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Classification of *BRCA2* variants of uncertain significance (VUS) using an ACMG/AMP model incorporating a homology directed repair (HDR) functional assay

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Abstract

Purpose: The identification of variants of uncertain significance (VUS) in the *BRCA1* and *BRCA2* genes by hereditary cancer testing poses great challenges for the clinical management of variant carriers. The ACMG/AMP variant classification framework, which incorporates multiple sources of evidence, has the potential to establish the clinical relevance of many VUS. We sought to classify the clinical relevance of 133 single nucleotide substitution variants encoding missense variants in the DNA binding domain (DBD) of *BRCA2* by incorporating results from a validated functional assay into an ACMG/AMP variant classification model from a hereditary cancer testing laboratory.

Experimental Design: The 133 selected VUS were evaluated using a validated homology-directed double strand DNA break repair (HDR) functional assay. Results were combined with clinical and genetic data from variant carriers in a rules-based variant classification model for BRCA2.

Results: Of 133 missense variants, 44 were designated as non-functional and 89 were designated as functional in the HDR assay. When combined with genetic and clinical information from a single diagnostic laboratory in an ACMG/AMP variant classification framework, 66 variants previously classified by the diagnostic laboratory were correctly classified, and 62 of 67 VUS (92.5%) were reclassified as likely pathogenic (n=22) or likely benign (n=40). In total, 44 variants

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were classified as pathogenic/likely pathogenic, 84 as benign/likely benign, and 5 remained as VUS.

Conclusions: Incorporation of HDR functional analysis into an ACMG/AMP framework model substantially improves *BRCA2* VUS re-classification and provides an important tool for determining the clinical relevance of individual *BRCA2* VUS.

Introduction:

Genetic testing has been integrated into clinical management due to advances in molecular genetics and sequencing technology. This testing benefits patients and society through enhanced cancer surveillance, prevention measures, and therapeutic options. However, the clinical relevance of many inherited variants (variants of uncertain significance (VUS)) in disease-related genes identified through genetic testing has not been determined. The American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) has developed variant classification guidelines based on a general framework that includes information on variant frequency, computational prediction, functional analysis, and segregation studies(1). However, these general ACMG/AMP framework guidelines lack a uniform classification framework for specific genes, which can lead to discrepancies in variant classification among groups conducting testing.

BRCA2 (MIM: 600185) is a frequently mutated gene in the general population (1 in 1000 in unaffected individuals)(2), for which inherited pathogenic variants have been associated with high risks of breast, ovarian, prostate and pancreatic cancer(2,3). Current clinical management of individuals with *BRCA2* pathogenic variants can involve specific cancer screening and surveillance measures, surgical prevention, and therapeutic options for patients and family members. However, individuals found to carry VUS cannot benefit from these clinical management strategies due to uncertainty about the clinical relevance of the VUS.

As of 5/1/2022 there were 6176 *BRCA2* VUS listed in the ClinVar database including 5534 missense VUS. Furthermore, of all 6506 *BRCA2* missense variants in ClinVar, 5534 (85%) were VUS, 667 (10.4%) had conflicting interpretations, and 153 (2.4%) were benign. Another 85 were considered Pathogenic/Likely Pathogenic, but most appeared to influence splicing. Only 21 missense variants that do not influence splicing have been established as Pathogenic/Likely Pathogenic and 35 as Benign of Likely Benign by the ENIGMA expert panel for BRCA1/2 variant classification (prior to development of ACMG/AMP rules-based analysis) (4–6). All of these are located in the *BRCA2* DNA binding domain (DBD) (amino acids 2479–3192). *BRCA2* VUS characterization is an area of active research with recent efforts focused on functional evaluation(4,6–10), computational algorithm development(11,12), and expanded case-control analysis of variants(5). Among efforts focused on functional evaluation of *BRCA2* VUS, a Homology Directed Repair (HDR) cell-based DNA repair assay has demonstrated high sensitivity and specificity for established pathogenic and benign missense variants from the *BRCA2* DBD(4,6). This assay has been applied only to missense variants in the *BRCA2* DBD because this hotspot region contains all established non-splice P/LP missense variants in BRCA2, and because it is not known if

the HDR assay can measure the functional effects of variants in other domains of BRCA2. Results from this functional assay have recently been integrated into an ACMG/AMP framework VUS classification model developed by Ambry Genetics(6). In the current study, we report on re-standardization of the HDR assay and systematic evaluation and classification of 133 *BRCA2* DBD missense variants using an alternative ACMG/AMP model, as applied by the GeneDx clinical genetic testing lab, that incorporates the HDR assay results.

Material and Methods:

Variants evaluated

A total of 133 missense variants in the *BRCA2* DBD domain (amino acids position 2479–3192) that were evaluated for effects on *BRCA2* activity using an HDR functional assay and were clinically observed by GeneDx (up to 12/22/2020) were included in the current study. These 133 were among 450 BRCA2 DBD missense variants selected for evaluation by the HDR assay (data not shown) because of high prediction scores for deleterious/non-functional variants from the BayesDel (13) and BRCA-ML (12) *in silico* prediction models and presence in the NIH supported ClinVar database (133 of 1413 (9.4%). Variants with observed splicing effects or predicted to have potential splicing effects by *in silico* models were excluded. Variants were annotated according to HGVS recommendations and RefSeq transcript NM_000059.3 (Supplementary Table 1).

