BRIEF COMMUNICATION

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Solving the enigma of POLD1 p.V295M as a potential cause of increased cancer risk

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Germline variants that affect the proofreading activity of polymerases epsilon (*POLE*) and delta (*POLD1*) predispose to colorectal adenomas and carcinomas, among other cancers. All cancer-associated pathogenic variants reported to date consist of non-disruptive genetic changes affecting the sequence that codifies the exonuclease domain (ED). Generally, disruptive (frameshift, stop-gain) *POLE* and *POLD1* variants and missense variants outside the ED do not predispose to cancer. However, this statement may not be true for some, very specific variants that would indirectly affect the proofreading activity of the corresponding polymerase. We evaluated, by using multiple approaches, the possibility that *POLD1* c.883G>A; p.(Val295Met), -a variant located 9 amino acids upstream the ED and present in ~0.25% of hereditary cancer patients-, affects POLD1 proofreading activity. Our findings show cumulative evidence that support no alteration of the proofreading activity and lack of association with cancer. The variant is classified as likely benign according to the ACMG/AMP guidelines.

INTRODUCTION
Germline pathogenic non-disruptive variants in the region coding for the exonuclease domain (ED) of polymerases epsilon and delta, cause increased risk to colorectal cancer (CRC), adenomatous polyposis and other tumor types, including endometrial, breast, ovarian and brain cancers; which defines the polymerase proofreading-associated polyposis (PPAP) [1, 2]. The alteration of *POLE* or *POLD1* proofreading activity, either in the germline or in a tumor (somatic), causes defective DNA repair during replication, which translates into an accumulation of specific genetic changes in associated tumors (>100 variants per Mb (var/Mb) and COSMIC mutational signature SBS10, or SBS14 and SBS20 when combined with mismatch repair (MMR) deficiency) [1, 3]. Available data indicate that there is no association of loss-of-function variants and of variants located outside the ED with cancer, as they do not alter the polymerase proofreading [2, 4, 5]. Nevertheless, although never empirically proven, it has been speculated that some variants located outside the ED might indirectly affect proof-reading, thus having a similar effect than ED pathogenic variants. Such is the case of *POLE* c.1420G>A; p.(Val474Ile), which affects a residue three amino acids downstream the ED, and causes an effect on proofreading when tested in a yeast model [6]. However, exome sequencing of a carrier's CRC revealed neither hyper/ultra-mutation nor accumulation of the transversions observed in proofreading-defective tumors. Here we present the results of multiple studies performed to elucidate the potential pathogenicity of *POLD1* c.883G>A; p.(Val295Met), a recurrently identified variant affecting a residue in close proximity to POLD1 ED.

METHODS
POLD1 p.Val295Met carriers
Variant carriers were identified among: (i) 2,309 unrelated familial/early-onset cancer patients subjected to a multi-gene hereditary cancer panel [5]; (ii) 504 unrelated cancer patients that include high risk breast and/or ovarian families, patients with personal or familial history of different tumor types previously associated with PPAP (CRC and polyposis excluded), patients with other multiple tumors, and patients fulfilling the criteria for *TP53* genetic testing [5]; and (iii) 529 families with familial/early onset CRC and/or polyposis and no germline pathogenic variants in other known high-penetrance CRC genes [7]. The characteristics of the cohorts are detailed elsewhere [5, 7]. Informed consent was obtained from all subjects and the study received the approval of IDIBELL Ethics Committee.

In silico pathogenicity prediction
In silico predictions were extracted from Varsome [8], which uses BayesDel_addAF, DEOGEN2, EIGEN, FATHMM-MKL, M-CAP, MVP, Mutatio-nAssessor, MutationTaster, PrimateAI, REVEL and SIFT for pathogenicity predictions and GERP++ for conservation.

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Tumor mutational signatures

Mutational signature analysis from tumor exome sequencing data was performed with DeconstructSigs [5].

