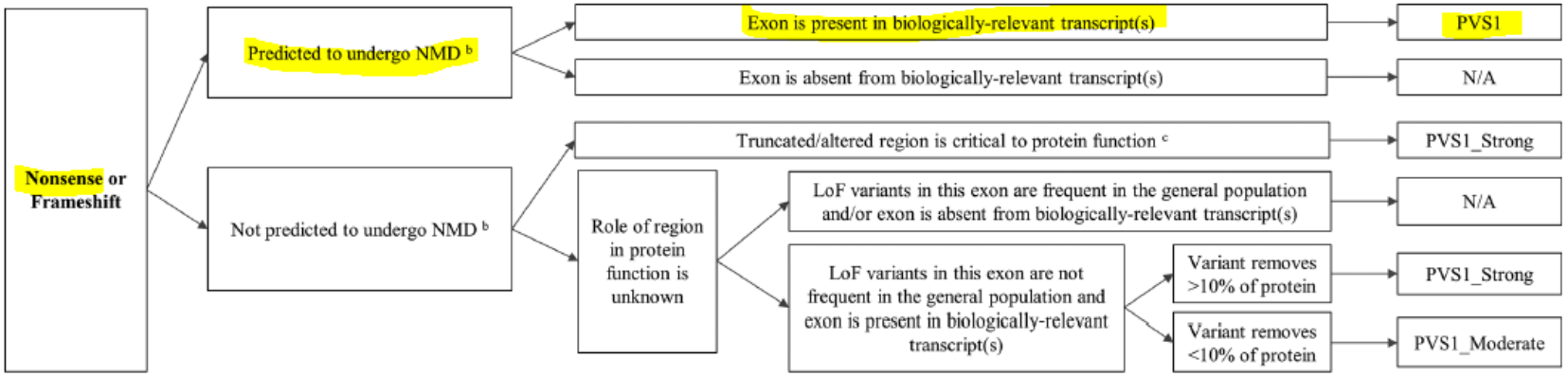


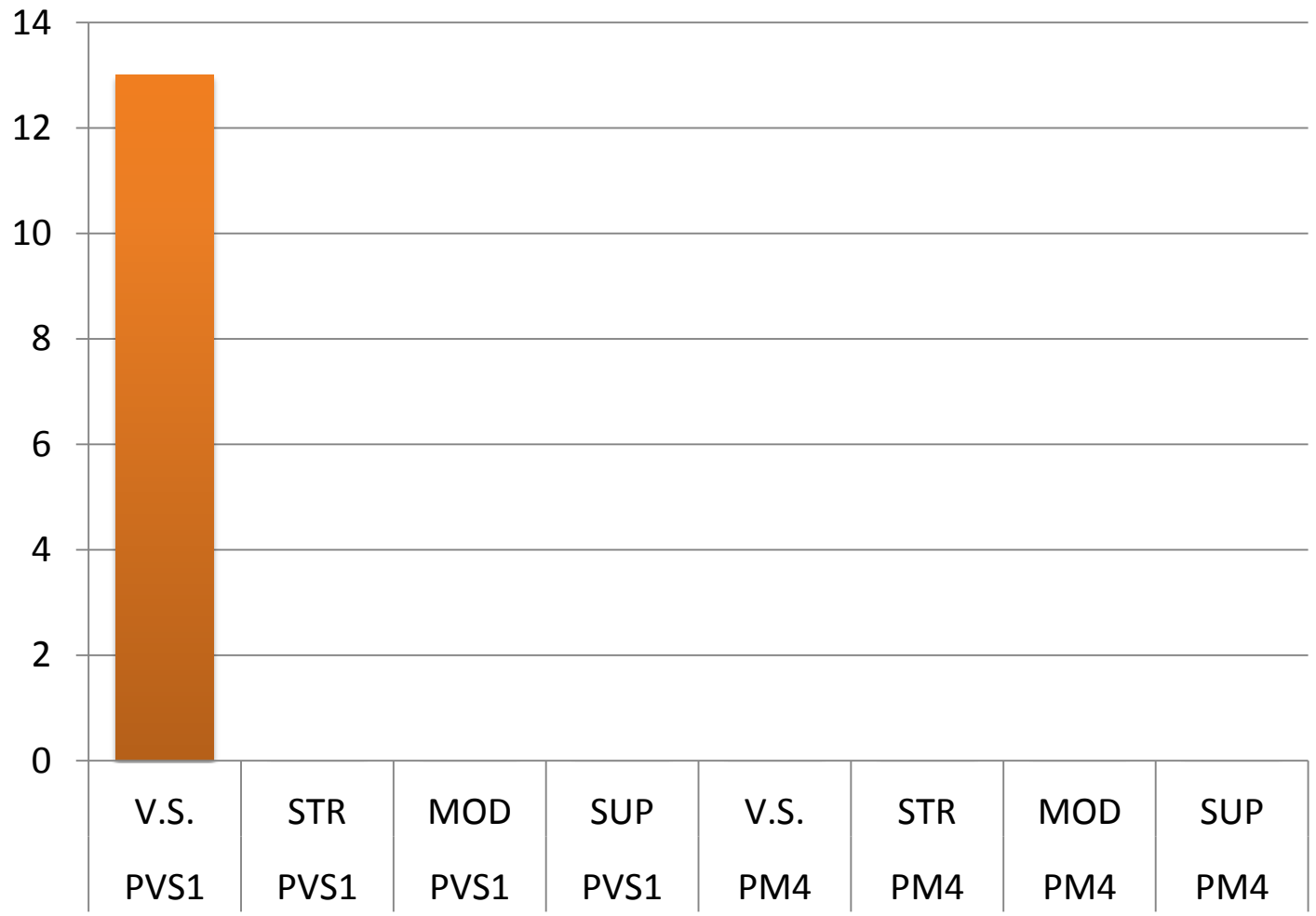
FIGURE 1 PVS1 decision tree. Refer to text for detailed description. (a) This criterion should not be applied in combination with in silico splicing predictions (PP3). Additionally, splice site variants must have no detectable nearby (+/- 20 nts) strong consensus splice sequence that may reconstitute in-frame splicing. (b) NMD prediction based on the premature termination codon not occurring in the 3′ most exon or the 3′-most 50 bp of the penultimate exon. (c) Relevant domain indicated by experimental evidence proving a critical role of the domain and/or presence of non-truncating pathogenic variants in the region. (d) For a full gene deletion of a known haploinsufficient gene, a pathogenic classification is warranted (in the absence of conflicting data) even though application of PVS1 alone would not reach a pathogenic classification using the combining rules in Richards et al. (2017). NMD, nonsense-mediated decay; LoF, loss of function

Variant is in exon 4 of 13 and results in premature termination codon -> NMD

PVS1_very strong

So this is very straightforward, as would a frameshift variant with a premature termination codon prior to the last two exons

PALB2 c.1240C>T p.(Arg414*)

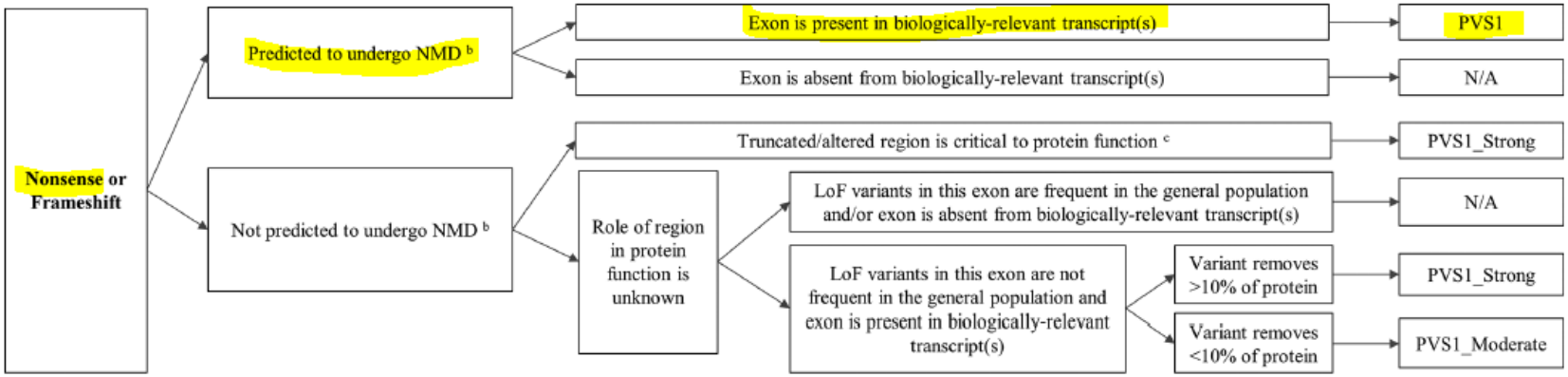


PALB2 c.1240C>T p.(Arg414*)

Comments (consensus statement):

- *Very strong - Nonsense variant in exon 4 (of 13). The mRNA is predicted to undergo Nonsense Mediated Decay (NMD). Loss of function variants have previously been reported as disease causing.*

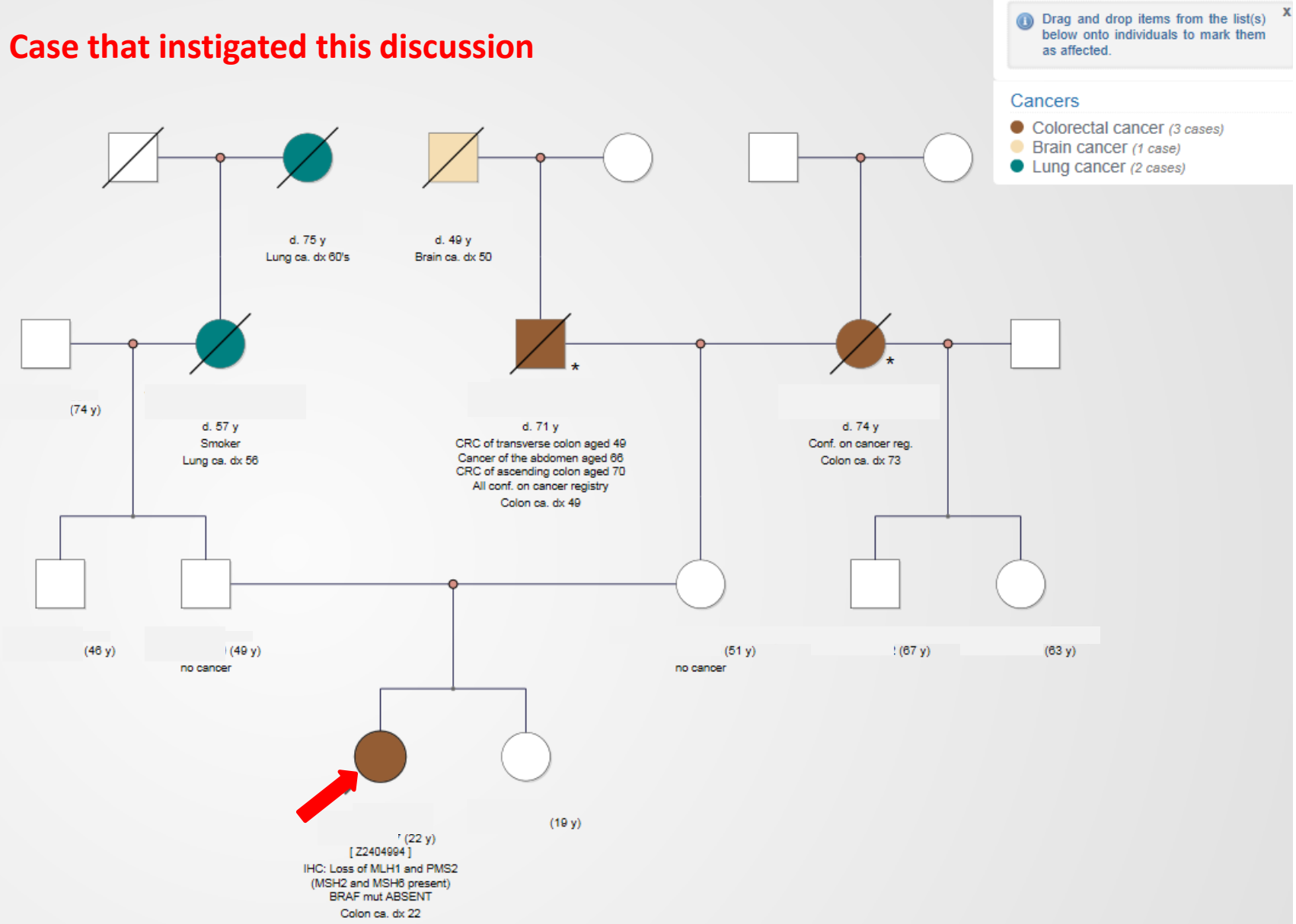
PALB2 c.1240C>T p.(Arg414Ter)



So far so good!

However, using PVS1 can be more problematic when frameshifts occur late in gene…

Case that instigated this discussion



Colonic adenocarcinoma – IHC loss MLH1 & PMS2, BRAF negative

Germline MLH1 screen requested (NGS & MLPA)

Variant identified: MLH1 c.2163_2164dupTA, p.(Lys722IlefsTer62)

Frameshift in last exon

Variant classification:

Based on Garrett et al 2020 (https://jmg.bmj.com/content/jmedgenet/suppl/2020/03/15/jmedgenet-2019-106759.DC1/jmedgenet-2019-106759supp001_data_supplement.pdf).

Appendix 1: CanVIG-UK Consensus Specification for Cancer Susceptibility Genes of ACGS Best Practice Guidelines for Variant Classification (v1.2 03/03/20)

Guidance notes:

- For the following evidence items, CanVIG-UK have no additional specification for CSGs to add beyond that provided in ACGS Best Practice Guidelines for Variant Classification 2020[1]: PVS1, PS1, PS2, PM4, PM5, PM6, PP1, BS4, BP3, BP7 **(shaded white).**
- For the remaining evidence items, whilst remaining consistent with ACGS Best Practice Guidelines for Variant Classification 2020[1], there are more specific recommendations pertaining to CSGs contained within the CanVIG-UK Consensus Specification (**shaded grey).**
- A number of disease-specific expert panels have been established by the USA ClinGen Sequence Variant Interpretation (SVI) Working Group, generating disease/gene specific variant interpretation guidelines. Following evaluation within the CanVIG-UK group, in subsequent updates to the CanVIG-UK specification, we shall include specific recommendations regarding adoption and implementation of these disease/gene specific-guidance.