HDR functional analysis

HDR functional analysis of *BRCA2* DBD missense variants was carried out as described previously(4). Briefly, variants were introduced into a full-length FLAG-tagged *BRCA2* mammalian expression plasmid by site-directed mutagenesis. The presence of variants was verified by Sanger sequencing and related protein expression was verified by Western blot using anti-FLAG antibodies. Individual plasmids expressing *BRCA2* and the *iSceI* restriction endonuclease were co-transfected into *brca2*-deficient V-C8 cells containing a stably integrated DR-GFP reporter with an *iSceI* recognition site. Repair of the *iSceI* induced double strand break by *BRCA2*-dependent homologous recombination resulted in green fluorescent protein (GFP) expression. The proportion of GFP expressing cells for each transfection was quantified by flow cytometry. The established pathogenic missense variant p.Asp2723His and wild-type *BRCA2* were used as internal controls for normalizing the number of DNA repair-dependent GFP positive cells for each *BRCA2* construct to a 1 to 5 scale. All variants were evaluated using at least two independent clones in duplicate experiments.

HDR assay calibration

A Bayesian regression model was used to estimate the distribution of HDR scores and 95% confidence intervals (2.5 and 97.5 percentiles of the posterior distributions) for all variants. All calculations were performed in R using the rstanarm package. Based on the log-normal distribution of HDR scores for 21 pathogenic/likely pathogenic and 35 benign/likely benign missense variant standards from the *BRCA2* DBD (previously classified by the ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles)

expert panel or reported in previous publications(4–6) (Supplementary Table 1), 99.9% probability thresholds for both pathogenic and benign variants were calculated.

ACMG/AMP framework for classification of *BRCA2* DBD missense variants

The ACMG/AMP framework is a combination of 27 sources of evidence from population, computational and predictive, functional, segregation, and other relevant data, in which each contributing variable is weighted as very strong (PVS1), strong (PS1, PS2, PS3, PS4), moderate (PM1, PM2, PM3, PM4, PM5, PM6), and supporting (PP1, PP2, PP3, PP4, PP5) for pathogenicity, or stand-alone (BA1), strong (BS1, BS2, BS3, BS4), and supporting (BP1, BP2, BP3, BP4, BP5, BP6, BP7) for benign effects(14). The five variant classifications based on the ACMG/AMP framework are pathogenic, likely pathogenic, benign, likely benign, and VUS. In this study ACMG/AMP scoring rules(14) modified by GeneDx were used for clinical classification of *BRCA2* variants. The study was conducted in accordance with guidelines set forth by the Western Institutional Review Board (WIRB), which waived authorization for use of de-identified data. The strength of all pathogenic and benign ACMG/AMP criteria were considered. Thus, variant-specific functional data in support of neutrality (BS3) outweighed less specific evidence such as rarity in populations, which might otherwise support pathogenicity (PM2). For *BRCA2* missense variants with no reported effects on RNA splicing, the very strong evidence of pathogenicity (PVS1) rule was not applied. ACMG/AMP criteria used for evaluation of *BRCA2* missense variants were as follows:

Stand-alone evidence—BA1- This is the only stand-alone evidence applied within the ACMG/AMP framework and is used to assign benign impact based on variant frequency in populations. GeneDx evaluated population frequency relative to the disease incidence as stand-alone data for classifying a variant as benign. For rare disorders, proportionally lower allele frequencies are accepted as stand-alone criteria relative to the disease incidence(14).

Strong evidence—PS3/BS3- These two strong forms of evidence for either pathogenicity (PS3) or benign impact (BS3) are based on well-established *in vitro* or *in vivo* functional studies supportive of a deleterious (PS3) or neutral (BS3) effect on protein function. We applied results from the HDR assay, with demonstrated high sensitivity and specificity for 21 pathogenic and 35 benign variants, as strong functional evidence.

BS1- This evidence is applied when allele frequency is greater than expected for disorders(1). In addition, GeneDx utilizes a gene-specific lower threshold with supporting evidence weight (BS1_Supporting) (>0.1%).

Moderate evidence—PM1- Characterized by location of a variant in a mutational hot spot and/or critical and well-established functional domain without benign variation. Because all evaluated variants were in the *BRCA2* DBD domain, PM1 was applied to all evaluated variants.(15)

PM2- Absent or very rare in controls (gnomAD, ExAC).

PM3- Variant observed *in trans* with a *BRCA2* pathogenic variant in individual(s) with phenotypes consistent with Fanconi anemia (FA). As *BRCA2* bi-allelic pathogenic mutations are associated with FA, a variant that occurs *in trans* with a PVS1 variant in an FA case is attributed PM3 evidence. PM3 is applied as strong (PM3_Strong) evidence with multiple independent occurrences (≥2), or supporting (PM3_Supporting) evidence when with incomplete segregation (phase is not confirmed for one or more observations).