3D structure modeling and predictions

The cryo-EM structure of human POLD1 determined at 3.08 angstroms resolution (PDB ID: 6tny, chain A) and a 3D model based on the crystallographic structure of the homologous yeast protein Pol3 (PDB ID: 3iay, chain A) [7], were used in this study. 3D stability predictions were performed with I-Mutant 3.0 (http://gpcr.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi), CUP-SAT (http://cupsat.tu-bs.de), PoPMuSiC (http://dezyme.com), MAESTRO (https://biwww.che.sbg.ac.at/maestro/web/), INPS-3D (http://inpsmd.biocomp.unibo.it/inpsSuite/default/index3D), DeepDDG (http://protein.org.cn/ddg.html) and DynaMut (http://biosig.unimelb.edu.au/dynamut/).

Case-control studies

POLD1 p.(Val295Met) allele frequencies in breast cancer patients, CRC patients and controls were obtained from a population-based multi case-control series (MCC-Spain, www.mccspain.org).

Variant repository

Variant and phenotype information of the families carrying POLD1 p.Val295Met has been submitted to LOVD (https://www.lovd.nl/3.0/home).

RESULTS AND DISCUSSION

By searching for pathogenic variants in POLE and POLD1 affecting the proofreading activity of polymerases epsilon and delta, our group identified POLD1 (LRG_785, t1) c.883G>A; p.(Val295Met), a variant located 9 amino acids upstream the ED, in a total of 16 families (19 carriers). Of those, two families belonged to the series of 529 familial/early onset colorectal cancer (CRC) and/or polyposis families [7] (Families 1 & 2; Table 1); 11 to the 2,309 familial/early-onset cancer patients [5] (Families 3–13); and three to the 504 unrelated cancer patients with selected phenotypes [5] (Materials and Methods for details) (Families 14–16).

The tumor spectrum of POLD1 p.(Val295Met) carriers mainly included breast and/or ovarian cancer (11/19 carriers) and CRC (8/19). In two families, POLD1 p.(Val295Met) co-occurred with a BRCA2 pathogenic variant, and in one family, with the likely pathogenic variant POLD1 p.(Asp316Gly) in trans (Table 1).

POLD1 c.883G>A; p.(Val295Met) was not predicted pathogenic (benign computational verdict based on 10 benign predictions vs. 1 pathogenic prediction by FATHMM-MKL) and affected a non-conserved amino acid (GERP + + = 3.64). Nevertheless, further analyses were performed to elucidate its actual involvement in cancer predisposition, due to its recurrence (16 families) and its proximity to the ED.

Exome sequencing data from an MMR-proficient CRC developed at age 48 by a POLD1 p.(Val295Met) carrier (Family 2) revealed no hypermutation (~5 var/Mb). Mutational signature analysis revealed a subtle presence of proofreading defective-associated signatures SBS10 (2% signature contribution), and SBS20 (3.5% contribution), associated with combined MMR deficiency and POLD1 pathogenic variant, despite the absence of detectable MMR deficiency (Supplementary Fig. 1). No somatic POLE or POLD1 ED variants were identified. These findings led us to study the mutational burden and signatures in other MMR-proficient tumors harboring the POLD1 variant. We identified one tumor, among 42 non-treated, stage II, MMR-proficient CRCs with exome sequencing data [9], with a somatic POLD1 p.(Val295Met) and no additional suspicious POLE or POLD1 variants. The tumor harbored ~50 var/Mb, and no trace of POLE/D1-associated signatures (Supplementary Fig. 1).

Being the valine 295 not conserved in yeast, we were not able to perform a yeast-based proofreading assay to assess the effect of the variant [5]. The suspicion of a potential (weak) effect of the variant on the proofreading activity of polymerase δ, -based on the results of the analysis of tumor mutational signatures in one of the tumors and the proximity of the variant to the ED-, led us to perform an in-depth analysis of the effect of the variant on the structural 3D conformation changes of the ED and/or the alteration of the DNA binding cleft, which has been observed to be the ED structural (3D) region most directly associated with the proofreading activity of the polymerases [5, 10, 11].