PVS1	_VSTR	_STR	_MOD	_SUP
Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where LOF is a known mechanism of disease				
See LOF decision tree and criteria (Tayoun *et al* 2018)[2]				

Affected proteins - 1

```
NM_000249.3(MLH1_i001):p.(Lys722Ilefs*62)
```

Reference protein

```
  1  MSFVAGVIRR LDETVVNRIA AGEVIQRPAN AIKEMIENCL DAKSTSIQVI VKEGGLKLIQ
 61  IQDNGTGIRK EDLDIVCERF TTSKLQSFED LASISTYGFR GEALASISHV AHVTITTKTA
121  DGKCAYRASY SDGKLKAPPK PCAGNQGTQI TVEDLFYNIA TRRKALKNPS EEYGKILEVV
181  GRYSVHNAGI SFSVKKQGET VADVRTLPNA STVDNIRSIF GNAVSRELIE IGCEDKTLAF
241  KMNGYISNAN YSVKKCIFLL FINHRLVEST SLRKAIETVY AAYLPKNTHP FLYLSLEISP
301  QNVDVNVHPT KHEVHFLHEE SILERVQQHI ESKLLGSNSS RMYFTQTLLP GLAGPSGEMV
361  KSTTSLTSSS TSGSSDKVYA HQMVRTDSRE QKLDAFLQPL SKPLSSQPQA IVTEDKTDIS
421  SGRARQQDEE MLELPAPAEV AAKNQSLEGD TTKGTSEMSE KRGPTSSNPR KRHREDSDVE
481  MVEDDSRKEM TAACTPRRRI INLTSVLSLQ EEINEQGHEV LREMLHNHSF VGCVNPQWAL
541  AQHQTKLYLL NTTKLSEELF YQILIYDFAN FGVLRLSEPA PLFDLAMLAL DSPESGWTEE
601  DGPKEGLAEY IVEFLKKKAE MLADYFSLEI DEEGNLIGLP LLIDNYVPPL EGLPIFILRL
661  ATEVNWDEEK ECFESLSKEC AMFYSIRKQY ISEESTLSGQ QSEVPGSIPN SWKWTVEHIV
721  YKALRSHILP PKHFTEDGNI LQLANLPDLY KVFERC*
```

Protein predicted from variant coding sequence

```
  1  MSFVAGVIRR LDETVVNRIA AGEVIQRPAN AIKEMIENCL DAKSTSIQVI VKEGGLKLIQ
 61  IQDNGTGIRK EDLDIVCERF TTSKLQSFED LASISTYGFR GEALASISHV AHVTITTKTA
121  DGKCAYRASY SDGKLKAPPK PCAGNQGTQI TVEDLFYNIA TRRKALKNPS EEYGKILEVV
181  GRYSVHNAGI SFSVKKQGET VADVRTLPNA STVDNIRSIF GNAVSRELIE IGCEDKTLAF
241  KMNGYISNAN YSVKKCIFLL FINHRLVEST SLRKAIETVY AAYLPKNTHP FLYLSLEISP
301  QNVDVNVHPT KHEVHFLHEE SILERVQQHI ESKLLGSNSS RMYFTQTLLP GLAGPSGEMV
361  KSTTSLTSSS TSGSSDKVYA HQMVRTDSRE QKLDAFLQPL SKPLSSQPQA IVTEDKTDIS
421  SGRARQQDEE MLELPAPAEV AAKNQSLEGD TTKGTSEMSE KRGPTSSNPR KRHREDSDVE
481  MVEDDSRKEM TAACTPRRRI INLTSVLSLQ EEINEQGHEV LREMLHNHSF VGCVNPQWAL
541  AQHQTKLYLL NTTKLSEELF YQILIYDFAN FGVLRLSEPA PLFDLAMLAL DSPESGWTEE
601  DGPKEGLAEY IVEFLKKKAE MLADYFSLEI DEEGNLIGLP LLIDNYVPPL EGLPIFILRL
661  ATEVNWDEEK ECFESLSKEC AMFYSIRKQY ISEESTLSGQ QSEVPGSIPN SWKWTVEHIV
721  YIKPCAHTFC LLNISQKMEI SCSLLTCLIY TKSLRGVKYG YLCTVGCVLL SLYSDTKCCI
781  KV*
```

Normal protein
757aa

Abnormal and extended protein
784aa

36/757 = ~5 wt lost%

Different insertion variant that is same length on HGMD

codon 722 + 62 = 784

codon 752 + 32 = 784 (same length)

CI085163	ATCTA^[750]TACAAaaAGTCTTTGAG	c.2252_2253dupAA	p.(Val752Lysfs*32)	DM	Colorectal cancer, non-polyposis

Looks pathogenic? Actual detail in record, not so convincing…

HGMD® Professional 2019.4

Gene Mutation Phenotype Reference Batch Advanced | Statistics Information Support | Home Logout

HGMD accession	Reported disease/phenotype	Variant class	Gene symbol	Insertion	Codon (^)
CI085163	Colorectal cancer, non-polyposis	DM	MLH1	ATCTA^TACAAaaAGTCTTTGAG	750

Literature citation	Citation type	Support	Comments/notes
1. Sheng (2008) *Cytogenet Genome Res* **122:** 22 PubMed: 18931482 Mismatch repair gene mutations in Chinese HNPCC patients.	Primary literature report	☑	No comments
2. Pistorius (2000) *Int J Colorectal Dis* **15:** 255 PubMed: 11151427 Clinical consequences of molecular diagnosis in families with mismatch repair gene germline mutations.	Additional literature report	☐	unclassified variant
3. Wolf (2005) *Wien Klin Wochenschr* **117:** 269 PubMed: 15926618 Spectrum of germ-line MLH1 and MSH2 mutations in Austrian patients with hereditary nonpolyposis colorectal cancer.	Additional literature report	☐	uncertain pathogenicity
4. Rossi (2017) *BMC Cancer* **17:** 623 PubMed: 28874130 A survey of the clinicopathological and molecular characteristics of patients with suspected Lynch syndrome in Latin America.	Additional phenotype	☐	Lynch syndrome; Reported as class 3 (VUS)

Extra information	
Coding strand genomic sequence (GRCh38)	CAGCTTGCTAACCTGCCTGATCTATACAAA(-/aa)GTCTTTGAGAGGTGTTAAATATGGTTATTT
Genomic coordinate (GRCh38)	chr3:37050635-37050636
Genome viewers	UCSC; NCBI MapViewer; NCBI SeqViewer
HGVS nomenclature	NM_000249.3: c.2252_2253dupAA; NP_000240.1: p.(Val752Lysfs*32)
Variant Call Format (VCF)	CHROM POS ID REF ALT 3 37050632 CI085163 C CAA
ClinVar ID	90102
Clinical significance	Uncertain_significance

And InSiGHT lists as VUS

MLH1	c.2252_2253dup View InSiGHT Database	p. (Val752Lysfs*32)	Class 3: uncertain	2019/06/21 v2.4	Insufficient evidence: extends protein by 26 amino acids	0.86 (used data from Sheng et al., 2008, ORCID:Leung et al, Wolf et al., 2005)

Affected transcripts - 1

Our variant

NM_000249.3(MLH1_v001):c.2163_2164dup

Affected proteins - 1

NM_000249.3(MLH1_i001):p.(Lys722Ilefs*62)

Reference protein

```
  1  MSFVAGVIRR LDETVVNRIA AGEVIQRPAN AIKEMIENCL DAKSTSIQVI VKEGGLKLIQ
 61  IQDNGTGIRK EDLDIVCERF TTSKLQSFED LASISTYGFR GEALASISHV AHVTITTKTA
121  DGKCAYRASY SDGKLKAPPK PCAGNQGTQI TVEDLFYNIA TRRKALKNPS EEYGKILEVV
181  GRYSVHNAGI SFSVKKQGET VADVRTLPNA STVDNIRSIF GNAVSRELIE IGCEDKTLAF
241  KMNGYISNAN YSVKKCIFLL FINHRLVEST SLRKAIETVY AAYLPKNTHP FLYLSLEISP
301  QNVDVNVHPT KHEVHFLHEE SILERVQQHI ESKLLGSNSS RMYFTQTLLP GLAGPSGEMV
361  KSTTSLTSSS TSGSSDKVYA HQMVRTDSRE QKLDAFLQPL SKPLSSQPQA IVTEDKTDIS
421  SGRARQQDEE MLELPAPAEV AAKNQSLEGD TTKGTSEMSE KRGPTSSNPR KRHREDSDVE
481  MVEDDSRKEM TAACTPRRRI INLTSVLSLQ EEINEQGHEV LREMLHNHSF VGCVNPQWAL
541  AQHQTKLYLL NTTKLSEELF YQILIYDFAN FGVLRLSEPA PLFDLAMLAL DSPESGWTEE
601  DGPKEGLAEY IVEFLKKKAE MLADYFSLEI DEEGNLIGLP LLIDNYVPPL EGLPIFILRL
661  ATEVNWDEEK ECFESLSKEC AMFYSIRKQY ISEESTLSGQ QSEVPGSIPN SWKWTVEHIV
721  YKALRSHILP PKHFTEDGNI LQLANLPDLY KVFERC*
```

Protein predicted from variant coding sequence

```
  1  MSFVAGVIRR LDETVVNRIA AGEVIQRPAN AIKEMIENCL DAKSTSIQVI VKEGGLKLIQ
 61  IQDNGTGIRK EDLDIVCERF TTSKLQSFED LASISTYGFR GEALASISHV AHVTITTKTA
121  DGKCAYRASY SDGKLKAPPK PCAGNQGTQI TVEDLFYNIA TRRKALKNPS EEYGKILEVV
181  GRYSVHNAGI SFSVKKQGET VADVRTLPNA STVDNIRSIF GNAVSRELIE IGCEDKTLAF
241  KMNGYISNAN YSVKKCIFLL FINHRLVEST SLRKAIETVY AAYLPKNTHP FLYLSLEISP
301  QNVDVNVHPT KHEVHFLHEE SILERVQQHI ESKLLGSNSS RMYFTQTLLP GLAGPSGEMV
361  KSTTSLTSSS TSGSSDKVYA HQMVRTDSRE QKLDAFLQPL SKPLSSQPQA IVTEDKTDIS
421  SGRARQQDEE MLELPAPAEV AAKNQSLEGD TTKGTSEMSE KRGPTSSNPR KRHREDSDVE
481  MVEDDSRKEM TAACTPRRRI INLTSVLSLQ EEINEQGHEV LREMLHNHSF VGCVNPQWAL
541  AQHQTKLYLL NTTKLSEELF YQILIYDFAN FGVLRLSEPA PLFDLAMLAL DSPESGWTEE
601  DGPKEGLAEY IVEFLKKKAE MLADYFSLEI DEEGNLIGLP LLIDNYVPPL EGLPIFILRL
661  ATEVNWDEEK ECFESLSKEC AMFYSIRKQY ISEESTLSGQ QSEVPGSIPN SWKWTVEHIV
721  YIKPCAHTFC LLNISQKMEI SCSLLTCLIY TKSLRGVKYG YLCTVGCVLL SLYSDTKCCI
781  KV*
```

Affected transcripts - 1

HGMD/InSiGHT variant

NM_000249.3(MLH1_v001):c.2252_2253dup

Affected proteins - 1

NM_000249.3(MLH1_i001):p.(Val752Lysfs*32)

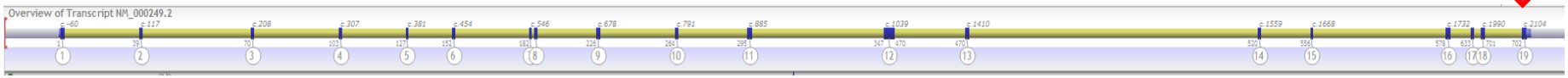
Reference protein

```
  1  MSFVAGVIRR LDETVVNRIA AGEVIQRPAN AIKEMIENCL DAKSTSIQVI VKEGGLKLIQ
 61  IQDNGTGIRK EDLDIVCERF TTSKLQSFED LASISTYGFR GEALASISHV AHVTITTKTA
121  DGKCAYRASY SDGKLKAPPK PCAGNQGTQI TVEDLFYNIA TRRKALKNPS EEYGKILEVV
181  GRYSVHNAGI SFSVKKQGET VADVRTLPNA STVDNIRSIF GNAVSRELIE IGCEDKTLAF
241  KMNGYISNAN YSVKKCIFLL FINHRLVEST SLRKAIETVY AAYLPKNTHP FLYLSLEISP
301  QNVDVNVHPT KHEVHFLHEE SILERVQQHI ESKLLGSNSS RMYFTQTLLP GLAGPSGEMV
361  KSTTSLTSSS TSGSSDKVYA HQMVRTDSRE QKLDAFLQPL SKPLSSQPQA IVTEDKTDIS
421  SGRARQQDEE MLELPAPAEV AAKNQSLEGD TTKGTSEMSE KRGPTSSNPR KRHREDSDVE
481  MVEDDSRKEM TAACTPRRRI INLTSVLSLQ EEINEQGHEV LREMLHNHSF VGCVNPQWAL
541  AQHQTKLYLL NTTKLSEELF YQILIYDFAN FGVLRLSEPA PLFDLAMLAL DSPESGWTEE
601  DGPKEGLAEY IVEFLKKKAE MLADYFSLEI DEEGNLIGLP LLIDNYVPPL EGLPIFILRL
661  ATEVNWDEEK ECFESLSKEC AMFYSIRKQY ISEESTLSGQ QSEVPGSIPN SWKWTVEHIV
721  YKALRSHILP PKHFTEDGNI LQLANLPDLY KVFERC*
```

Protein predicted from variant coding sequence

```
  1  MSFVAGVIRR LDETVVNRIA AGEVIQRPAN AIKEMIENCL DAKSTSIQVI VKEGGLKLIQ
 61  IQDNGTGIRK EDLDIVCERF TTSKLQSFED LASISTYGFR GEALASISHV AHVTITTKTA
121  DGKCAYRASY SDGKLKAPPK PCAGNQGTQI TVEDLFYNIA TRRKALKNPS EEYGKILEVV
181  GRYSVHNAGI SFSVKKQGET VADVRTLPNA STVDNIRSIF GNAVSRELIE IGCEDKTLAF
241  KMNGYISNAN YSVKKCIFLL FINHRLVEST SLRKAIETVY AAYLPKNTHP FLYLSLEISP
301  QNVDVNVHPT KHEVHFLHEE SILERVQQHI ESKLLGSNSS RMYFTQTLLP GLAGPSGEMV
361  KSTTSLTSSS TSGSSDKVYA HQMVRTDSRE QKLDAFLQPL SKPLSSQPQA IVTEDKTDIS
421  SGRARQQDEE MLELPAPAEV AAKNQSLEGD TTKGTSEMSE KRGPTSSNPR KRHREDSDVE
481  MVEDDSRKEM TAACTPRRRI INLTSVLSLQ EEINEQGHEV LREMLHNHSF VGCVNPQWAL
541  AQHQTKLYLL NTTKLSEELF YQILIYDFAN FGVLRLSEPA PLFDLAMLAL DSPESGWTEE
601  DGPKEGLAEY IVEFLKKKAE MLADYFSLEI DEEGNLIGLP LLIDNYVPPL EGLPIFILRL
661  ATEVNWDEEK ECFESLSKEC AMFYSIRKQY ISEESTLSGQ QSEVPGSIPN SWKWTVEHIV
721  YKALRSHILP PKHFTEDGNI LQLANLPDLY KKSLRGVKYG YLCTVGCVLL SLYSDTKCCI
781  KV*
```