PM5- Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before.

Supporting evidence—PP1- Co-segregation with disease in multiple affected family members. PP1 is also applied as moderate evidence (PP1_Moderate) with increasing segregation data.

PP3/BP4- Multiple lines of computational evidence support a deleterious effect (PP3) or no impact (BP4) on the gene or gene product. The *in silico* algorithm PROVEAN was used for protein prediction.

BP2- Observed in *trans* or phase unknown with a *BRCA2* pathogenic variant, in the absence of reported phenotypes consistent with FA.

BP5- Variant observed in a case with an alternate molecular basis for disease. Variants co-occurring with established pathogenic variants are considered BP5. Strong evidence is applied (BP5_Strong) when multiple co-occurrences (>10) were observed.

Supplementary evidence—Multifactorial prediction was applied as supporting (PP_Multifactorial/BP_Multifactorial) or strong (PP_Multifactorial_Strong) evidence based on multifactorial probability models that incorporate evidence from segregation, co-occurrence, and pathology (5,16,17). This Multifactorial prediction was not identified as a source of evidence in the original ACMG/AMP classification model(1).

Comparison with other reported functional analysis

Results from other functional studies including mouse embryonic-stem cell (m-ESC) based functional analysis(7,10,18–20), a *BRCA2* deficient cell line-based drug assay (MANO-B) (8), and a prime editing-based saturation genome editing (SGE) analysis(9) were compared to results for the HDR assay.

ClinVar classification

Variant classification from ClinVar submitted by clinical laboratories meeting ClinGen minimum requirements for data sharing to support quality assurance and the ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) consortium expert panel was used for comparison with the GeneDx ACMG/AMP model (including functional data).

Data availability

All data are presented in the manuscript and/or are available directly from the authors.

Results

HDR functional results of 133 *BRCA2* DBD missense variants observed clinically

The HDR functional assay for *BRCA2* DBD missense variants was previously shown to have 100% sensitivity (95%CI: 79%–100%) and 100% specificity (95%CI: 93%–100%) for pathogenic variants based on 18 known Benign/Likely Benign and 12 known Pathogenic/Likely Pathogenic variants.(4) However, a subset of these “standards” are now known to influence RNA splicing. Here the HDR assay was recalibrated using a larger set of 21 known Pathogenic/Likely Pathogenic and 35 known Benign/Likely Benign variants, none of which were predicted to cause aberrant splicing by Splice AI prediction analysis or shown to cause aberrant splicing by RT-PCR (Supplementary Table 1). Thresholds for 99.9% probability for pathogenic and benign effects were estimated based on these standards. An HDR score <1.49 was considered non-functional with probability of pathogenicity >0.99, whereas variants with HDR score >2.50 were considered functional, with probabilities of neutrality >0.99. *BRCA2* missense variants with HDR scores between 1.49 and 2.50 were considered to have potential hypomorphic/partial effects on function with uncertain clinical significance and were excluded from the current study.

The HDR scores for 133 clinically observed *BRCA2* DBD missense variants evaluated in this study ranged from 0.83 (95%CI: 0.76–0.91) for p.His2623Arg to 5.67 (95%CI: 5.05–6.36) for p.Asp3112Asn (Table 1, Table 2, Figure 1). These included 67 VUS evaluated for the first time in this study and 66 missense variants previously evaluated by the HDR assay and other functional assays (Tables 1 and 2) (4,11,12,21). Of the 133 variants, 44 were designated as non-functional with HDR scores <1.49 and 89 were designated as functional with HDR scores >2.50. Both non-functional and functional variants were evenly distributed within the DBD domain, except for a reduced number of observed variants in the OB2 domain (Figure 1). Multiple residues contained more than one amino acid alteration. Of these, substitutions in 14 of 15 residues had the same functional impact (all functional or all non-functional), such as the p.Arg2488Gly/Ser functional and the p.Asp2723His/Ala/Val non-functional variants. However, p.Arg2625Lys was functional, whereas p.Arg2625Ile was non-functional.

Comparison with other *BRCA2* functional studies

We compared results from the HDR assay for the 133 variants with available results from other functional studies (Supplementary Table 2). The other functional studies included mouse embryonic stem cell (mESC)-based survival and homologous recombination (HR) assays(7,10,18–20), drug response assays for DLD1 *BRCA2* deficient cells reconstituted with *BRCA2* (MANO-B)(8), and prime editing-based SGE[9]. Among 39 variants evaluated with the mESC-based methods, 19 variants that were functional in the HDR assay showed full complementation in the mESC-based survival analysis with 50% to 116% BRCA2 protein activity in mESC-based HR assays. All 20 variants that were non-functional in the HDR assay showed complete loss or reduced mESC survival and low or no HR activity in the mESC-based HR assays. Among 41 variants also evaluated for effects on drug response in the MANO-B study, which rated variants as class 1–5 for the sensitivity to four drugs (Olaparib, niraparib, rucaparib and cisplatin), 12 of 18 variants considered