We used the cryo-EM structure of human POLD1 and a 3D model based on the crystallographic structure of the homologous yeast protein [7], to study the effect of p.Val295Met. While the cryo-EM structure and the 3D model superpose perfectly with a root mean square deviation of less than 1.9 angstroms (PDBeFold method) (Fig. 1A), the DNA binding site is placed at different positions (Fig. 1B and C). Single-stranded DNA from the 3D model is in the same position as in bacteriophage T4 polymerase complex (PDB ID: 1NOY), which fits with the position of the DNA when the exonuclease is working. Despite the proximity of Val295 to the ED in the linear sequence (9 amino acids upstream), both the cryo-EM structure and the 3D model show that residue Val295 is distant from the DNA binding pocket of the exonuclease, suggesting lack of effect on the proofreading activity.

Compared to the wildtype residue (Val295), Met295 was predicted, by 4 out of 7 different methods, to locally alter the 3D stability of the protein (Supplementary Table 1). As predicted by DynaMut, this local structure destabilization is caused by direct steric clashes with neighboring residues (Supplementary Fig. 2). However, when comparing the 3D conformation changes of the whole protein by Normal Mode Analysis (NMA; implemented in DynaMut) between POLD1 wildtype and POLD1 p.(Val295Met), the two variants showed similar structural profiles; i.e. low fluctuation for the whole protein except for the C-terminal region in the cryo-EM structure (Supplementary Fig. 3); indicating that Met295 has no effect on the global protein dynamics.

Based on the carriers' phenotypes, we evaluated the allele frequency of POLD1 c.883G>A in CRC and breast cancer patients and matched controls from the same geographic area (source: MCC_Spain [12]). The case-control study showed lack of association with either cancer type (Table 2). Moreover, the observed allele frequencies (0.19% for CRC patients, 0.18% for breast cancer patients and 0.16%-0.20% for controls) were highly similar to those observed in the prospective hereditary cancer cohort (0.24%) and in Spanish individuals (0.22%; source: http://csvs.babelomics.org/) (Table 2). To investigate the potentially associated phenotypes, we separately analyzed the allele frequencies in hereditary/early-onset breast and/or ovarian cancer patients, and in hereditary/early-onset CRC and/or polyposis patients included in the prospective hereditary cancer cohort. While the p.(Val295Met) allele frequency in breast and/or ovarian phenotypes (0.17%) was similar to the one observed in the above-mentioned cases and controls, the frequency in patients with colorectal phenotypes was higher (0.50%), suggesting a potential association with the disease (Table 2). To validate this observation in an independent series of patients, we accessed the exome sequencing data obtained from 1,006 familial/early-onset CRC probands via Canvar (https://canvar.icr.ac.uk; accessed October 2020). The allele frequency for POLD1 p.(Val295Met) was 0.05% (1/1,778), same that those observed in European non-cancer population (MAF: 0.05%; 119/239,056; source: gnomAD 2.1.1), arguing against its involvement in colorectal cancer predisposition. Interestingly, the frequency of the variant was higher in Spanish population (0.22%), compared to that observed in Europeans (0.05%), suggesting an enrichment in that geographical area.

Our findings suggest that POLD1 p.(Val295Met) does not affect the proofreading activity of POLD1 and is not associated with cancer predisposition. Considering the gathered evidence,

Table 1. Phenotypic features of families carrying POLD1 c.883G>A; p.(Val295Met). Phenotypes of the same individual are separated by commas, while phenotypes from different individuals are separated by a semicolon.