Identical over highlighted residues



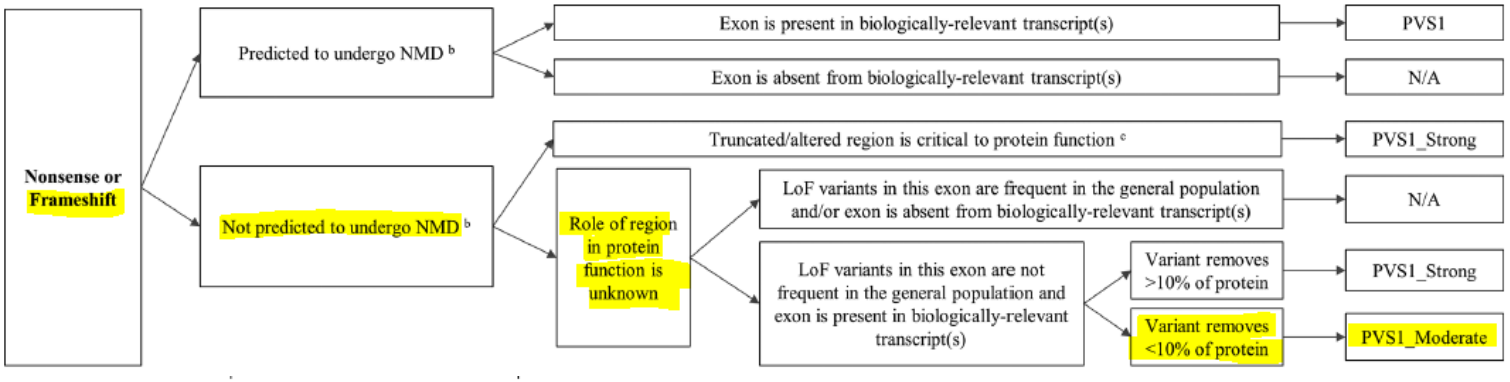
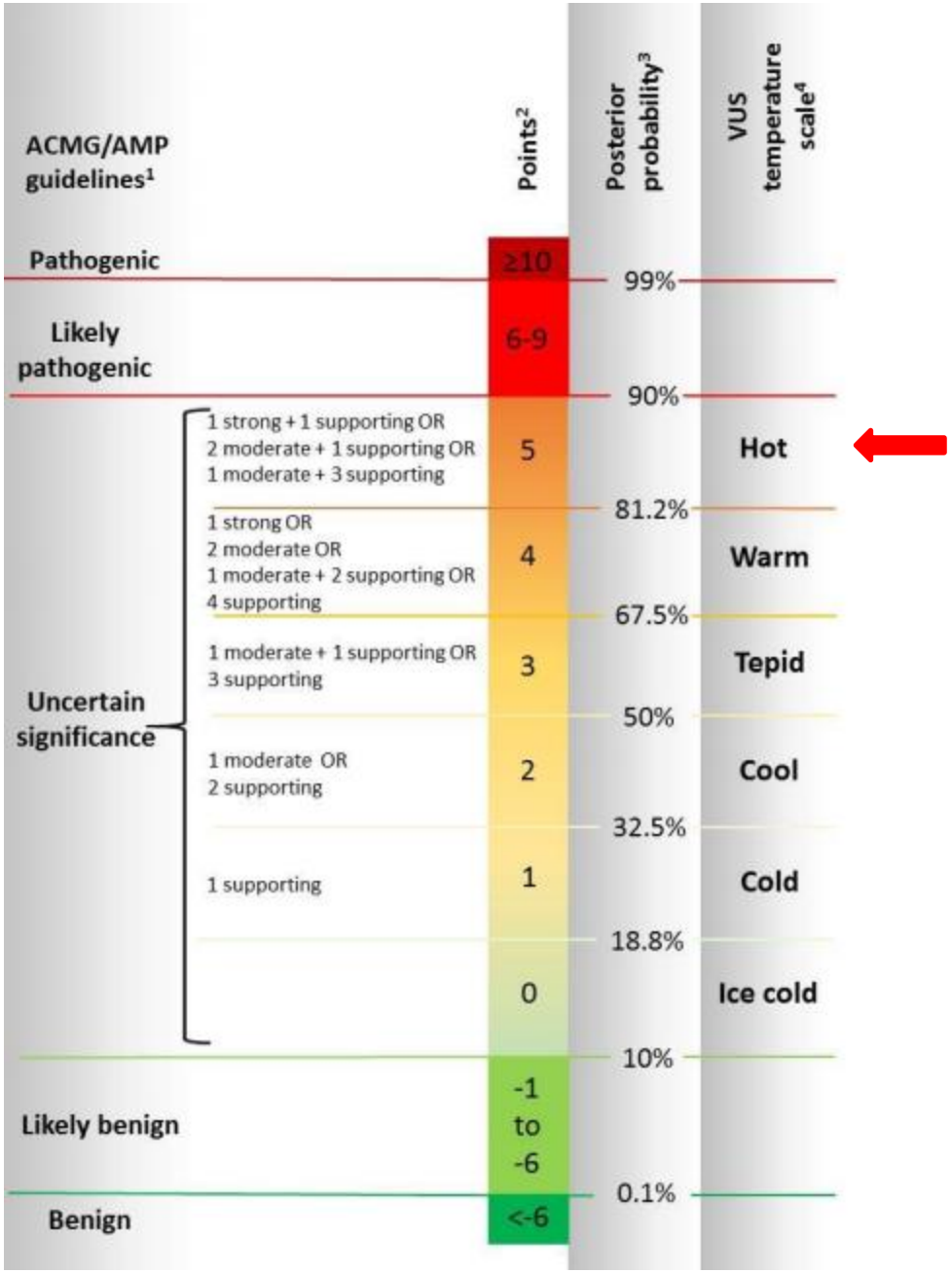


FIGURE 1 PVS1 decision tree. Refer to text for detailed description. (a) This criterion should not be applied in combination with in silico splicing predictions (PP3). Additionally, splice site variants must have no detectable nearby (+/- 20 nts) strong consensus splice sequence that may reconstitute in-frame splicing. (b) NMD prediction based on the premature termination codon not occurring in the 3′ most exon or the 3′-most 50 bp of the penultimate exon. (c) Relevant domain indicated by experimental evidence proving a critical role of the domain and/or presence of non-truncating pathogenic variants in the region. (d) For a full gene deletion of a known haploinsufficient gene, a pathogenic classification is warranted (in the absence of conflicting data) even though application of PVS1 alone would not reach a pathogenic classification using the combining rules in Richards et al. (2017). NMD, nonsense-mediated decay; LoF, loss of function

Because of frameshift and abnormal extended protein we went with role of protein function unknown (particularly in light of other similar variant on InSiGHT as VUS). Not a "hotspot" or clearly defined functional domain? (like BRCA BRCT or RING)

PVS1_mod: see above – score 2
PM2_mod: absent from gnomAD v2.1.1 (non-cancer cohort) – score 2
PP4_supp: IHC & BRAF – score 1
Total score = 5


ACMG/AMP guidelines[1]
Points[2]
Posterior probability[3]
VUS temperature scale[4]
Pathogenic
≥10
99%
Likely pathogenic
6-9
90%
1 strong + 1 supporting OR
2 moderate + 1 supporting OR
1 moderate + 3 supporting
5
Hot
81.2%
1 strong OR
2 moderate OR
1 moderate + 2 supporting OR
4 supporting
4
Warm
67.5%
1 moderate + 1 supporting OR
3 supporting
3
Tepid
Uncertain significance
50%
1 moderate OR
2 supporting
2
Cool
32.5%
1 supporting
1
Cold
18.8%
0
Ice cold
10%
Likely benign
-1 to -6
0.1%
Benign
<-6

REFERRAL REASON **MLH1 screening. Diagnosed with bowel cancer, IHC shows loss of MLH1 and PMS2. Diagnostic.**

REPORT **Heterozygous variant of uncertain clinical significance detected in MLH1: c.2163_2164dupTA p.(Lys722IlefsTer62)**

This patient has clinical symptoms suggestive of Lynch Syndrome. Immunohistochemical analysis of a colonic adenocarcinoma from Sophie showed loss of expression of MLH1 and PMS2 (Histopathology report HS19-06489) whilst BRAF testing was negative (HODS report HMG19-01984).

DNA from this patient has been screened for pathogenic variants and large scale deletions/duplications of the MLH1 gene by next generation sequencing and multiplex ligation-dependent probe amplification (MLPA).

Fluorescent sequencing has confirmed the presence of a heterozygous MLH1 variant identified by next generation sequencing, c.2163_2164dupTA. This frameshift variant is predicted to result in an abnormal MLH1 protein that is longer than the normal by 27 amino acids: the first 721 amino acids are preserved but 62 novel amino acids are subsequently introduced: p.(Lys722IlefsTer62). This variant has not been previously reported on InSiGHT, ClinVar or HGMD and based on current guidelines is classified as a variant of uncertain clinical significance, albeit one that may prove to be clinically relevent.

We do not recommend that DNA testing is offered to unaffected relatives until there is further evidence for the pathogenicity of this base change.

Therefore we are unable to confirm a diagnosis of Lynch syndrome in this patient.

No other clearly pathogenic variant nor large scale deletion/duplication in the MLH1 gene was detected.

Tumour testing in Manchester performed:

SOMATIC MISMATCH REPAIR GENE ANALYSIS ON PATHOLOGY TISSUE

NAME:
DATE OF BIRTH:
SEX:
NHS No:

POSTCODE:
YOUR REF:
OUR REF:
DATE: 20-Jul-2020

REASON FOR REFERRAL: This patient has been diagnosed with moderately differentiated adenocarcinoma. Previous testing showed loss of MLH1 and PMS2 on IHC analysis (information supplied with referral). Germline MLH1 testing identified the c.2163_2164dupTA p.(Lys722IleextTer26)* variant of unknown clinical significance (information supplied with referral). Screening of a panel of 13 colorectal cancer genes and loss-of-heterozygosity (LOH) analysis across the three mismatch repair genes (MLH1, MSH2, MSH6) has been requested on DNA extracted from this patient's pathology sample (Path Ref HS19-06489 A1). The neoplastic cell content of the tumour pathology sample was not stated.

RESULTS:

NAME (DoB)	PATH LAB REF NO.	RESULT
		see comments

COMMENTS: DNA extracted from this patient's tumour pathology sample (Path Ref HS19-06489 A1) has been screened for variants in a panel of 13 genes associated with inherited predisposition to colorectal cancers (84% of the target coding region was sequenced to a minimum depth of 350X with an average read depth of 3140x). (percentage of the coding region covered at 350X read depth; MLH1 100%, MSH2 96%, MSH6 92%, for coverage of other genes in the panel please contact the lab).

The MLH1 c.2163_2164dupTA p.(Lys722IleextTer26) and c.2140T>C p.(Trp714Arg) variants were identified, in 81% and 10% of sequence reads, respectively. These variants have been assessed as being variants of unknown clinical significance (ACGS Class 3) using published guidelines[1]. Loss-of heterozygosity (LOH) analysis of MLH1 was uninformative (a proportion of patients are homozygous for the MLH1 region SNVs in our assay).

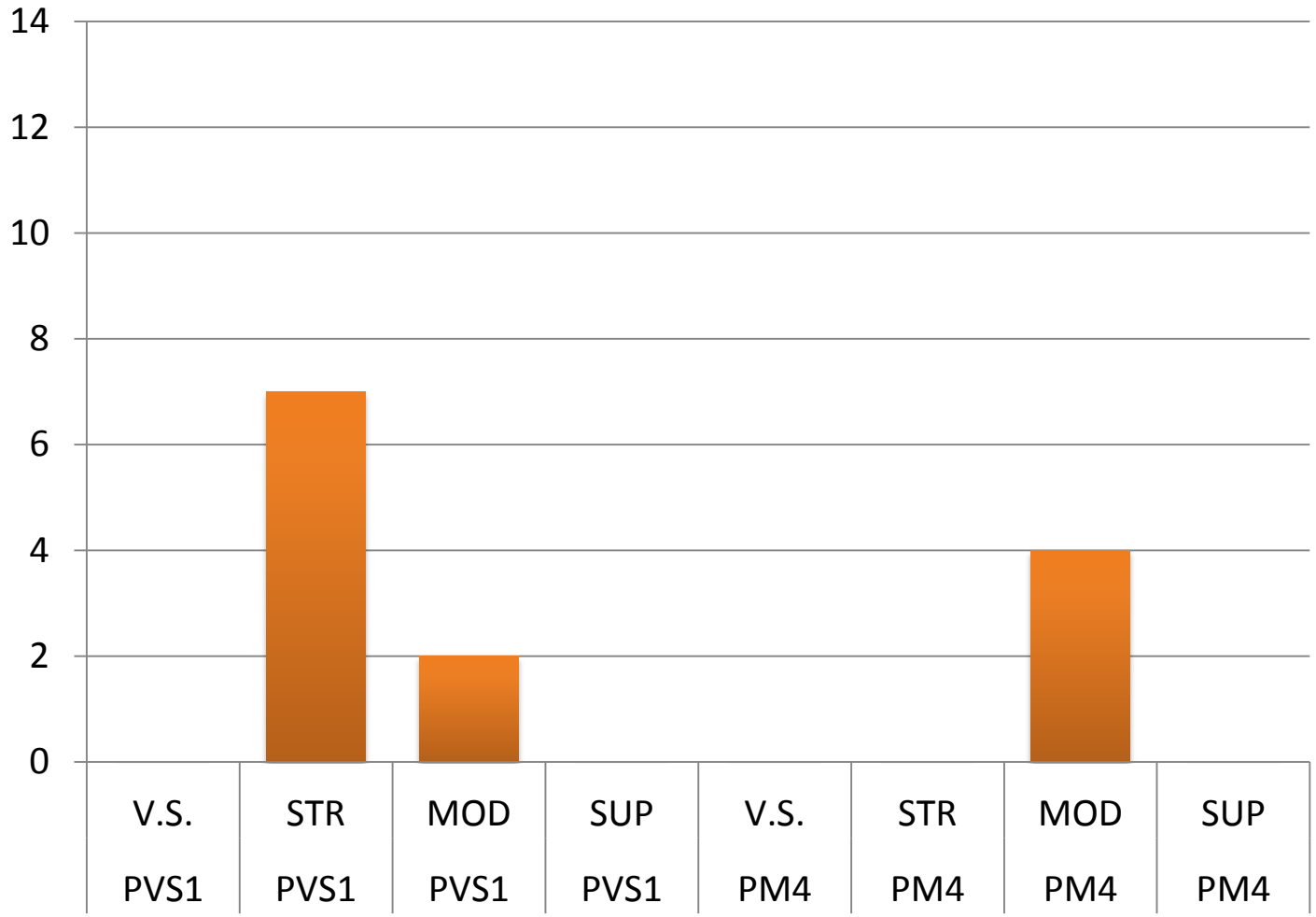
Please note the method of analysis used does not reliably detect large genomic re-arrangements (large deletions/duplications >40bp in size). Therefore we cannot exclude the possibility that there may be a germline or somatic genomic rearrangement of a mismatch repair gene in this patient's tissue sample on the basis of this analysis.

In conclusion, no evidence of a somatic pathogenic variant was detected, although we did identify two variants of unknown clinical significance in MLH1. The MLH1 c.2163_2164dupTA p.(Lys722IleextTer26) variant was previously identified in this patient's germline DNA sample. The MLH1 c.2140T>C p.(Trp714Arg) variant is presumed somatically acquired.

Also identified the same variant and classified it as Class 3 (VUS)
PM4 used instead of PVS1_mod (both moderate bits of evidence).

PM4	_MOD	_SUP
Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants		
Use at **Moderate** for • In-frame insertions/deletions for which PVS1 is not applicable (Tayoun et al 2018[2]) Use at **Supporting** for • In-frame insertions/deletions of a single amino-acid		

MLH1 c.2163_2164dupTA p.(Lys722Ilefs*62)



So 6 moderate and 7 strong – difference between class 3 and 4 in this case

Some discordance in use of guidelines (PVS1 vs PM4) - Fortunately for this presentation !

Result caused some discussion…

Question – did we use PVS1 correctly, should we have used PM4?