functional in the HDR assay were consistently resistant (class 1 or 2) in the four drug-response assays (Supplementary Table 2). However, of the remaining 6 functional HDR variants, p.Val2908Gly (HDR: 2.53, 95%CI: 2.36–2.72) was class 4/5 (non-functional) for all 4 drug assays; p.Gly2508Ser (HDR:3.22, 95%CI: 2.95–3.52) was class 3/4 (uncertain/non-functional) for the 4 drug assays; p.Leu2972Trp (HDR:2.78, 95%CI:2.47–3.12) and p.Pro2771Leu (HDR:2.81, 95%CI:2.44–3.24) were class 3 (uncertain) for the 4 drug assays; p.Arg2520Pro (HDR:3.54, 95%CI:3.08–4.08) and p.Leu3011Pro (HDR:3.12, 95%CI:2.89–3.36) were mixed class 3 (uncertain) and class 2 (non-functional) in the drug assays. In contrast, 22 of 23 variants designated non-functional by HDR assay were class 4 or 5 (non-functional) in the MANO-B study. Only p.Trp2626Arg was class 3 (uncertain) for Olaparib and Rucaparib response (Supplementary Table 2).

Comparisons with the prime editing SGE analysis(9) were less informative because only variants in part of exon 15 (c.7545_7552, residues 2515–2518) and portions of exon 17 ((c.7826_7842, residues 2609–2614); (c.7916_7924, residues 2639–2642), and (c.7930_7960, residues 2644–2654)) of the BRCA2 DBD were evaluated by prime editing. Of 5 variants evaluated by HDR and by prime editing, 4 were non-functional by both methods (p.Gly2609Val (HDR=1.08, 95%CI:0.97–1.22), p.Leu2647Pro (HDR:1.00, 95%CI:0.89–1.12), p.Val2652Gly (HDR:1.14, 95%CI:0.99–1.32), and p.Leu2653Pro (HDR:0.92, 95%CI:0.80–1.06)), and one was functional by both methods (p.Cys2646Trp (HDR:3.39, 95%CI:2.95–3.91)) (Supplementary Table 2). This suggests a potentially strong correlation between the two assays, but more variants must be analyzed by both methods for a detailed comparison.

Incorporation of HDR functional data into an ACMG/AMP variant classification model

The ClinGen SVI recommendations for applying weight to functional studies within the ACMG/AMP ClinGen framework suggest that the PS3 and BS3 rules can be applied as strong lines of evidence when an assay has sensitivity and specificity for known pathogenic and benign variants that yields an odds of pathogenicity (oddspath) > 18.7(6,22). The HDR assay in this study was assigned strong evidence of pathogenicity under the PS3/BS3 functional assay rule because the assay yielded an oddspath of 35.0 on the basis of widely accepted standard variants (21 pathogenic and 35 benign), that were correctly identified by the assay as deleterious or neutral, respectively (Supplementary Table 1). This was consistent with a previous study of the HDR assay that yielded an oddspath of 46.0 (6). Thus, among the 133 variants under evaluation, those found to be non-functional in the HDR assay were assigned PS3 (strong evidence of pathogenicity) in the ACMG/AMP model, whereas variants considered functional were assigned BS3 (strong evidence of benign).

Of the 133 variants, 66 (49.3%) had previously been classified as pathogenic/likely pathogenic (n=22) or benign/likely benign (n=44) by GeneDx based on an internal ACMG/AMP framework model. Results from previously reported HDR assays and other functional studies contributed to classification of 43 of these 66 variants (16/22 pathogenic/likely pathogenic and 27/44 benign/likely benign). No changes in classification occurred after applying new HDR functional data for 61 of these 66 variants previously classified as benign, likely benign, pathogenic, or likely pathogenic. However, 5 variants had stronger

evidence of pathogenicity and changed from likely pathogenic to pathogenic (p.Leu2510Pro, p.Leu2686Pro, p.Asp2723Val, p.Gly2724Trp, and p.Leu3101Arg) (Table 1, Supplementary Table 2, Figure 2). In addition, 67 variants had a classification of VUS before the current study. After evaluation by the HDR assay (Figure 1, Figure 2), 92.5% (62/67) of the VUS were reclassified as likely benign (n=40) or likely pathogenic (n=22) by the ACMG/AMP classification model that included the BS3 or PS3 rules (Figure 1, Table 1, Table 2, Supplementary Table 2). Although 5 VUS (p.Cys2646Trp, p.Pro2735Arg, p.Cys2765Gly, p.Pro2771Leu, and p.Pro2785Leu) had strong evidence of benign effects in the HDR assay (BS3-strong), these variants were not reclassified as benign or likely benign due to lack of other supporting benign criteria (Supplementary Table 2). Other than the PS3/BS3 functional data, moderate evidence from variant location in a functional domain (PM1) (100.0% (133/133)) and population frequency (PM2/BA1/BS1_Supporting) (86.5% (115/133)), and supporting evidence from computational prediction (PP3/BP4) (100% (133/133)) were the most utilized forms of evidence for classification (Table 2, Supplementary Table 2).