Family	# carriers/relatives tested	Cancer diagnosis in carriers (age at diagnosis)	Cancer diagnosis in non-carriers (age at diagnosis)	Cancer in non-tested relatives
1	2/3	1.1. Breast ca. (46); [a]1.2. EC (57), breast ca. (65; [d]75)	[a]CRC (44), EC (54)	Skin ca. (56)
2	2/2	2.1. CRC (70); 2.2. CRC (48)	n.a.	Cervical cancer (41); [d]Prostate (63); CRC (68); [d]Lung ca. (80); [d]Skin ca. (56); Liver ca. (44); [d]Gastric ca. (56)
3	1/2	[b]3.1. Breast ca. (55), ovarian ca. (44)	[b]Breast ca. (26)	[d]Ovarian ca. (66), [d]ovarian ca. (53)
4	2/2	4.1. CRC (32); 4.2. Ovarian ca. (67)	n.a.	Ovarian ca. (67); Prostate ca. (68), polyps (61); Polyps (65); Polyps (47); CRC (73); Leukemia (3)
5	1/1	5.1. CRC (38)	n.a.	[d]Breast ca. (40)
6	1/1	6.1. CRC (62), 43 polyps	n.a.	No
7	1/1	7.1. Breast ca. (37)	n.a.	Breast ca. (47)
8	1/1	[c]8.1. Ovarian ca. (64)	n.a.	Skin (64); [d]Breast ca. (76)
9	1/1	9.1. CRC (31)	n.a.	Ovarian ca. (50)
10	1/1	10.1. Ovarian ca. (50)	n.a.	[d]Breast ca. (26); [d]Breast ca. (50); [d]Esophageal ca. (52); [d]Prostate ca. (70); [d]Prostate ca. (66); [d]Prostate ca. (56)
11	1/1	11.1. CRC (34), 1 LG-TA	n.a.	CRC (40); Breast ca. (46), 1 LG-TA; Skin (70), lung ca. (71); Prostate ca. (75)
12	1/1	12.1. Breast ca. (55)	n.a.	Breast ca. (46); Breast ca. (56); Skin ca. (47); Esophageal ca. (51); Lung ca. (58)
13	1/1	13.1. CRC (70)	n.a.	[d]Prostate ca. (70); [d]Lung ca. (52); [d]Brain ca. (40); [d]Brain ca. (40)
14	1/2	14.1. Breast ca. (60)	Breast ca. (50)	Breast ca. (64); [d]Skin ca. (88)
15	1/1	15.1. Leiomyosarcoma (54), renal ca. (61), breast ca. (65)	n.a.	Breast ca. (30), [d]gastric ca. (60); [d]Head and neck ca. (66)
16	1/1	16.1. Breast ca.(39)	n.a.	Breast ca. (32); Breast ca. (38); Head and neck ca. (73); Myeloid leukemia (52)

ca cancer, *CRC* colorectal cancer, *EC* endometrial cancer, *LG-TA* tubular colorectal adenoma with low-grade dysplasia, *n.a.* not available information because there are no non-carriers identified in the family.

[a]Carrier of POLD1 c.947A>G; p.(Asp316Gly) (catalytic residue of the exonuclease). In trans with POLD1 p.(Val295Met).

[b]Carrier of BRCA2 c.1310_1313delAAGA; p.(Lys437Ilefs*22).

[c]Carrier of BRCA2 c.3500_3501del; p.(Ile1167Asnfs*16).