George B and I realised Cancer guidelines in isolation may be issue…

ACGS 2020 Rare disease guidelines…contain more detail on PVS1

Table 4: Supplementary information for classifying pathogenic (P) or benign (B) variants

Evidence criteria (level) supplementary notes
PVS1 – (Very Strong) null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where LOF is a known mechanism of disease
The evidence strength level can be modified depending upon the variant type, location within the gene or any additional evidence for the likelihood of a true null effect. A PVS1 decision tree has been developed by the ClinGen Sequence Variant Interpretation group to support the interpretation of loss of function variants (Tayoun *et al* 2018).
PVS1 can also be used for stop loss variants that abolish the canonical termination codon. In the absence of an in-frame termination codon in the 3' UTR the mRNA transcript is likely to undergo nonstop mediated decay and PVS1_Very strong can be used. If there is an in-frame termination codon within the 3'UTR then the predicted consequence is a protein with additional amino acids and PM4 (protein length change) can be used (see Figure 2).
Note that caution is required when interpreting 3' nonsense or frameshift variants predicted to escape nonsense mediated decay and consensus spice donor/acceptor site variants predicted to lead to in frame deletions or affecting alternative transcripts. For example the *BRCA2* nonsense variant, p.(Lys3326Ter) c.9976A>T, results in loss of the last 93 amino acids of the BRCA2 protein but does not confer a high risk of familial breast cancer

Copyright © ACGS 2020

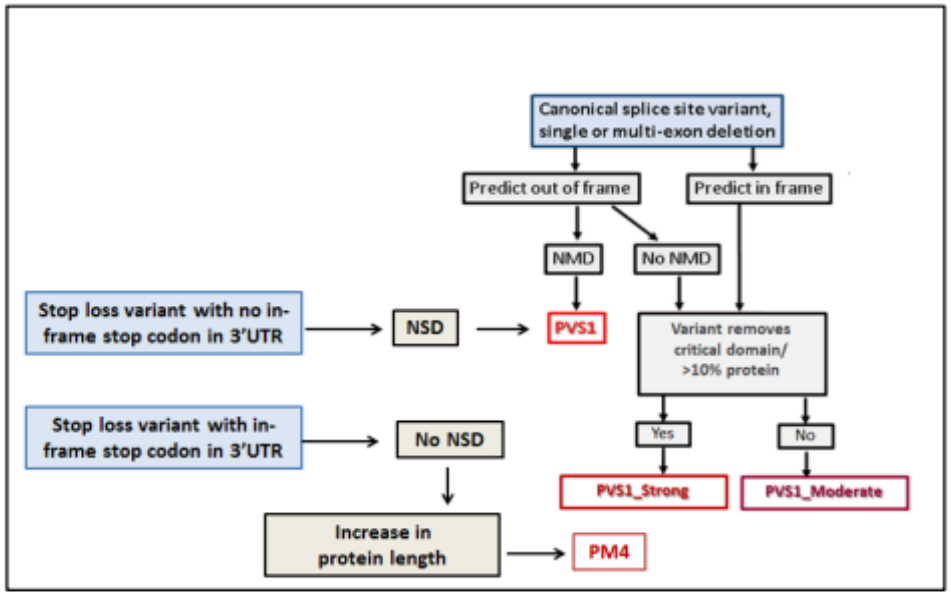
(Mazoyer *et al* 1996). Nor does the *BRCA1* c.594-2A>C slice acceptor site variant (de la Hoya *et al* 2016).

We note that use of the PVS1 decision tree (Tayoun *et al* 2018) "assumes that the gene/disease association is at a Moderate, Strong, or Definitive clinical validity level (Strande *et al* 2017)" in addition to LOF being a known mechanism of disease. Gene-disease validity curations at these levels are only available for ~500 genes (https://search.clinicalgenome.org/kb/gene-validity). It is not essential to perform a formal curation for every gene not yet on this list, but laboratories are expected to establish that there is sufficient evidence for the gene/disease association in addition to the LOF mechanism before applying the PVS1 criterion.

Should we use PVS1 (NSD) or PM4?

And a nice picture of this

Figure 2: Use of PVS1 and PM4 for stop loss variants (courtesy of Kevin Colclough, Royal Devon & Exeter NHS Foundation Trust and including part of the PVS1 decision tree re-drawn from https://www.biorxiv.org/content/early/2018/05/09/313718). NMD=nonsense mediated decay; NSD = nonstop mediated decay



Should we use PVS1 or PM4? NSD? PVS1 strength?

Wrote to Kevin Colclough for advice

Hi James,

So how you use PVS1 will depend upon where the frameshift starts and whether the transcript will be predicted to undergo non-stop decay, which in turn is dependent on whether you have a PTC in the 3'UTR which will trigger ribosome disassociation and escape from the decay process. If there is no predicted PTC in the UTR and the ribosome stalls at the PolyA site, then it won't disassociate and this will trigger non-stop decay.

If in your examples the PTC is occurring upstream of the polyA site then stop-loss decay isn't predicted. You'll therefore predict the transcript to be retained and generate a protein with an extended C terminus. If it was just a stop-loss variant (i.e. loss of the canonical stop codon) then the normal protein sequence is retained and just extended, so PM4 would be used. But you also have a frameshift which is changing the normal protein sequence encoded by the penultimate and/or last exon. So you have protein extension but also sequence alteration. So I would probably treat this is a frameshift not undergoing a decay mechanism and apply the Tayoun et al 2018 rules accordingly – i.e. if the sequence in the penultimate/last exon is critical for protein function use PVS1_Strong, or if there are other definite pathogenic frameshift or nonsense variants that would not undergo decay but change this sequence then use PVS1_Strong. Or if frameshift/nonsense variants in last exon are very rare/absent in gnomAD and the frameshifted protein sequence represents >10% of protein and then use PVS1_Strong. If less than 10% use at moderate. Although use your judgement as to the significance of protein change that would be caused as a result of the frameshifted sequence.

BW Kev

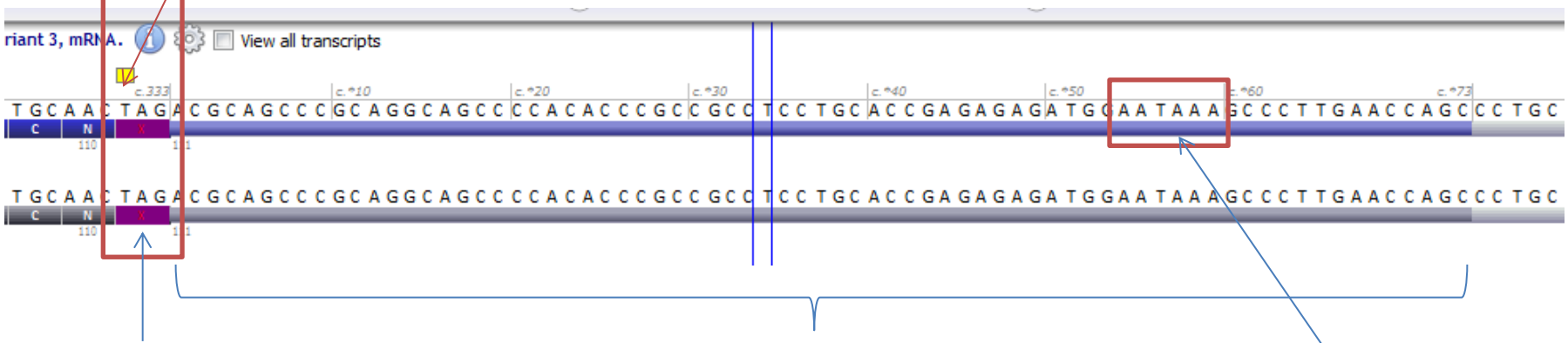
Kevin Colclough
Principal Clinical Scientist

Exeter Genomics Laboratory
RILD Building
Royal Devon & Exeter Hospital
Barrack Road
Exeter
Devon
EX2 5DW

We have already excluded NMD, what about NSD?

Novel stop-loss variant in *INS*

c.331T>G variant abolishes the TAG termination codon at the end of the ORF (p.*111Gluext*?)



Stop codon

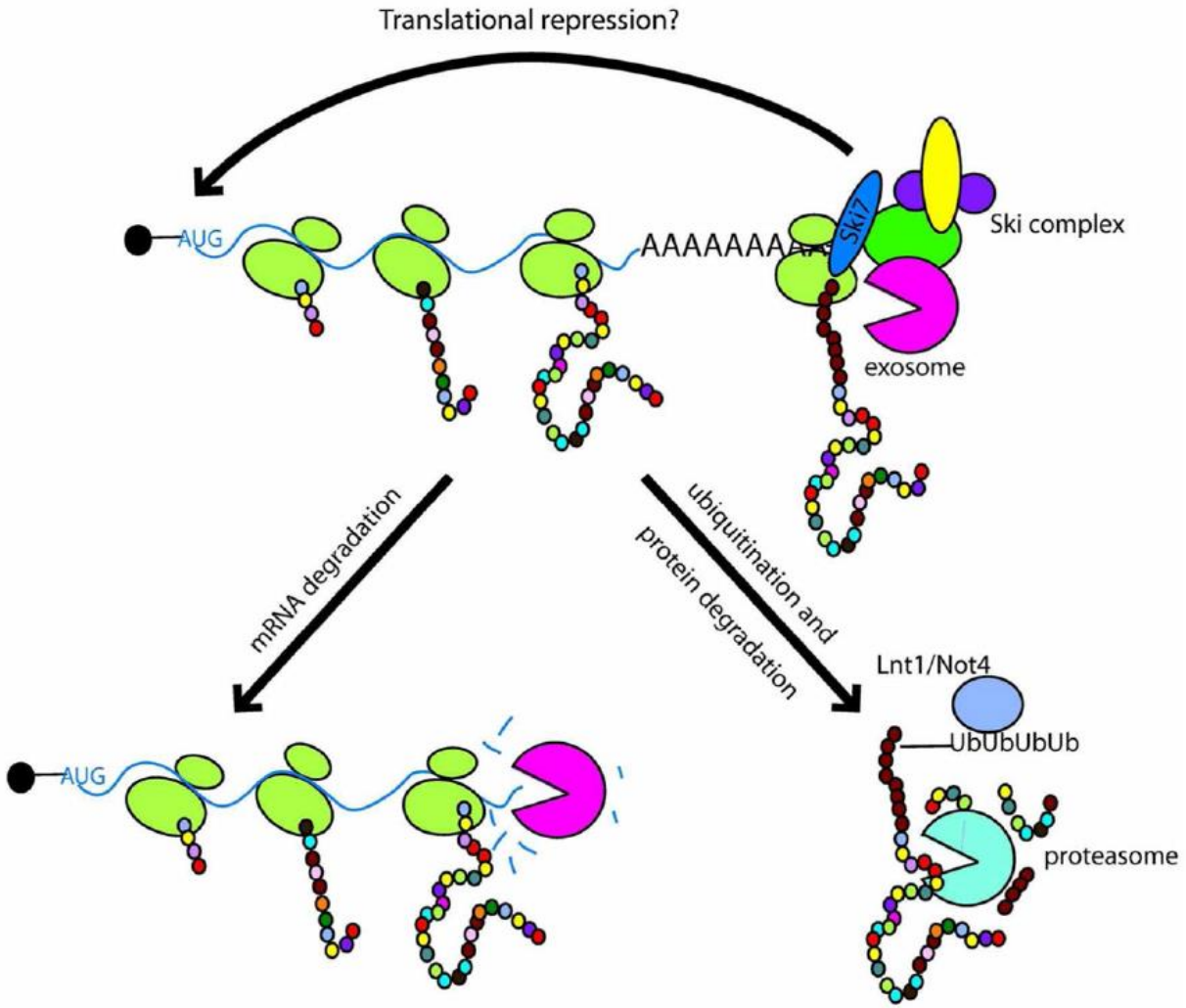
3’ UTR lacking an in-frame termination codon

polyadenylation signal sequence AATAAA

- Recognised by CPSF enzyme
- Cleaves RNA
- Allows polyadenylation to start

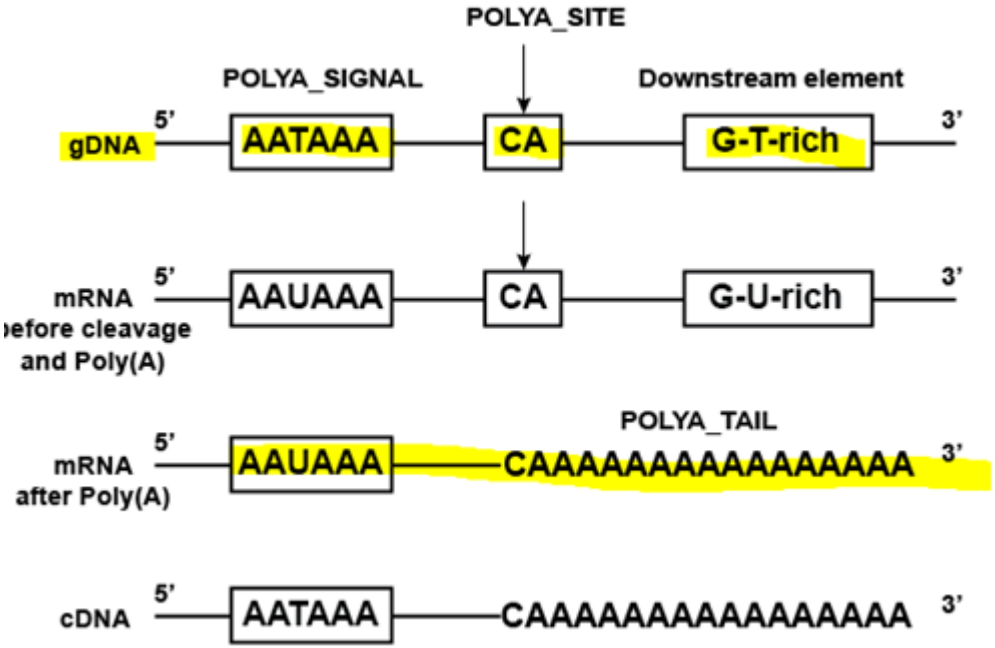
Slide kindly provided by Kevin Colclough

What happens when a ribosome doesn't encounter a stop codon?



Slide kindly provided by Kevin Colclough

Is NSD occurring in this case?



Shouldn't be NSD – novel termination codon in fs protein is upstream of PolyA region, so ribosome should be able to dissociate from mRNA

Hi James,

So how you use PVS1 will depend upon where the frameshift starts and whether the transcript will be predicted to undergo non-stop decay, which in turn is dependent on whether you have a PTC in the 3'UTR which will trigger ribosome disassociation and escape from the decay process. If there is no predicted PTC in the UTR and the ribosome stalls at the PolyA site, then it won't disassociate and this will trigger non-stop decay.

If in your examples the PTC is occurring upstream of the polyA site then stop-loss decay isn't predicted. You'll therefore predict the transcript to be retained and generate a protein with an extended C terminus. If it was just a stop-loss variant (i.e. loss of the canonical stop codon) then the normal protein sequence is retained and just extended, so PM4 would be used. But you also have a frameshift which is changing the normal protein sequence encoded by the penultimate and/or last exon. So you have protein extension but also sequence alteration. So I would probably treat this is a frameshift not undergoing a decay mechanism and apply the Tayoun et al 2018 rules accordingly – i.e. if the sequence in the penultimate/last exon is critical for protein function use PVS1_Strong, or if there are other definite pathogenic frameshift or nonsense variants that would not undergo decay but change this sequence then use PVS1_Strong. Or if frameshift/nonsense variants in last exon are very rare/absent in gnomAD and the frameshifted protein sequence represents >10% of protein and then use PVS1_Strong. If less than 10% use at moderate. Although use your judgement as to the significance of protein change that would be caused as a result of the frameshifted sequence.

BW Kev

Kevin Colclough
Principal Clinical Scientist

Exeter Genomics Laboratory
RILD Building
Royal Devon & Exeter Hospital
Barrack Road
Exeter
Devon
EX2 5DW

So not NSD, what about using PM4?

An example of PM4

c.2269T>A p.(*757Lysext*36)

In this case the Termination codon T>A with an in frame read through to another stop codon upstream of the upstream of PolyA region, so ribosome should be able to dissociate from mRNA. No NSD.

Simple protein extension so PM4

But our variant, c.2163_2164dupTA, p.(Lys722IlefsTer62) is a frameshift and Extension

Hi James,

So how you use PVS1 will depend upon where the frameshift starts and whether the transcript will be predicted to undergo non-stop decay, which in turn is dependent on whether you have a PTC in the 3'UTR which will trigger ribosome disassociation and escape from the decay process. If there is no predicted PTC in the UTR and the ribosome stalls at the PolyA site, then it won't disassociate and this will trigger non-stop decay.

If in your examples the PTC is occurring upstream of the polyA site then stop-loss decay isn't predicted. You'll therefore predict the transcript to be retained and generate a protein with an extended C terminus. If it was just a stop-loss variant (i.e. loss of the canonical stop codon) then the normal protein sequence is retained and just extended so PM4 would be used. But you also have a frameshift which is changing the normal protein sequence encoded by the penultimate and/or last exon. So you have protein extension but also sequence alteration. So I would probably treat this is a frameshift not undergoing a decay mechanism and apply the Tayoun et al 2018 rules accordingly – i.e. if the sequence in the penultimate/last exon is critical for protein function use PVS1_Strong, or if there are other definite pathogenic frameshift or nonsense variants that would not undergo decay but change this sequence then use PVS1_Strong. Or if frameshift/nonsense variants in last exon are very rare/absent in gnomAD and the frameshifted protein sequence represents >10% of protein and then use PVS1_Strong. If less than 10% use at moderate. Although use your judgement as to the significance of protein change that would be caused as a result of the frameshifted sequence.