Comparison with variant classifications by other clinical testing laboratories

The classifications for 133 variants based on incorporation of the HDR functional assay results into the GeneDx ACMG/AMP classification model were compared to the classifications submitted to ClinVar by other clinical testing laboratories (Supplementary Table 2). Among the 129 variants reported to ClinVar, 14 consistently classified as likely benign or benign by ClinGen-designated high quality testing groups were reclassified as benign/likely benign by the GeneDx ACMG/AMP model. A further 31 consistently classified as VUS in ClinVar were reclassified as benign/likely benign (n=10), pathogenic/likely pathogenic (n=16) and VUS (n=5). All 15 variants consistently classified as likely pathogenic or pathogenic in ClinVar were reclassified as likely pathogenic or pathogenic using the GeneDx model. However, 69 variants displayed conflicting classifications in ClinVar. After incorporating the HDR functional assay results, 95.0% (95 out of 100) variants with at least one entry as VUS in ClinVar were reclassified as either pathogenic/likely pathogenic or benign/likely benign by the GeneDx ACMG/AMP classification model, and 83.9% (26 out of 31) variants consistently classified as VUS in ClinVar were reclassified as either pathogenic/likely pathogenic or benign/likely benign by GeneDx (Figure 2, Supplementary Table 2).

Discussion

We evaluated 133 clinically observed *BRCA2* DBD missense variants with an HDR functional assay and then incorporated the findings into an ACMG/AMP framework variant classification model. Of these, 128 variants were classified as pathogenic/likely pathogenic or benign/likely benign, including 62 VUS that were re-classified as likely pathogenic (n=22) or likely benign (n=40). These results suggest that over 30% of VUS in the *BRCA2* DBD hotspot may be reclassified as pathogenic/likely pathogenic 32.8% (22 of 67) and that approximately 60% may be downgraded to benign/likely benign (59.7% (40 of 67)). This is consistent with results for *BRCA2* DBD VUS evaluated using the separate Ambry Genetics ACMG/AMP classification model (25.3% pathogenic/likely pathogenic, 60.4% benign/likely benign) (6). However, these findings contrast with studies showing that <10%

of unique VUS from the entire *BRCA2* gene are upgraded to pathogenic/likely pathogenic (23), suggesting that very few VUS outside of the DBD impact BRCA2 function and predispose to cancer.

Carriers of the *BRCA2* variants classified as pathogenic/likely pathogenic will benefit from these studies by now qualifying for the same clinical management strategies as carriers of known pathogenic protein truncating variants in *BRCA2*, such as enhanced screening with MRI and/or consideration for risk reducing prophylactic mastectomy/oophorectomy. For those already diagnosed with cancer the reclassifications provide eligibility for PARP inhibitor therapy. Furthermore, the information can be used to provide risk assessment and management for relatives. Carriers of *BRCA2* variants classified as benign/likely benign also benefit by eliminating the uncertainty associated with a genetic testing VUS diagnosis.

Publicly accessible data and computational predictions (PM1, PM2, and PP3/BP4) have been applied most frequently to variants within the ACMG/AMP framework rules-based classification model. However, these sources provide only mainly moderate or supporting evidence and do not resolve the classification of numerous VUS, which pose challenges to clinical management. In contrast, the HDR functional assay provides strong evidence of pathogenic or benign effects and leads to the classification of the great majority (128 of 133) of *BRCA2* DBD missense variants observed clinically by GeneDx. These findings suggest that the majority of missense VUS observed in this domain of *BRCA2* can be reclassified when adding this or other validated functional assays to ACMG/AMP framework rules-based classification models.

The HDR assay results were consistent with those from an mESC-based functional assay and CRISPR prime editing based SGE, but discrepancies were observed between the HDR assays and the MANO-B drug response assays. As the HDR assay has been validated using a large number of known pathogenic (n=21) and benign missense variants (n=35) (Supplementary Table 1), the suggestion is that the drug response analysis may need to be fine-tuned for variant classification. In addition, the implications of the inconsistencies for PARP inhibitor therapy remains to be determined.

The ACMG/AMP framework BP1 rule providing evidence for benign effects of missense variants in a gene that primarily has disease causing truncating variants was not used in the current study because established pathogenic missense variants in *BRCA2* have exhibited similar risks for breast cancer as truncating variants. Similarly, the PP2 rule providing evidence of pathogenicity that applies to a gene with a low rate of benign missense variation, in which missense variants are a common mechanism of disease, was not applicable to *BRCA2*. Among the very strong and strong sources of evidence for pathogenicity within the ACMG/AMP framework, PVS1 was not applicable for the majority of missense variants unless there was a demonstrated splicing effect by RNA analyses. The PS1 rule for a variant that causes the same amino acid change as a known pathogenic variant was rarely applicable. The PS2 rule for *de novo* variant was limited by availability of family data, and the PS4 rule for enrichment in cases over controls in a population was not applicable for the majority of these rare VUS. In addition, the PM5 rule for pathogenic missense hotspots, should be applied with caution. For example, observed amino acid alterations in the same

residue can have different clinical impact (p.Arg2625Lys as likely benign vs p.Arg2625Ile as likely pathogenic). PS3/BS3 functional evidence was the major strong source of evidence applied in the current study. This was sufficient, when combined with other moderate and supporting evidence, to classify 128 of 133 variants.

