S1 Table: Summary of previous functional measurements and current InSiGHT classifications

The table lists functional results of previously performed MLH1 variant measurements, sorted by stability (expression of WT in %). All variants with **stability measurements** lower than the clinical stability reference variant for pathogenicity p.(Ala681Thr) are indicated with red colour, those with expression higher than the neutral stability reference variant p.(V716M) are indicated by green colour. For **MMR activity** measurements, activity close to WT (>75%) was considered proficient, activity close to negative control (<25%) was considered deficient. Functional result summary can be compared to current variant classifications of the InSiGHT (retrieved October 2022).

Variant	Results functional analysis in Hinrichsen *et al.* (2013)[1]			Classification InSiGHT (10/2022)[2]	
	Expression % of WT >V716M=proficient <A681T=deficient	MMR activity % of WT >75=proficient <25=deficient	Functional result summary	Class	Class in words
WT	100	100	proficient (MMR and stability)		
I219V	94	97	proficient (MMR and stability)	1	not pathogenic
R265H	91	91	proficient (MMR and stability)	-	no classification
L749P	85	15	MMR deficient	5	pathogenic
E663G	85	95	proficient (MMR and stability)	-	no classification
D601G	82	96	proficient (MMR and stability)	3	uncertain
K618A	78	92	proficient (MMR and stability)	1	not pathogenic
K84E	76	9	MMR deficient	4	likely pathogenic
H718Y	73	110	proficient (MMR and stability)	1	not pathogenic
R755W	72	10	MMR deficient	3	uncertain
E578G	71	91	proficient (MMR and stability)	1	not pathogenic
H109Q	71	71	unclear (MMR)	3	uncertain
V716M	64	88	STABILITY REFERENCE VARIANT 1[5]	1	not pathogenic
R522W	62	105	unclear	-	no classification
R659Q	61	85	unclear	3	uncertain
V506A	58	88	unclear	3	uncertain
N551T	53	80	unclear	3	uncertain
G54E	52	44	unclear	-	no classification
A681T	52	99	STABILITY REFERENCE VARIANT 2[5]	5	pathogenic
A586P	50	21	Stability and MMR deficient	3	uncertain
L559R	45	79	Stability deficient	3	uncertain
T117M	44	5	Stability and MMR deficient	5	pathogenic
L622H	42	80	Stability deficient	5	pathogenic
T82I	41	6	Stability and MMR deficient	5	pathogenic
P640S	30	80	Stability deficient	4	likely pathogenic
R659L	28	8	Stability and MMR deficient	-	no classification
L574P	27	16	Stability and MMR deficient	4	likely pathogenic
R725C	26	101	Stability deficient	3	uncertain
P654L	25	67	Stability deficient	5	pathogenic
L676R	24	12	Stability and MMR deficient	3	uncertain
P648L	22	92	Stability deficient	4	likely pathogenic
T662P	22	93	Stability deficient	-	no classification
D41G	20	10	Stability and MMR deficient	5	pathogenic
G65D	17	19	Stability and MMR deficient	4	likely pathogenic
G101D	16	11	Stability and MMR deficient	4	likely pathogenic
N38H	14	15	Stability and MMR deficient	5	pathogenic
R659P	13	99	Stability deficient	5	pathogenic
L653R	11	5	Stability and MMR deficient	4	likely pathogenic
E102K	2	27	Stability deficient	-	no classification

Variant	Results functional analysis in Hinrichsen *et al.* (2014)[3]			Classification InSiGHT (10/2022)[2]	
	Expression % of WT >V716M=proficient <A681T=deficient	MMR activity % of WT >75=proficient <25=deficient	Functional result summary	Class	Class in words
Q689R	106	99	proficient (MMR and stability)	1	not pathogenic
L507F	101	91	proficient (MMR and stability)	-	no classification
V716M	83	-	STABILITY REFERENCE VARIANT 1[5]	1	not pathogenic
D41H	70	11	MMR deficient	5	pathogenic
E605del	70	89	unclear	-	no classification
A681T	52	-	STABILITY REFERENCE VARIANT 2[5]	5	pathogenic
E605del+V716M	44	87	Stability deficient	-	no classification

Variant	Results functional analysis in Köger *et al.* (2018)[4]			Classification InSiGHT (10/2022)[2]	
	Expression % of WT >V716M=proficient <A681T=deficient	MMR activity % of WT >75=proficient <25=deficient	Functional result summary	Class	Class in words
N338S	130	95	proficient (MMR and stability)	3	uncertain
R575K	121	101	proficient (MMR and stability)	-	no classification
I500del	105	104	proficient (MMR and stability)	-	no classification
H112Q	103	72	unclear (reduced MMR)	-	no classification
P141A	92	111	proficient (MMR and stability)	3	uncertain
V716M	84	-	STABILITY REFERENCE VARIANT 1[5]	1	not pathogenic
Y97D	81	27	unclear	-	no classification
A681T	61	-	STABILITY REFERENCE VARIANT 2[5]	5	pathogenic
R265P	60	29	Stability deficient	4	likely pathogenic
L676P	41	49	Stability deficient	3	uncertain
K616del	40	35	Stability deficient	-	no classification

[1] Hinrichsen I, Brieger A, Trojan J, Zeuzem S, Nilbert M, Plotz G. **Expression defect size among unclassified MLH1 variants determines pathogenicity in Lynch syndrome diagnosis**. *Clin Cancer Res*. 2013; 19:2432–41. doi: 10.1158/1078-0432.CCR-12-3299 PMID: 23403630.

[2] MLH1 variant pathogenicity classifications performed by experts according to current guidelines in 5-tiered system, retrieved from https://www.insight-database.org/classifications/ in October 2022.

[3] Hinrichsen I, Schäfer D, Langer D, Köger N, Wittmann M, Aretz S, et al. **Functional testing strategy for coding genetic variants of unclear significance in MLH1 in Lynch syndrome diagnosis**. *Carcinogenesis*. 2015; 36:202–11. doi: 10.1093/carcin/bgu239 PMID: 25477341.

[4] Köger N, Paulsen L, López-Kostner F, Della Valle A, Vaccaro CA, Palmero EI, et al. **Evaluation of MLH1 variants of unclear significance**. *Genes Chromosomes Cancer*. 2018; 57:350–8. doi: 10.1002/gcc.22536 PMID: 29520894.

[5] **MLH1 protein stability reference variants** were extensively established in reference [1]. **Stability referece variant 1** (**V716M=p.(Val716Met**)) represents a MLH1 polymorphism that displays reduced protein stability, but does not entail any effects on human health. It is therefore used to judge if a stability defect in an unclassified MLH1 variant is clinically neutral (when expression is higher than V716M). **Stability reference variant 2** (**A681T=p.(Ala681Thr)**) is a variant that displays WT MMR activity, but a protein stability stronger reduced than V716M. A681T is pathogenic, and carriers of the A681T variant have Lynch syndrome (although this variant has a lower penetrance). For these reasons, the variant is used as reference variant for protein stability defects that confer a pathogenic phenotype.