BW Kev

Kevin Colclough
Principal Clinical Scientist

Exeter Genomics Laboratory
RILD Building
Royal Devon & Exeter Hospital
Barrack Road
Exeter
Devon
EX2 5DW

Clarified that PM4 is also not appropriate…so back to Tayoun for PVS1 but with Kevin's additional sage words of guidance

Hi James,

So how you use PVS1 will depend upon where the frameshift starts and whether the transcript will be predicted to undergo non-stop decay, which in turn is dependent on whether you have a PTC in the 3'UTR which will trigger ribosome disassociation and escape from the decay process. If there is no predicted PTC in the UTR and the ribosome stalls at the PolyA site, then it won't disassociate and this will trigger non-stop decay.

If in your examples the PTC is occurring upstream of the polyA site then stop-loss decay isn't predicted. You'll therefore predict the transcript to be retained and generate a protein with an extended C terminus. If it was just a stop-loss variant (i.e. loss of the canonical stop codon) then the normal protein sequence is retained and just extended, so PM4 would be used. But you also have a frameshift which is changing the normal protein sequence encoded by the penultimate and/or last exon. So you have protein extension but also sequence alteration. So I would probably treat this is a frameshift not undergoing a decay mechanism and apply the Tayoun et al 2018 rules accordingly – i.e. if the sequence in the penultimate/last exon is critical for protein function use PVS1_Strong, or if there are other definite pathogenic frameshift or nonsense variants that would not undergo decay but change this sequence then use PVS1_Strong. Or if frameshift/nonsense variants in last exon are very rare/absent in gnomAD and the frameshifted protein sequence represents >10% of protein and then use PVS1_Strong. If less than 10% use at moderate. Although use your judgement as to the significance of protein change that would be caused as a result of the frameshifted sequence.

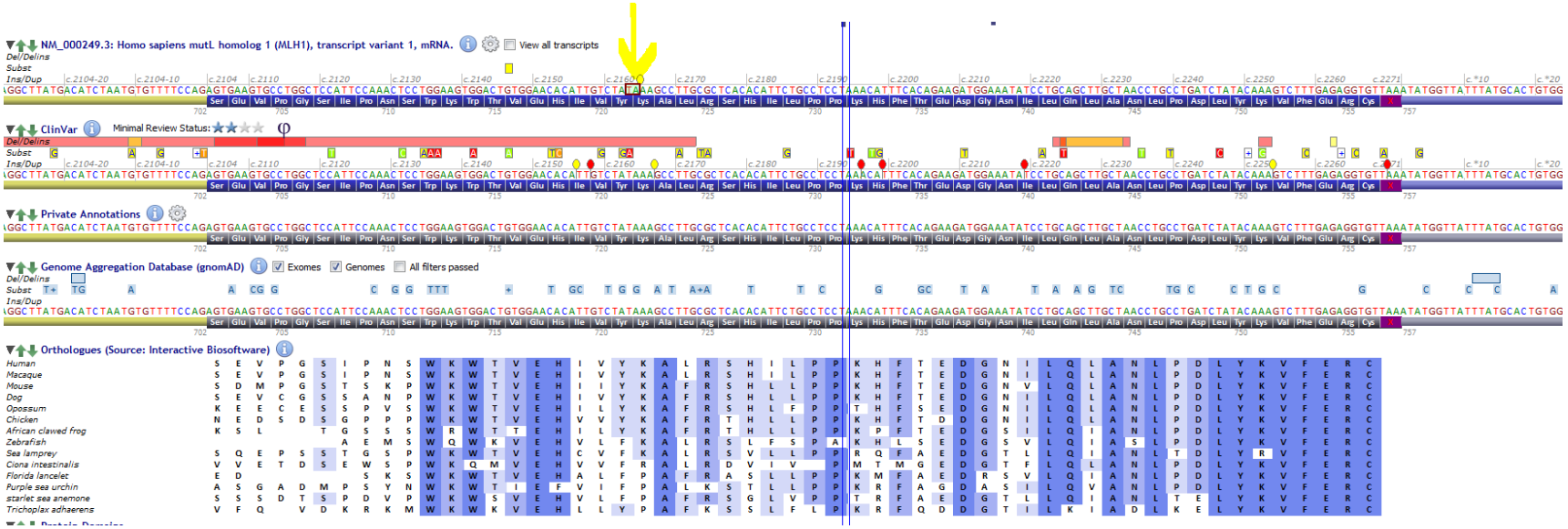
BW Kev

Kevin Colclough
Principal Clinical Scientist

Exeter Genomics Laboratory
RILD Building
Royal Devon & Exeter Hospital
Barrack Road
Exeter
Devon
EX2 5DW

We have already excluded NMD & NSD for our variant, so are there other variants that also evade NMD & NSD that are classified as pathogenic in the region?

MLH1 c.2163_2164dupTA, p.(Lys722IlefsTer62)



Region of protein is highly conserved

Multiple pathogenic ClinVar entries in region – confirmed on InSiGHT

NM_000249.3(MLH1):c.2135G>A (p.Trp712Ter) AND Lynch syndrome
RCV000075550.2

Clinical Significance:	Pathogenic
Last evaluated:	05/09/2013
Review status:	☆☆☆
Record status:	current

NM_000249.3(MLH1):c.2194A>T (p.Lys732Ter) AND Lynch syndrome
RCV000075573.3

Clinical Significance:	Pathogenic
Last evaluated:	05/09/2013
Review status:	☆☆☆
Record status:	current

NM_000249.3(MLH1):c.2246T>C (p.Leu749Pro) AND Lynch syndrome
RCV000075583.3

Clinical Significance:	Pathogenic
Last evaluated:	05/09/2013
Review status:	☆☆☆
Record status:	current

NM_000249.3(MLH1):c.2163T>A (p.Tyr721Ter) AND Lynch syndrome
RCV000075565.2

Clinical Significance:	Pathogenic
Last evaluated:	05/09/2013
Review status:	☆☆☆
Record status:	current

NM_000249.3(MLH1):c.2224C>T (p.Gln742Ter) AND Lynch syndrome
RCV000075580.3

Clinical Significance:	Pathogenic
Last evaluated:	05/09/2013
Review status:	☆☆☆
Record status:	current

NM_000249.3(MLH1):c.2263A>G (p.Arg755Gly) AND Lynch syndrome I
RCV000206724.1

Clinical Significance:	Pathogenic
Last evaluated:	24/11/2015
Review status:	☆☆☆
Record status:	current

Gene	Variant	Protein	Concensus InSiGHT Classification	Classification Date	Summary Justification
MLH1	c.2218dup View_InSiGHT_Database	p. (Ile740Asnfs*6)	Class 5: pathogenic	2013/09/05 v1.9	Frameshift interrupting C-terminus interaction domain

Gene	Variant	Protein	Concensus InSiGHT Classification	Classification Date	Summary Justification
MLH1	c.2195_2198dup View_InSiGHT_Database	p.His733Glnfs*14	Class 5: pathogenic	2013/09/05 v1.9	Frameshift interrupting C-terminus interaction domain. Multifactorial likelihood analysis posterior probability >0.99

These should also evade NMD & NSD

InSiGHT Variant Classifications

We encourage submission of relevant unpublished information to assist in the classification of variants via LOVD or this template
Contact: InSiGHT curator John-Paul Plazzer

Select gene: MLH1

Search variant: 2252
Example c.123A>G or 123

or Search protein:
Example Thr123Arg

Search

Join the community following this variant to receive updates on classification or add new information you have by clicking on 'Follow variant'

Follow Variant

NOTICE: Click here to view new and reclassified variants - UPDATED JANUARY 2019 !!!

InSiGHT Classification criteria are available here

InSiGHT Classifications can be batch downloaded from ClinVar

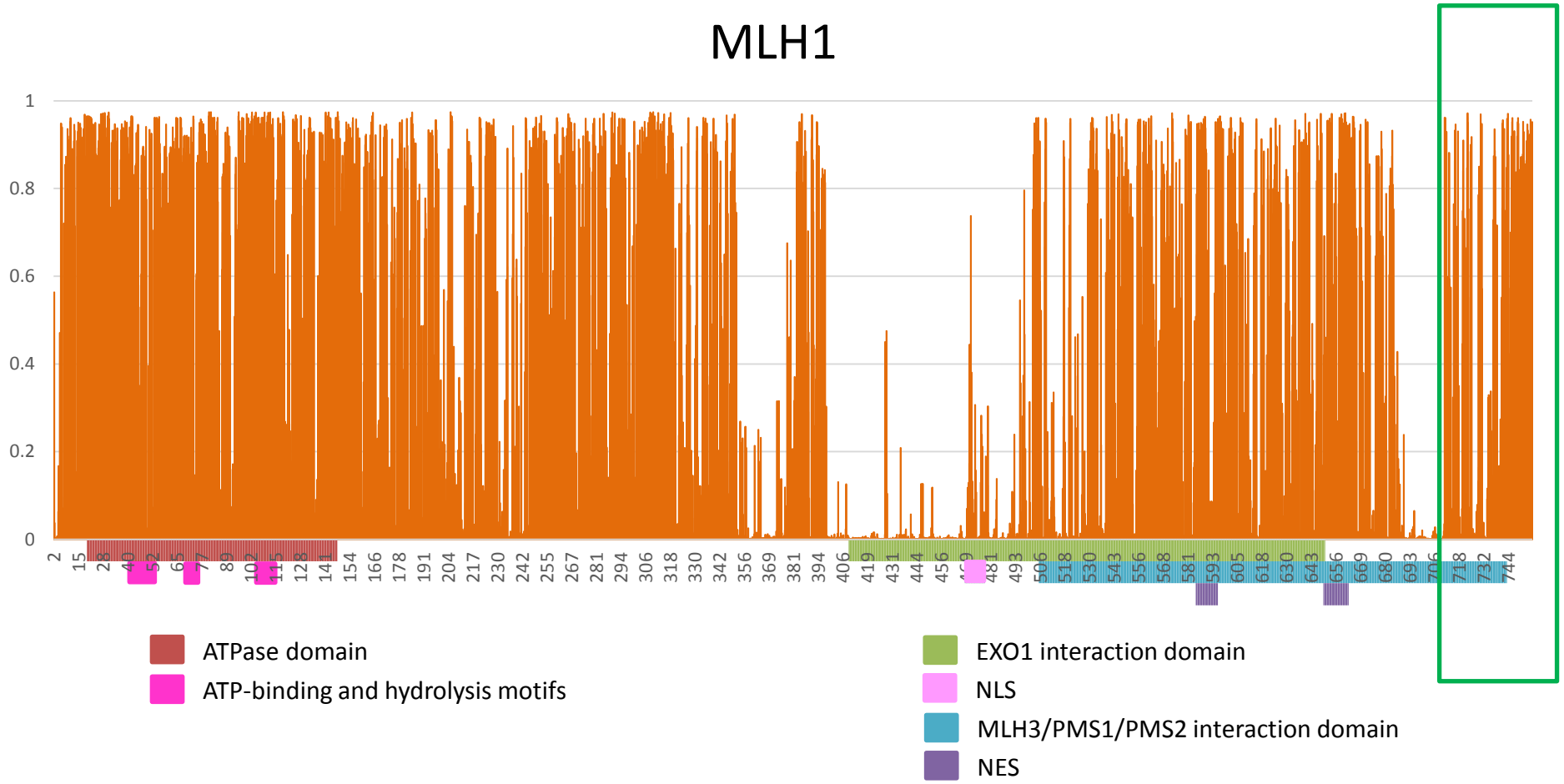
Gene	Variant	Protein	Concensus InSiGHT Classification	Classification Date	Summary Justification	Posterior Probability of Pathogenicity	Splicing/transcript expression	Co-occurr
MLH1	c.2252_2253del View InSiGHT Database	p.(Lys751Serfs*3)	Class 5: pathogenic	2014/10/10 v1.9	Multifactorial likelihood analysis posterior probability >0.99	>0.999 (used data from Borelli et al., 2014)		
MLH1	c.2252_2253dup View InSiGHT Database	p.(Val752Lysfs*32)	Class 3: uncertain	2019/06/21 v2.4	Insufficient evidence: extends protein by 26 amino acids	0.86 (used data from Sheng et al., 2008, ORCID:Leung et al, Wolf et al., 2005)		
MLH1	c.2266_2269dup View InSiGHT Database	p.*757Leuext*34	Class 5: pathogenic	2013/09/05 v1.9	Multifactorial likelihood analysis posterior probability >0.99	0.997 (used data from Lynch et al., 1990, Boyd et al., 1999, Papadopoulos et al., 1994)	Extended protein identified using IVSP Liu et al., 1996.	
MLH1	c.2269dup View InSiGHT Database	p.*757Leuext*33	Class 5: pathogenic	2013/09/05 v1.9	Multifactorial likelihood analysis posterior probability >0.99	1.000 (used data from Viel et al., 1998, Capozzi et al., 1999, Caluseriu et al., 2004, Ponti et al., 2006, Ponti et al., 2008, [Monica Pedroni])	No effect on splicing or RNA expression (RT-PCR & restriction enzyme digestion of LCL RNA Caluseriu et al., 2004)	
MLH1	c.2269T>A View InSiGHT Database	p.(*757Lysext*36)	Class 3: uncertain	2019/06/21 v2.4	Insufficient evidence: elongates protein by 36 amino acids			