The five variants that were not reclassified and remained as VUS (p.Cys2646Trp, p.Pro2735Arg, p.Cys2765Gly, p.Pro2771Leu, and p.Pro2785Leu) were considered functional in the HDR assay, but no other evidence to support classification as benign variants was available. PROVEAN *in silico* prediction for all five variants yielded PP3 evidence, which conflicted with the BS3 functional results. Similarly, PROVEAN provided BP4 evidence in favor of a benign classification for 36 variants that were finally classified as pathogenic/likely pathogenic. Application of *in silico* prediction models optimized for the gene of interest such as BRCA-ML(11) (which has been developed specifically for *BRCA1* and *BRCA2* variants), correctly predicted the five VUS as functional with BP4 evidence (Supplementary Table 2). However, generalized *in silico* models as opposed to gene-specific *in silico* models are often preferred by clinical labs testing a wide variety of genes. Overall, the PROVEAN predictor used by GeneDx contributed effectively to classification of 83 benign variants with BP4 evidence and 8 pathogenic/Likely Pathogenic variants with PP3 evidence. The limitations of all *in silico* models further validate the importance of functional assays for classifying *BRCA2* VUS.

To date (5/1/2022), over 5500 missense *BRCA2* VUS have been reported to the ClinVar database. While the current study demonstrates the important contribution of a functional assay with defined high sensitivity and specificity for pathogenic variants to variant classification, more efforts in development, validation and application of functional assays are needed in order to keep pace with VUS identification. Classification of variants in *BRCA2* will likely benefit from development of high-throughput methodologies and subsequent validation of findings in the near future, with results having direct effects on patient care. This is especially important considering that

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance:

Variants of uncertain significance (VUS) in *BRCA2* prevent individuals with these alterations from benefitting from clinical management strategies for cancer risk reduction and targeted therapy. Characterization of BRCA2 VUS using a validated cell-based homology directed repair assay, and incorporation of the results into an ACMG/AMP framework model, led to reclassification of 92.5% of VUS studied as either pathogenic/likely pathogenic or benign/likely benign. This method can determine the clinical relevance of many VUS in BRCA2 leading to improved clinical care for variant carriers.