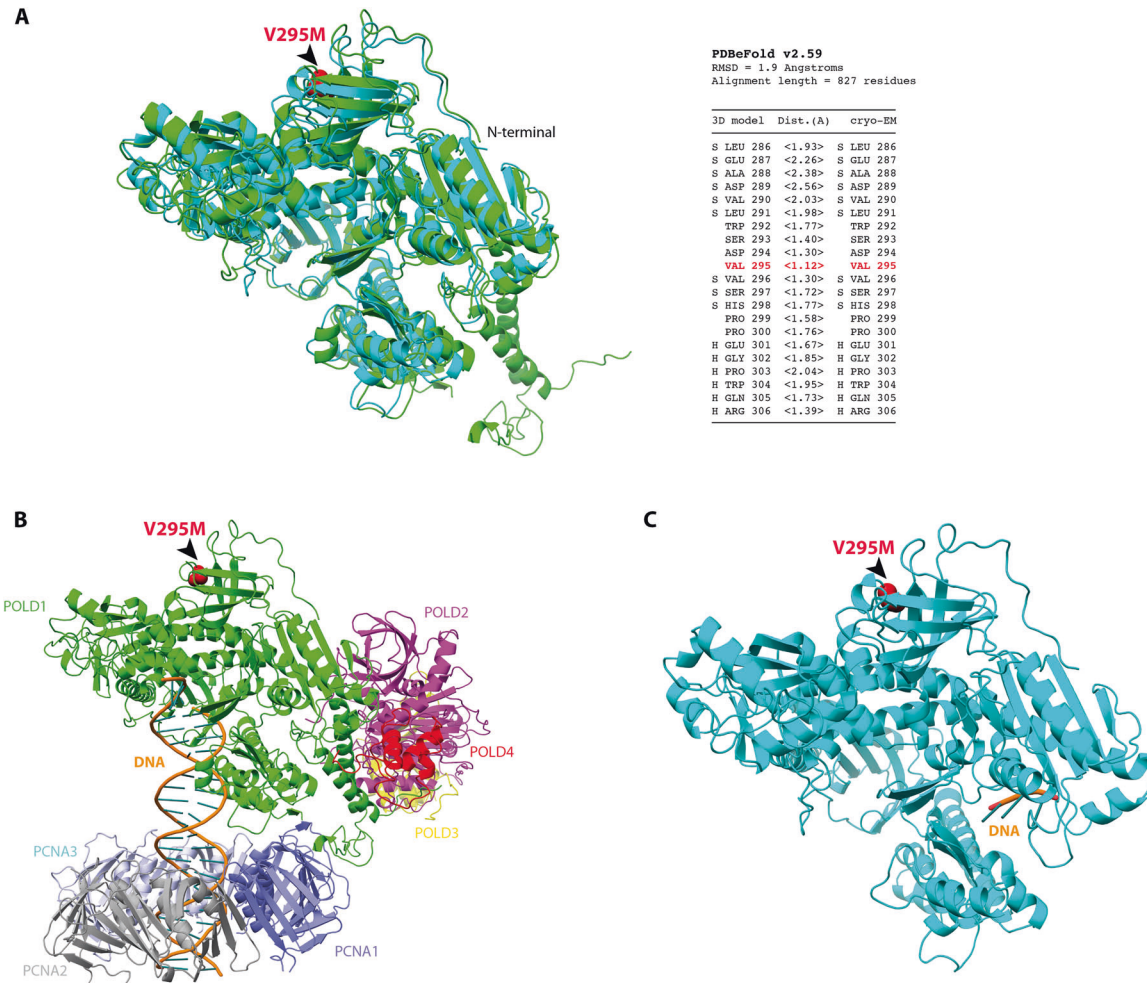[d]Cancer diagnosis not confirmed.

Fig. 1 Location of Valine 295 in the cryo-EM structure of human POLD1 determined at 3.08 Å resolution (PDB ID: 6tny, chain A) and the 3D model based on the crystallographic structure of the homologous yeast protein Pol3 (PDB ID: 3iay, chain A) [7]. Protein chains are represented in different colors. Location of Valine 295 is highlighted in red. **A** Structure comparison with PDBeFold v2.59. **B** DNA binding site in the cryo-EM structure. **C** DNA binding site in the 3D model.

Table 2. Allele frequency of rs199545019 (chr19:50905911 G>A; *POLD1* c.883G>A; p.Val295Met) assessed in cancer cases and controls from European populations, including data from the MCC_Spain case-control study, the Collaborative Spanish Variant Server (CSVS), and 2,309 hereditary cancer patients.

[a]MCC_Spain				[b]CSVS	Prospective hereditary cancer cohort		
CRC patients	**Controls**	**BrCa patients**	**Controls**	**Population controls**	**Total**	**Fam/EO CRC &/or polyposis**	**Fam/EO BrCa &/or OvCa**
5/2,672 (0.19%)	11/5,486 (0.20%)	4/2,276 (0.18%)	4/2,478 (0.16%)	7/3,217 (0.22%)	11/4,618 (0.24%)	6/1,202 (0.50%)	5/2,936 (0.17%)

BrCa breast cancer, *CRC* colorectal cancer, *CSVS* collaborative Spanish variant server, *Fam/EO* familial and/or early onset, *OvCa* ovarian cancer.
[a]Exome array data from MCC Spain includes 1,348 CRC patients and 2,744 controls; and 1,138 breast cancer patients and 1,240 controls (https://shiny.snpstats.net.exome/). Data included correspond to actual genotyping results (not imputed).
[b]Collaborative Spanish Variant Server (http://csvs.babelomics.org/); 31 cases belonging to the group "cancer" were excluded.

application of the ACMG/AMP guidelines [5, 13] resulted in the classification of *POLD1* p.Val295Met as likely benign (BS1, BP4; Supplementary Table 2).

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CONFLICT OF INTEREST
The authors declare no competing interest.

ADDITIONAL INFORMATION
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