Example we originally compared to our variant

PM4 example in slides above

Other fs variants in region are Class 5 in InSiGHT

MLH1

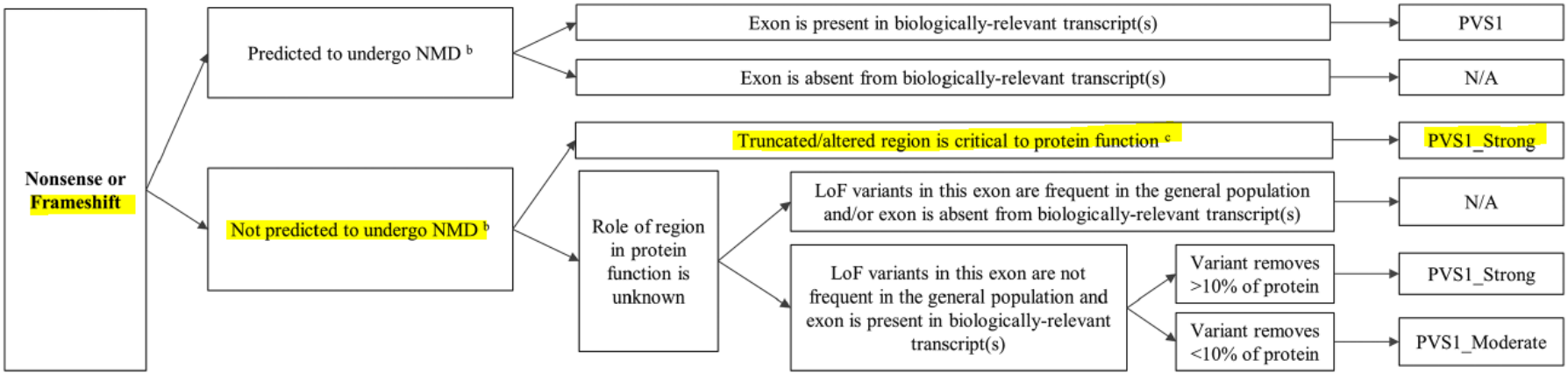


Slide courtesy of Ian Frayling

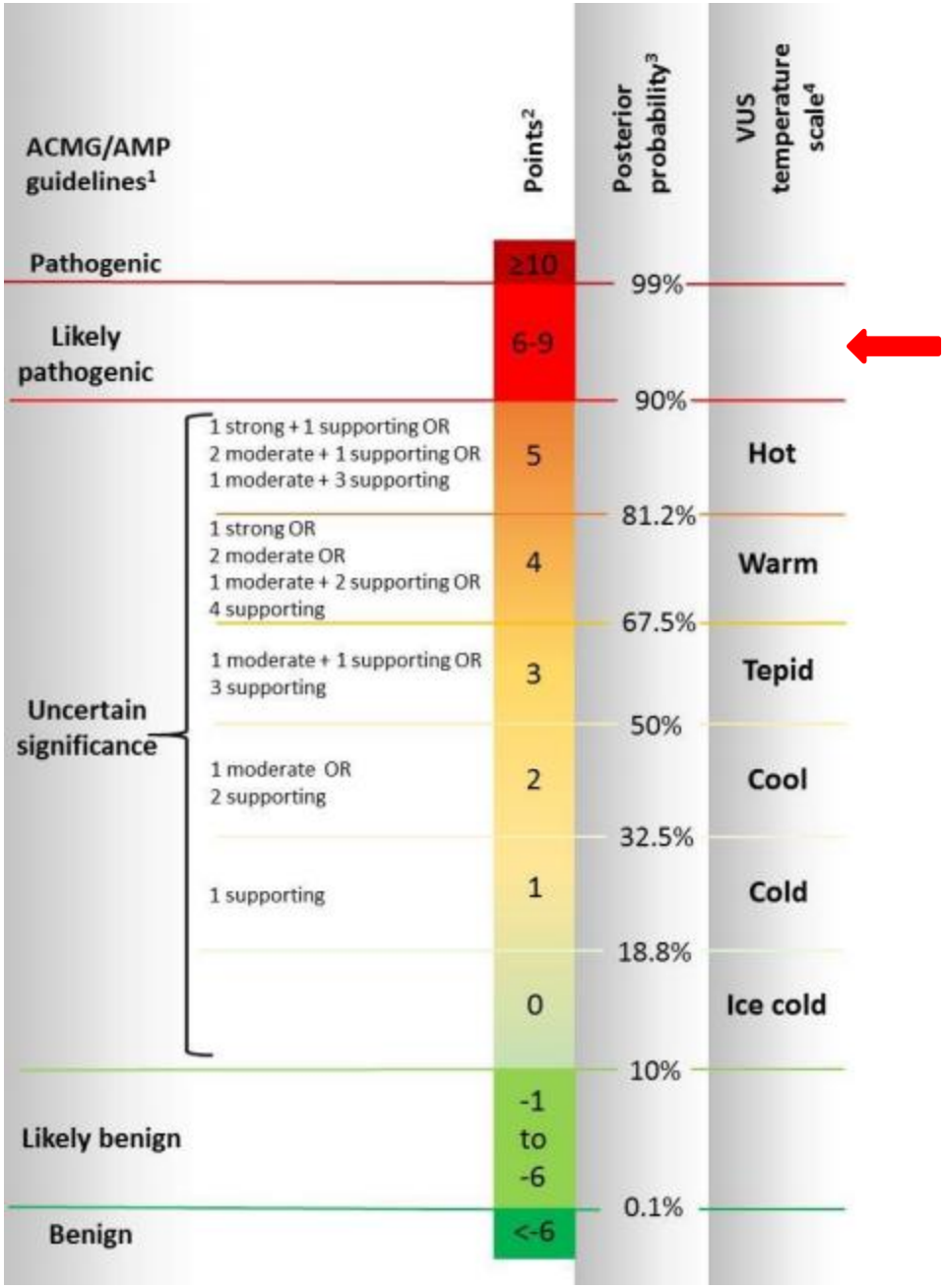
Courtesy: Thompson, Bryony A., et al. "Calibration of multiple in silico tools for predicting pathogenicity of mismatch repair gene missense substitutions." Human mutation 34.1 (2013): 255-265.
http://hci-lovd.hci.utah.edu/home.php?action=switch_db

InSiGHT

After further discussion with George B we agreed that PVS1_strong was appropriate based on Kevin's advice and available evidence



PVS1_strong: see above – score 4
PM2_mod: absent from gnomAD v2.1.1 (non-cancer cohort) – score 2
PP4_supp: IHC & BRAF – score 1
Total score = 7


ACMG/AMP guidelines[1]
Points[2]
Posterior probability[3]
VUS temperature scale[4]
Pathogenic
≥10
99%
Likely pathogenic
6-9
90%
1 strong + 1 supporting OR
2 moderate + 1 supporting OR
1 moderate + 3 supporting
5
Hot
81.2%
1 strong OR
2 moderate OR
1 moderate + 2 supporting OR
4 supporting
4
Warm
67.5%
1 moderate + 1 supporting OR
3 supporting
3
Tepid
Uncertain significance
50%
1 moderate OR
2 supporting
2
Cool
32.5%
1 supporting
1
Cold
18.8%
0
Ice cold
10%
Likely benign
-1 to -6
0.1%
Benign
<-6

REFERRAL REASON **MLH1 screening. Diagnosed with bowel cancer, IHC shows loss of MLH1 and PMS2. Diagnostic.**

REPORT

Consistent with a diagnosis of Lynch syndrome
Heterozygous likely pathogenic variant detected in MLH1:
c.2163_2164dupTA p.(Lys722IlefsTer62)

UPDATED REPORT: VARIANT RECLASSIFIED. This supersedes our previous report dated 24/01/20

This patient has clinical symptoms suggestive of Lynch Syndrome. Immunohistochemical analysis of a colonic adenocarcinoma from this patient showed loss of expression of MLH1 and PMS2 (Histopathology report HS19-06489) whilst BRAF testing was negative (HODS report HMG19-01984).

DNA from this patient has been screened for pathogenic variants and large scale deletions/duplications of the MLH1 gene by next generation sequencing and multiplex ligation-dependent probe amplification (MLPA).

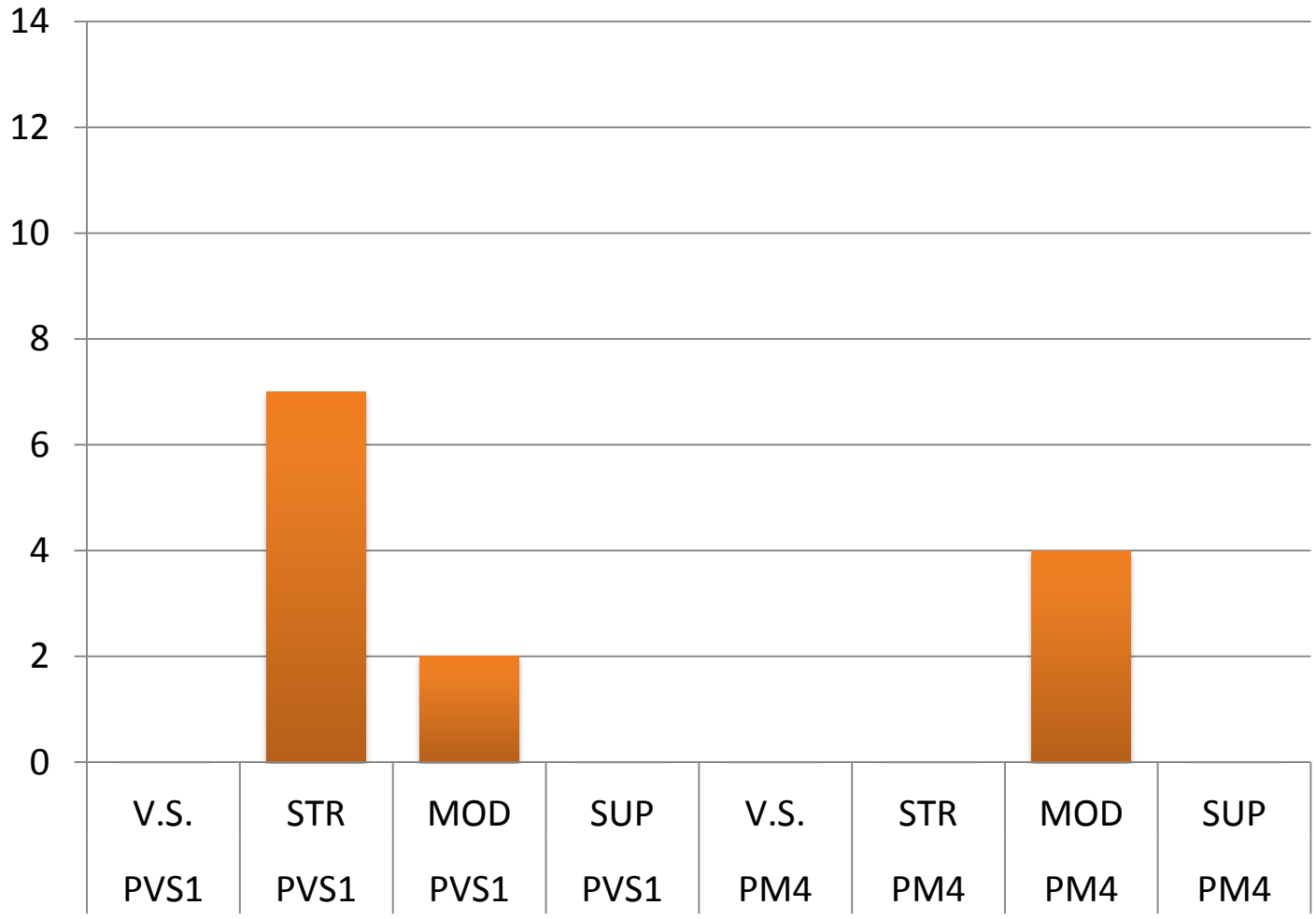
As noted in our previous report (dated 24/01/20) a variant was identified by this testing: MLH1 c.2163_2164dupTA p.(Lys722IlefsTer62). Based on the available information at the time this variant was considered to be of uncertain clinical significance.

This variant has been recently reassessed and is now classified as likely pathogenic.

This result is consistent with this patient's clinical status and she is at risk of developing additional MLH1 associated tumours.

This patient's children are at 50% risk of inheriting this likely pathogenic variant and developing a Lynch syndrome related cancer. Presymptomatic testing can now be offered to close relatives, if required. We recommend that genetic counselling is given prior to DNA testing.

MLH1 c.2163_2164dupTA p.(Lys722Ilefs*62)



So 6 moderate and 7 strong

MLH1 c.2163_2164dupTA p.(Lys722Ilefs*62)

Comments (consensus statement):

- **PVS1_strong** (7 submissions)

  Frameshift in last exon (19/19), not expected to undergo NMD…There are lots of pathogenic LOF variants after this location in ClinVar and on INSIGHT…Literature suggesting C-terminal is functionally important.

- **PVS1_moderate** (2 submissions)

  Frameshift variant in last exon, not in a critical region, loss of <10% of protein

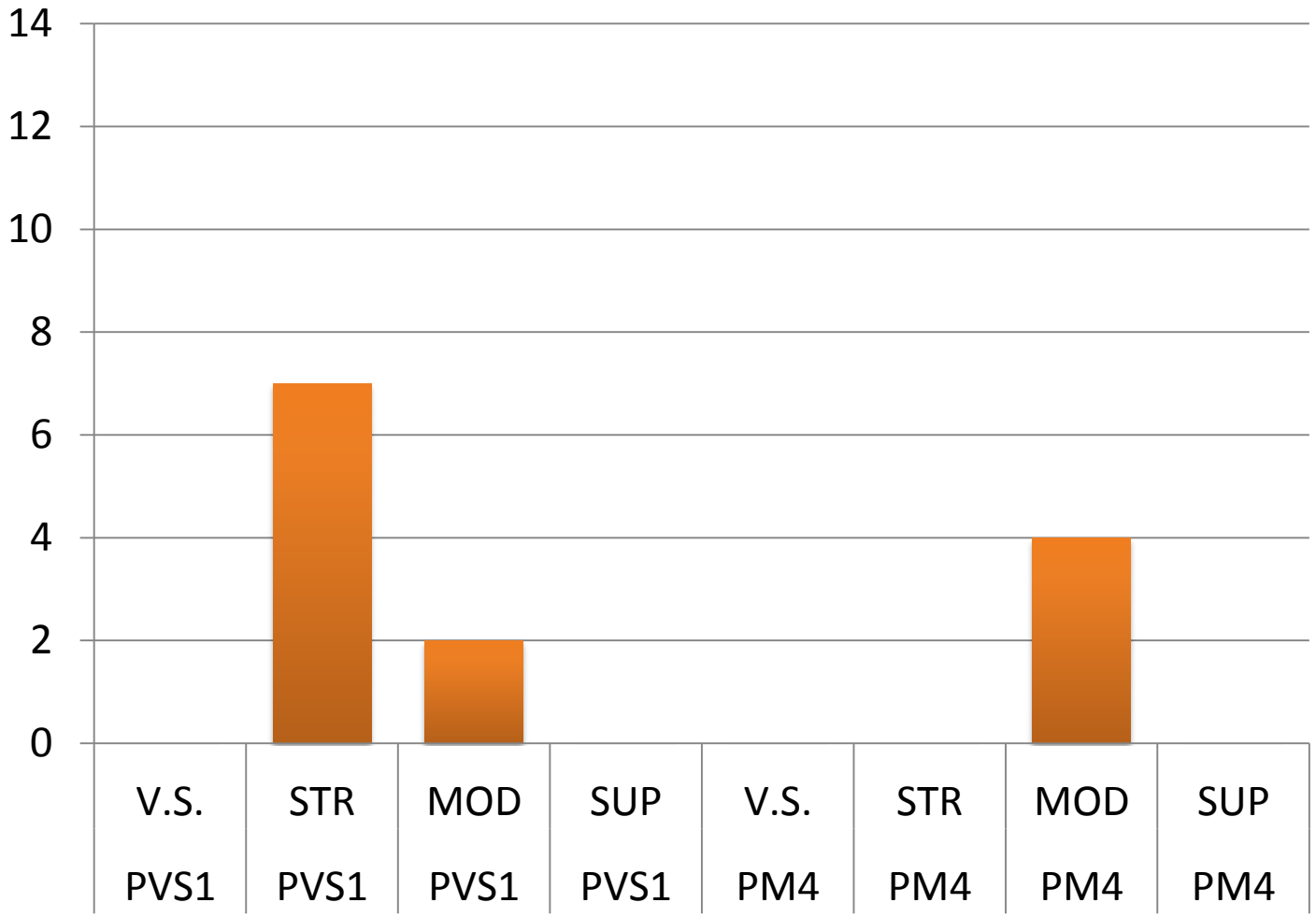
- **PM4_moderate** (4 submissions)

  New stop codon in 3'UTR (stop loss variant)… mRNA is predicted to escape Non-Stop mediated Decay (NSD) and be translated into elongated aberrant protein. Reference Ellard et al, 2020; Figure 2: Use of PVS1 and PM4 for stop loss variants.

Similar MLH1 variant (Manchester case)

Scoring	MLH1	c.2169_2175del	p.(Leu724HisfsTer57)	NM_000249.3
4	PVS1_Strong	Frameshift in last exon that is predicted to escape NMD		
		Causes extension of protein with abnormal amino acid tail	new coding from 724 to 781 - same terminal amino acids as other MLH1 var	
		Does not undergo NSD as termination codon in 3'UTR upstream of polyadenylation signal which will trigger ribosome dissociation		
		Plenty of other frameshift/nonsense pathogenic calls in region indicating region is critical to protein function		

MLH1 c.2169_2175del p.(Leu724Hisfs*57)



As expected – same split as before

MLH1 c.2169_2175del p.(Leu724Hisfs*57)

Comments (consensus statement):

- **PVS1_strong** (7 submissions)

  Located in last exon of MLH1, not predicted to undergo NMD. Altered region critical to protein function as evidenced by multiple pathogenic variants reported in this region

- **PVS1_moderate** (2 submissions)

  Frameshift variant in last exon, not in a critical region, loss of <10% of protein

- **PM4_moderate** (4 submissions)

  Frameshift variant in last exon (19/19) resulting in stop loss creating an inframe stop codon in the 3' UTR. The mRNA is predicted to escape Non-Stop mediated Decay (NSD) and be translated into elongated aberrant protein. Reference Ellard et al, 2020; Figure 2: Use of PVS1 and PM4 for stop loss variants.