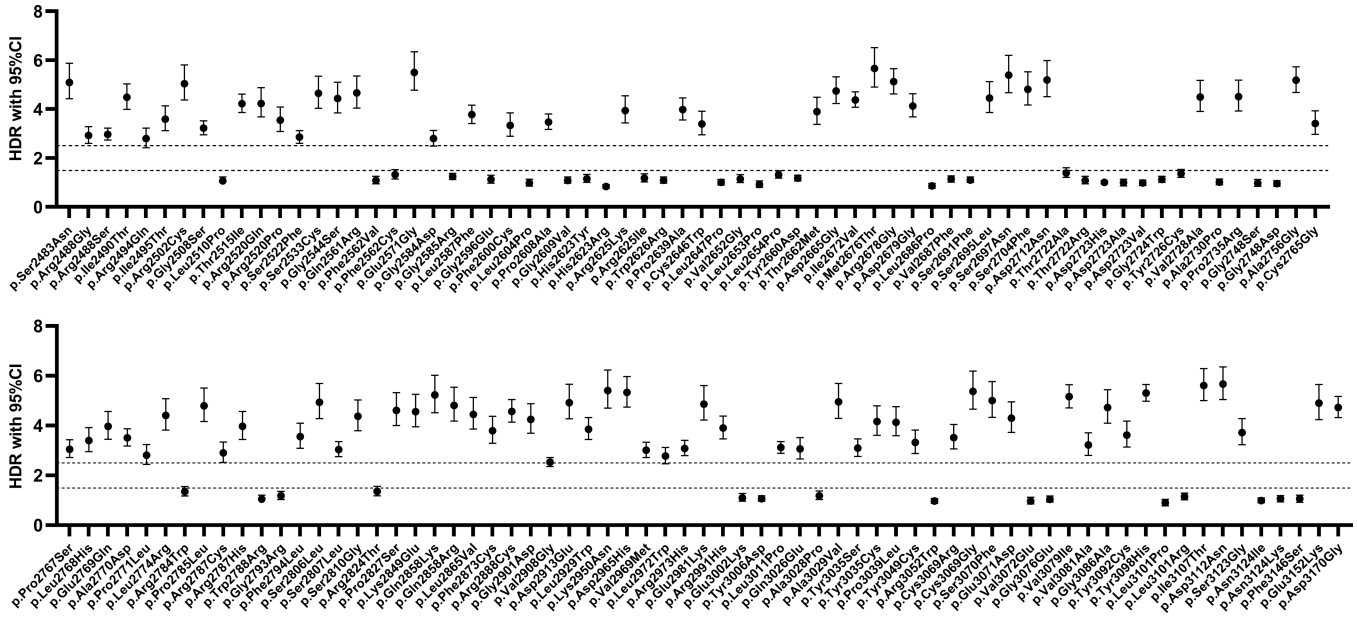

FIGURE 1: Homology Directed Repair (HDR) assay evaluation of clinically observed *BRCA2* DNA binding domain (DBD) missense variants.

HDR scores with 95% confidence intervals (CI) are presented on a 1 to 5 scale based on HDR activity of wild type *BRCA2* (HDR score=5) and the p.Asp2723His non-functional pathogenic variant (HDR score=1). HDR thresholds for functional (>2.50) and non-functional (<1.49) are indicated by horizontal dotted lines. Variants are ordered based on amino acid position (x-axis).

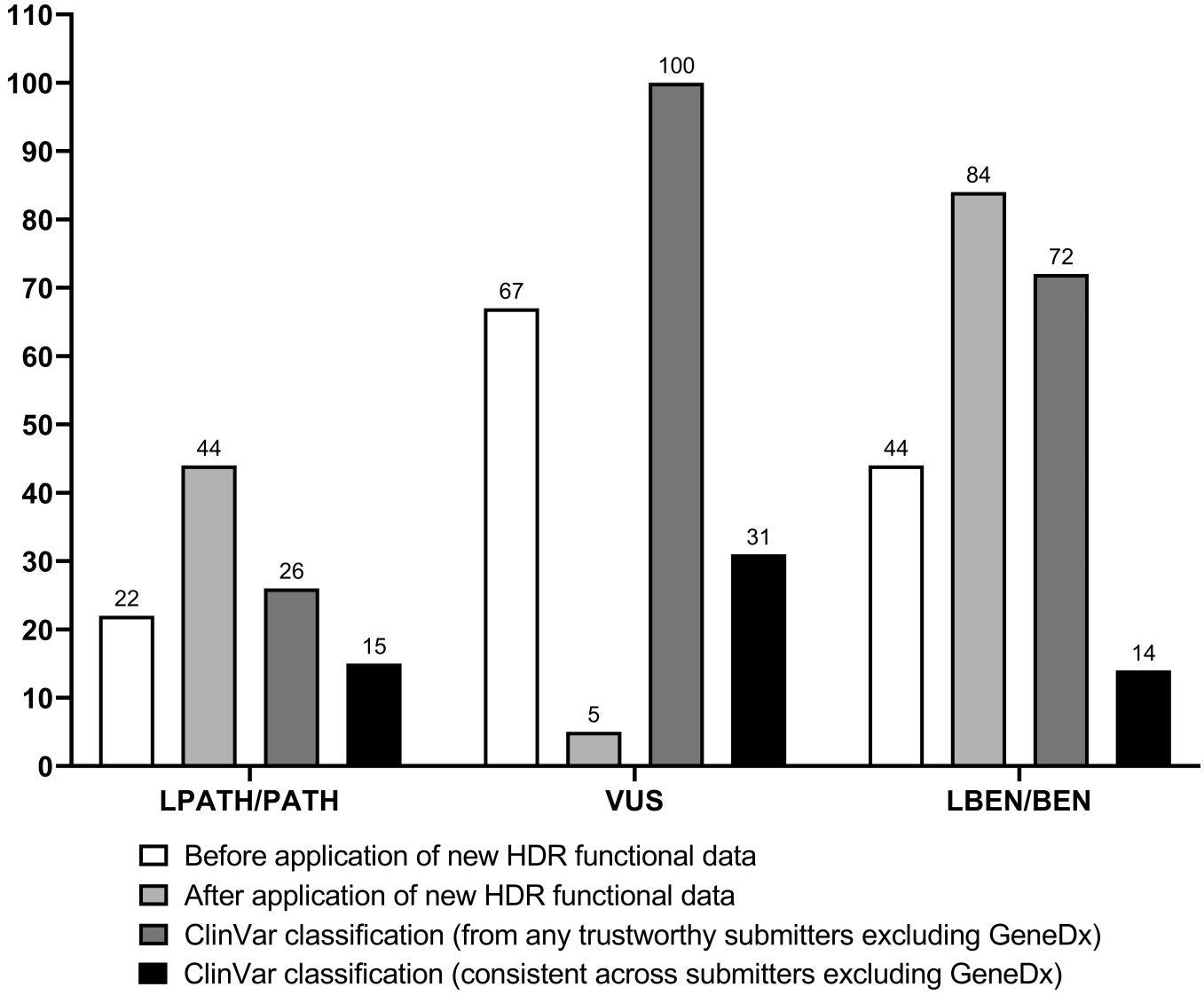

FIGURE 2: Incorporation of HDR functional data into a clinical ACMG/AMP framework for variant classification.