TSC2: c.5171delA p.(Gln1724ArgfsTer102)

Very similar to previous two examples

Novel termination at codon 1826 (usual at 1808)

TGA stop codon for extended protein – upstream of Poly A

No NSD, no NMD as frameshift and extension so stop codon in 3'UTR

FIGURE 1 PVS1 decision tree. Refer to text for detailed description. (a) This criterion should not be applied in combination with in silico splicing predictions (PP3). Additionally, splice site variants must have no detectable nearby (+/- 20 nts) strong consensus splice sequence that may reconstitute in-frame splicing. (b) NMD prediction based on the premature termination codon not occurring in the 3′ most exon or the 3′-most 50 bp of the penultimate exon. (c) Relevant domain indicated by experimental evidence proving a critical role of the domain and/or presence of

TSC2: c.5171delA p.(Gln1724ArgfsTer102) – termination at codon 1826

Penultimate exon but extension of protein beyond normal termination site

Other TSC2 variants classified as pathogenic on LOVD also terminating at 1826:

c.5161del p.(Met1721TrpfsTer105)
c.5173del p.(Val1725CysfsTer101) – de novo
c.5192del p.(Asn1731ThrfsTer95)
c.5196del p.(Thr1733ProfsTer93) – de novo
c.5247del p.(Leu1750SerfsTer76)

https://databases.lovd.nl/shared/variants/TSC2/unique

GAP domain affected – deleting terminal x aa and replacing them with novel seq

Plenty of other pathogenic changes in region:

LOVD 3 Leiden Open Variation Database — THE HUMAN VARIOME PROJECT

Global Variome shared LOVD

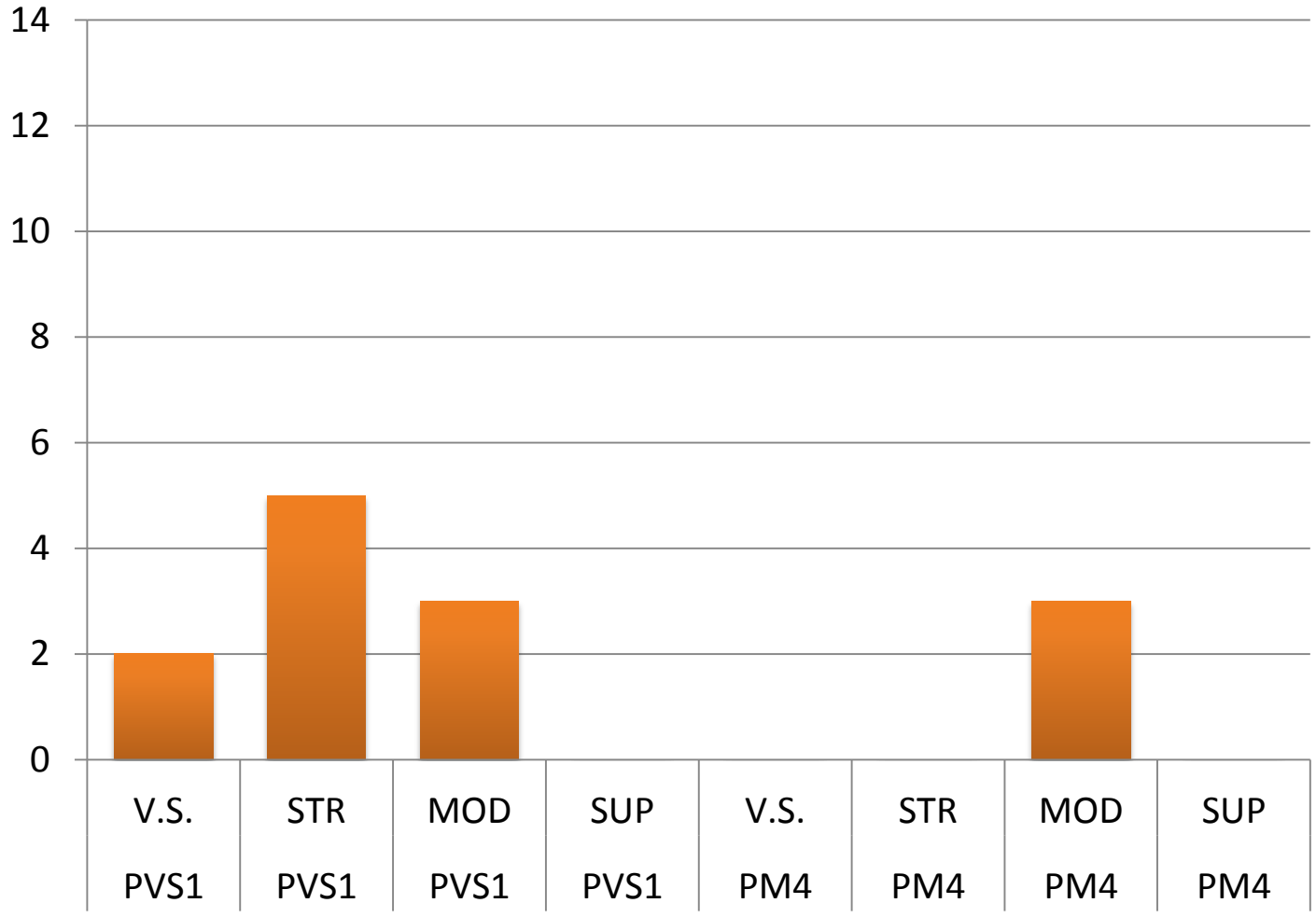
TSC2 (tuberous sclerosis 2)

+/+	39i	c.5069-18A>G	r.spl	p.?
-?/-?	39i	c.5069-11A>G	r.(?)	p.(=)
+/+	39i	c.5069-1G>C	r.spl	p?
+/+	40	c.5074dup	r.(?)	p.(Glu1692Glyfs*14)
?/?	40	c.5079_5088del	r.(?)	p.(Leu1694Profs*129)
-/-	40	c.5094C>A	r.(?)	p.(Ser1698Arg)
?/?	40	c.5101_5125del	r.(?)	p.(Lys1701Profs*117)
+/+	40	c.5116_5119dup	r.(?)	p.(Asn1707Thrfs*23)
+/+	40	c.5126C>T	r.(?)	p.(Pro1709Leu)
-/-	40	c.5131G>A	r.(?)	p.(Val1711Met)
?/?	40	c.5134_5135delinsTA	r.(?)	p.(Ala1712Tyr)
+/+	40	c.5138G>A	r.(?)	p.(Arg1713His)
+/+	40	c.5150_5154dup	r.(?)	p.(Ala1719Cysfs*109)
+?/+?	40	c.5155G>C	r.(?)	p.(Ala1719Pro)
+?/+?	40i	c.5160+5del	r.spl?	p.?
+/+	40i	c.5161-2A>C	r.spl	p.?
+/+	40i	c.5161-2A>G	r.spl	p.?
+/+	40i	c.5161-1G>T	r.spl	p.?
+/+	41	c.5168C>G	r.(?)	p.(Ser1723*)
+/+	41	c.5169dup	r.(?)	p.(Gln1724Thrfs*5)
+/+	41	c.5203_5204insG	r.(?)	p.(Ile1735Serfs*40)
+?/+?	41	c.5212del	r.(?)	p.(Ser1738Profs*?)
+/+	41	c.5219G>A	r.(?)	p.(Trp1740*)
+/+	41	c.5224dup	r.(?)	p.(Ala1742Glyfs*33)
+/+	41	c.5226_5243del	r.(?)	p.(His1746_Arg1751del)
+/+	41	c.5227C>T	r.(?)	p.(Arg1743Trp)
+/+	41	c.5228G>A	r.(?)	p.(Arg1743Gln)
+/+	41	c.5228G>C	r.(?)	p.(Arg1743Pro)
+/+	41	c.5228G>T	r.(?)	p.(Arg1743Leu)
?/?	41	c.5233_5238del	r.(?)	p.(Arg1745_His1746del)
+/+	41	c.5238_5255del	r.(?)	p.(His1746_Arg1751del)
?/?	41	c.5238_5255dup	r.(?)	p.(His1746_Arg1751dup)
+/+	41	c.5239_5249del	r.(?)	p.(Ile1747Profs*24)
+/+	41	c.5249T>C	r.(?)	p.(Leu1750Pro)
+/+	41_41i	c.5252_5259+19del	r.spl	p.?
?/?	41i	c.5259+8A>G	r.(=)	p.(=)
-/-	41i	c.5259+9T>C	r.(=)	p.(=)
?/?	41i	c.5260-34_5260-10del	r.(?)	p.?
+?/+?	42	c.5260_5262delinsTAA	r.5260_5262delinsTAA	p.Ile1754*
-/-	42	c.5266G>A	r.(?)	p.(Glu1756Lys)
-/-	42	c.5275G>A	r.(?)	p.(Ala1759Thr)
+?/+?	42	c.5278_5280delinsAG	r.(?)	p.(Tyr1760Serfs*66)

TSC2: c.5171delA p.(Gln1724ArgfsTer102)

scoring	TSC2	c.5171delA	p.(Gln1724ArgfsTer102)	NM_000548.3
4	PVS1_Strong	Frameshift in penultimate exon that is predicted to escape NMD		
		Causes extension of protein with abnormal amino acid tail		
		Does not undergo NSD as termination codon in 3'UTR upstream of polyadenylation signal which will trigger ribosome dissociation	Termination at codon 1826 (native is 1808)	
		Plenty of other frameshift/nonsense pathogenic calls in region indicating region is critical to protein function	GAP domain - there are multiple other different frameshift variants reported as pathogenic on LOVD TS database that also terminate at codon 1826	

TSC2 c.5171delA p.(Gln1724Argfs*102)



Even wider spread of returns on this one (Mod-v strong)!

TSC2 c.5171delA p.(Gln1724Argfs*102)

Comments (consensus statement):

- **PVS1_very strong** (2 submissions)

  Conflicting statements, possible error in submission- "Located in the penultimate exon of TSC2 but more than 50 nucleotides from the end of the exon therefore expected to trigger NMD" and "Frameshift variant likely escapes NMD… multiple variants in this region indicating critical role of region"

- **PVS1_strong** (5 submissions)

  Frameshift, not predicted to undergo NMD, altered region critical- pathogenic missense 3' of this change described and includes part of GTPase activating protein domain.

- **PVS1_moderate** (3 submissions)

  Frameshift variant in the second to last predicted to introduce a stop codon 102 codons downstream. The mRNA is predicted to escape Nonsense Mediated Decay (NMD) with less than 10% of protein lost.

- **PM4_moderate** (3 submissions)

  Variant occurs in exon 41 of 42, normal protein has 1808 codons so termination 102 codons after 1724 suggests protein length is actually extended by this change (stop loss variant) so use PM4.

Slides courtesy of Clare Maurer

MSH6 c.3978_3994del p.(Met1326IlefsTer9) – exon 9 of 10

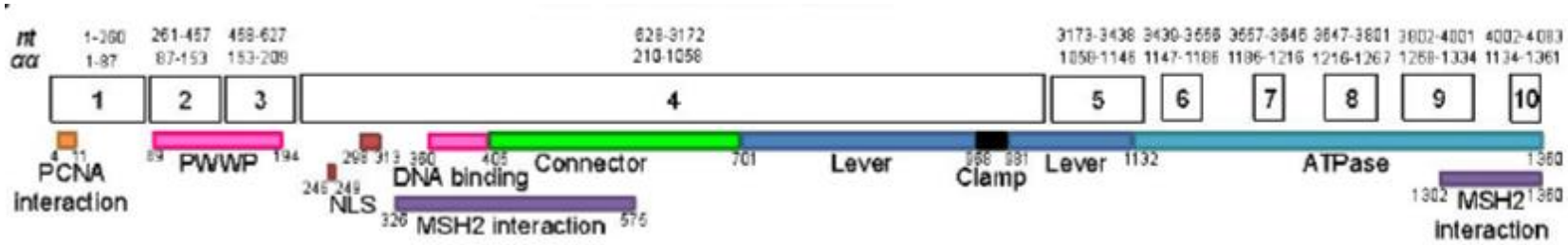
Reference protein

```
   1 MSRQSTLYSF FPKSPALSDA NKASARASRE GGRAAAAPGA SPSPGGDAAW SEAGPGPRPL
  61 ARSASPPKAK NLNGGLRRSV APAAPTSCDF SPGDLWAKM EGYPWPCLV YNHPFDGTFI
 121 REKGKSVRVH VQFFDDSPTR GWVSKRLLKP YTGSKSKEAQ KGGHFYSAKP EILRAMQRAD
 181 EALNKDKIKR LELAVCDEPS EPEEEEEMEV GTTYVTDKSE EDNEIESEEE VQPKTQGSRR
 241 SSRQIKKRRV ISDSESDIGG SDVEFKPDTK EEGSSDEISS GVGDSESEGL NSPVKVARKR
 301 KRMVTGNGSL KRKSSRKETP SATKQATSIS SETKNTLRAF SAPQNSESQA HVSGGGDDSS
 361 RPTVWYHETL EWLKEEKRRD EHRRRPDHPD FDASTLYVPE DFLNSCTPGM RKWMQIKSQN
 421 FDLVICYKVG KFYELYHMDA LIGVSELGLV FMKGNWAHSG FPEIAFGRYS DSLVQKGYKV
 481 ARVEQTETPE MMEARCRKMA HISKYDRVVR REICRIITKG TQTYSVLEGD PSENYSKYLL
 541 SLKEKEEDSS GHTRAYGVCF VDTSLGKFFI GQFSDDRHCS RFRTLVAHYP PVQVLFEKGN
 601 LSKETKTILK SSLSCSLQEG LIPGSQFWDA SKTLRTLLEE EYFREKLSDG IGVMLPQVLK
 661 GMTSESDSIG LTPGEKSELA LSALGGCVFY LKKCLIDQEL LSMANFEEYI PLDSDTVSTT
 721 RSGAIFTKAY QRMVLDAVTL NNLEIFLNGT NGSTEGTLLE RVDTCHTPFG KRLLKQWLCA
 781 PLCNHYAIND RLDAIEDLMV VPDKISEVVE LLKKLPDLER LLSKIHNVGS PLKSQNHPDS
 841 RAIMYEETTY SKKKIIDFLS ALEGFKVMCK IIGIMEEVAD GFKSKILKQV ISLQTKNPEG
 901 RFPDLTVELN RWDTAFDHEK ARKTGLITPK AGFDSDYDQA LADIRENEQS LLEYLEKQRN
 961 RIGCRTIVYW GIGRNRYQLE IPENFTTRNL PEEYELKSTK KGCKRYWTKT IEKKLANLIN
1021 AEERRDVSLK DCMRRLFYNF DKNYKDWQSA VECIAVLDVL LCLANYSRGG DGPMCRPVIL
1081 LPEDTPPFLE LKGSRHPCIT KTFFGDDFIP NDILIGCEEE EQENGKAYCV LVTGPNMGGK
1141 STLMRQAGLL AVMAQMGCYV PAEVCRLTPI DRVFTRLGAS DRIMSGESTF FVELSETASI
1201 LMHATAHSLV LVDELGRGTA TFDGTAIANA VVKELAETIK CRTLFSTHYH SLVEDYSQNV
1261 AVRLGHMACM VENECEDPSQ ETITFLYKFI KGACPKSYGF NAARLANLPE EVIQKGHRKA
1321 REFEKMWQSL RLFREVCLAS ERSTVDAEAV HKLLTLIKEL *
```

Arg is last aa of exon

Protein predicted from variant coding sequence

```
   1 MSRQSTLYSF FPKSPALSDA NKASARASRE GGRAAAAPGA SPSPGGDAAW SEAGPGPRPL
  61 ARSASPPKAK NLNGGLRRSV APAAPTSCDF SPGDLWAKM EGYPWPCLV YNHPFDGTFI
 121 REKGKSVRVH VQFFDDSPTR GWVSKRLLKP YTGSKSKEAQ KGGHFYSAKP EILRAMQRAD
 181 EALNKDKIKR LELAVCDEPS EPEEEEEMEV GTTYVTDKSE EDNEIESEEE VQPKTQGSRR
 241 SSRQIKKRRV ISDSESDIGG SDVEFKPDTK EEGSSDEISS GVGDSESEGL NSPVKVARKR
 301 KRMVTGNGSL KRKSSRKETP SATKQATSIS SETKNTLRAF SAPQNSESQA HVSGGGDDSS
 361 RPTVWYHETL EWLKEEKRRD EHRRRPDHPD FDASTLYVPE DFLNSCTPGM RKWMQIKSQN
 421 FDLVICYKVG KFYELYHMDA LIGVSELGLV FMKGNWAHSG FPEIAFGRYS DSLVQKGYKV
 481 ARVEQTETPE MMEARCRKMA HISKYDRVVR REICRIITKG TQTYSVLEGD PSENYSKYLL
 541 SLKEKEEDSS GHTRAYGVCF VDTSLGKFFI GQFSDDRHCS RFRTLVAHYP PVQVLFEKGN
 601 LSKETKTILK SSLSCSLQEG LIPGSQFWDA SKTLRTLLEE EYFREKLSDG IGVMLPQVLK
 661 GMTSESDSIG LTPGEKSELA LSALGGCVFY LKKCLIDQEL LSMANFEEYI PLDSDTVSTT
 721 RSGAIFTKAY QRMVLDAVTL NNLEIFLNGT NGSTEGTLLE RVDTCHTPFG KRLLKQWLCA
 781 PLCNHYAIND RLDAIEDLMV VPDKISEVVE LLKKLPDLER LLSKIHNVGS PLKSQNHPDS
 841 RAIMYEETTY SKKKIIDFLS ALEGFKVMCK IIGIMEEVAD GFKSKILKQV ISLQTKNPEG
 901 RFPDLTVELN RWDTAFDHEK ARKTGLITPK AGFDSDYDQA LADIRENEQS LLEYLEKQRN
 961 RIGCRTIVYW GIGRNRYQLE IPENFTTRNL PEEYELKSTK KGCKRYWTKT IEKKLANLIN
1021 AEERRDVSLK DCMRRLFYNF DKNYKDWQSA VECIAVLDVL LCLANYSRGG DGPMCRPVIL
1081 LPEDTPPFLE LKGSRHPCIT KTFFGDDFIP NDILIGCEEE EQENGKAYCV LVTGPNMGGK
1141 STLMRQAGLL AVMAQMGCYV PAEVCRLTPI DRVFTRLGAS DRIMSGESTF FVELSETASI
1201 LMHATAHSLV LVDELGRGTA TFDGTAIANA VVKELAETIK CRTLFSTHYH SLVEDYSQNV
1261 AVRLGHMACM VENECEDPSQ ETITFLYKFI KGACPKSYGF NAARLANLPE EVIQKGHRKA
1321 REFEKIISGS LPG*
```



Frameshift and loss of last exon (no ext)
NMD not predicted

Location & conservation

Absent from InSiGHT

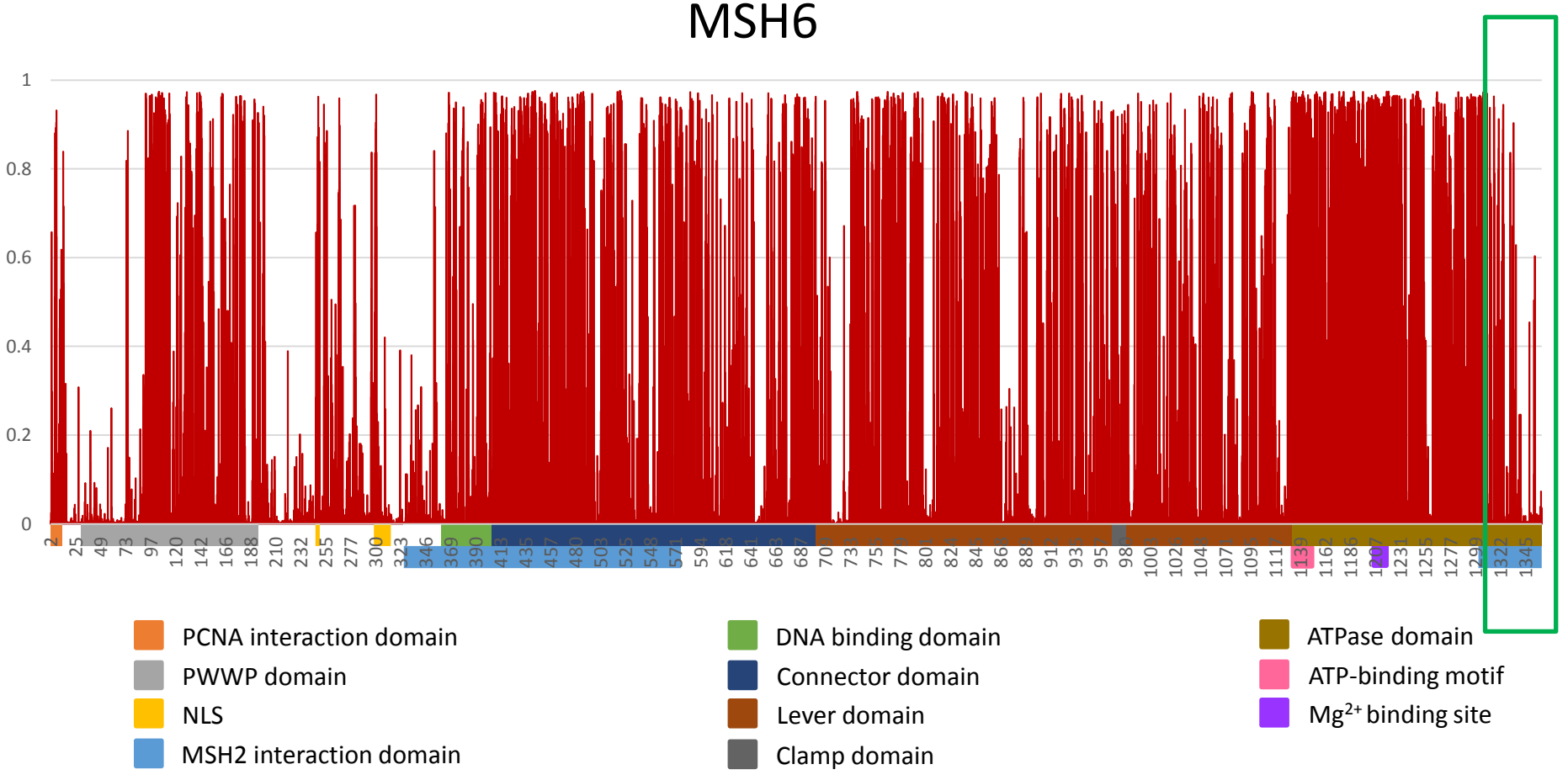
MSH6 c.3978_3994del p.(Met1326IlefsTer9)

Variants downstream:

c.3959_3962del	Class 3 on Insight	Path on ClinVar
c.3974_3976del	Class 3 on Insight	Single aa del
c.3969_3979del	Class 3 on Insight	Path on ClinVar
c.3991C>T p.(Ala1268GlyfsTer6)	Class 5 on Insight – different mechanism, shown to impact splicing (skipping of exon9)	
c.4028C>G p.(Ser1343Ter)	VUS on ClinVar	
c.3984_3985insATCA p.(Ser1329IlefsTer13)	Class 3 on Insight	Path on ClinVar
c.3984_3987dup p.(Leu1330ValfsTer12)	Class 3 on Insight	

NB Issue of ClinVar InSiGHT entries – need to check InSiGHT directly:
https://www.insight-database.org/classifications/

MSH6



- PCNA interaction domain
- PWWP domain
- NLS
- MSH2 interaction domain
- DNA binding domain
- Connector domain
- Lever domain
- Clamp domain
- ATPase domain
- ATP-binding motif
- Mg^{2+} binding site

Slide courtesy of Ian Frayling

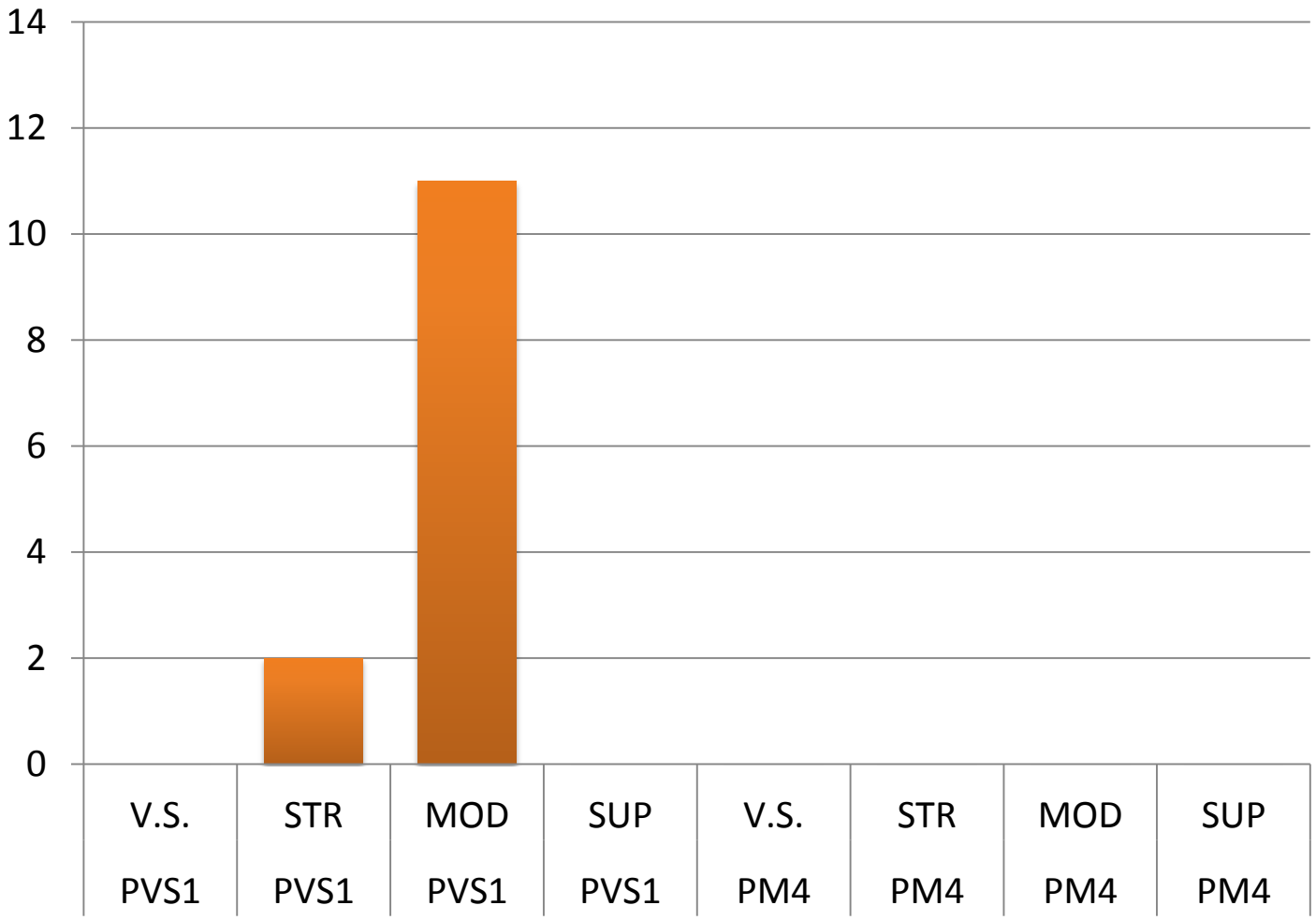
Courtesy: Thompson, Bryony A., et al. "Calibration of multiple in silico tools for predicting pathogenicity of mismatch repair gene missense substitutions." Human mutation 34.1 (2013): 255-265.
http://hci-lovd.hci.utah.edu/home.php?action=switch_db

InSiGHT

MSH6 variant – currently a live case needs input – PVS1_mod in this case?

Scoring	MSH6	c.3978_3994del	p.(Met1326IlefsTer9)	NM_000179.2		
2	PVS1_mod	exon 9 of 10 predicted to escape NMD				
		Premature termination codon within exon (no ext)				
		Region affected is not well conserved and lots of frameshift variants in InSIGHT in region are Class 3	NB some of these are Class 5 on ClinVar based on old entries but link out to InSiGHT which has Class 3			
		Variant removes less than 10% of protein				
2	PM2_mod	Absent from gnomAD v2.1.1 (non-cancer)	Not on ClinVar or HGMD			
1	PP4_sup	Loss of MSH6 on IHC	in index			
5	Class 3 (hot)					
Case info	Unaffected female born in 2000	Predictive test for var identified in father & grandmother		Index is female born in 1937. Ca jejunum aged 62. IHC done in 2000 showed normal IHC but due to FH MLH1 & MSH2 screening performed - normal result reported 2007. TAH & BSO in 40's. Colonoscopy - no polyps.	Repeat IHC at end 2016 showed loss of MSH6 so MSH6 gene screen performed and reported in early 2017. Classified as likely pathogenic.	Son of index born in 1963, has one sessile TVA with low grade dysplasia & I sessile serrated hyperplastic polyp. Tested positive for familial var in 2017 - classified as pathogenic.

MSH6 c.3978_3994del p.(Met1326Ilefs*9)



Much higher consensus on this case!

MSH6 c.3978_3994del p.(Met1326Ilefs*9)

Comments (consensus statement):

- **PVS1_strong** (2 submissions)

  Frameshift variant in the second to last exon so is likely to escape NMD. The last exon is poorly conserved although INSIGHT indicates 3' end of last exon is an MSH2 binding site...other pathogenic variants observed downstream in same exon

- **PVS1_moderate** (11 submissions)

  Not predicted to undergo NMD, role of region unknown, predict loss of <10% protein… loss of function variants in this region are rare in general population

Fresh this week:

Predictive for MSH6 c.3939_3957dup (probably now VUS if we used proposed schema above)

Predictives for MSH6 c.3847_3850dup & c.3753_3756dup (ok class clear)

Indicates these type of variants represent a fair proportion of our Lynch cases so clear guidance required

Summary

- This line of evidence in variant interpretation is often seen as straightforward, however recent cases (such as those outlined here) have raised important questions on their correct use within our lab
- Particularly worrying is the fact that moving between Mod and Strong will often result in significant changes in classification from Class 3 to Class 4
- The PVS1, PM4 guidelines seem to need clarification as the returns here show a high degree of variability in their use. PM4?
- Case studies help to tease out the fine detail in these cases
- Hopefully, the information here can help us to come to consensus agreement on how to use the rules and decide on clearer guidance
- Once happy, this affects both Cancer and rare disease – discuss with main ACMG
- Update of guidelines?

Thanks to:

George Burghel for discussion and prompting this review.

Massive thanks to Kevin Colclough for helping with this, agreeing to me using his email response in the slides and for sharing his slides with me.

Thanks to Clare Maurer & Ian Frayling for sharing slides

Thanks to CSTAG for feedback and additional info in compiling this presentation (particularly Laura King for compiling the feedback and comments for inclusion).