BRCA2 DBD missense variants observed clinically by GeneDx were classified by an ACMG/AMP-like framework into five categories: PATH (pathogenic), LPATH (likely pathogenic), VUS (variants of uncertain significance), LBEN (likely benign), BEN (benign). The PATH and LPATH categories were combined, LBEN and BEN categories were combined. The number of variants in each classification category defined by ClinVar (ClinGen approved laboratories excluding GeneDx) and by GeneDx before and after applying HDR functional data are shown.

Table 1:

HDR (non-functional score) of BRCA2 missense DBD variants and ACMG framework classification.

Variant (p.)	HDR Score	ACMG codes									Classification	
		PM1	PM5	PP1	PM3/BP2	PP3/BP4 (PROVEAN)	BA1/BS1/PM2 (Frequency)	BP5	PP_Multifac/ BP_Multifac	PS3/BS3	pre-HDR	post-HDR
p.His2623Arg	0.83	PM1	-	-	-	BP4	PM2	-	-	PS3-published*	LPATH	LPATH
p.Leu2686Pro	0.86	PM1	-	-	PM3	BP4	PM2	-	-	PS3	LPATH	PATH
p.Leu3101Pro	0.90	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Leu2653Pro	0.92	PM1	-	-	-	BP4	PM2	-	-	PS3-published*	PATH	PATH
p.Asp2723His	1.00	PM1	PM5	-	-	BP4	PM2	-	PP_Multifac	PS3-published*	PATH	PATH
p.Gly2748Asp	0.95	PM1	-	-	-	BP4	PM2	-	PP_Multifac _Strong	PS3-published*	PATH	PATH
p.Arg3052Trp	0.97	PM1	-	-	-	BP4	PM2	-	PP_Multifac _Strong	PS3-published*	PATH	PATH
p.Asp2723Val	0.98	PM1	PM5	-	-	PP3	PM2	-	-	PS3	LPATH	PATH
p.Asn3124Ile	0.99	PM1	-	-	-	PP3	PM2	-	PP_Multifac	PS3-published*	PATH	PATH
p.Gly2748Ser	0.97	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Val3072Glu	0.97	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Leu2647Pro	1.00	PM1	-	-	-	BP4	PM2	-	-	PS3-published^	LPATH	LPATH
p.Leu2604Pro	0.98	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Asp2723Ala	0.99	PM1	-	-	-	BP4	PM2	-	-	PS3-published^	LPATH	LPATH
p.Ala2730Pro	1.01	PM1	-	-	-	BP4	-	-	-	PS3	VUS	LPATH
p.Gly3076Glu	1.04	PM1	-	-	-	BP4	PM2	-	-	PS3-published^	LPATH	LPATH
p.Tyr3006Asp	1.06	PM1	-	-	-	PP3	PM2	-	-	PS3	VUS	LPATH
p.Asn3124Lys	1.06	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Trp2788Arg	1.05	PM1	-	-	-	PP3	PM2	-	-	PS3	VUS	LPATH
p.Phe3146Ser	1.06	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Gly2609Val	1.08	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Ser2691Phe	1.10	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Trp2626Arg	1.09	PM1	-	-	-	PP3	PM2	-	-	PS3	VUS	LPATH
p.Leu2510Pro	1.06	PM1	-	-	PM3	BP4	PM2	-	-	PS3	LPATH	PATH

Variant (p.)	HDR Score	ACMG codes									Classification	
		PM1	PM5	PP1	PM3/BP2	PP3/BP4 (PROVEAN)	BA1/BS1/PM2 (Frequency)	BP5	PP_Multifac/ BP_Multifac	PS3/BS3	pre-HDR	post-HDR
p.Thr2722Arg	1.08	PM1	PM5	-	-	BP4	PM2	-	PP_Multifac	PS3-published^	PATH	PATH
p.Phe2562Val	1.08	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Gly2724Trp	1.12	PM1	-	-	PM3	BP4	PM2	-	-	PS3	LPATH	PATH
p.Glu3002Lys	1.10	PM1	-	PP1_Moderate	-	BP4	PM2	-	-	PS3-published*	PATH	PATH
p.Val2687Phe	1.14	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Gly2596Glu	1.12	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Leu3101Arg	1.15	PM1	-	-	PM3_Strong	BP4	PM2	-	-	PS3	LPATH	PATH
p.Tyr2660Asp	1.17	PM1	-	PP1	-	PP3	PM2	-	-	PS3-published*	PATH	PATH
p.Val2652Gly	1.14	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.His2623Tyr	1.15	PM1	PM5	-	-	BP4	PM2	-	-	PS3	LPATH	LPATH
p.Arg2625Ile	1.17	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Gly2793Arg	1.18	PM1	-	-	-	PP3	PM2	-	PP_ Multifac	PS3-published^	PATH	PATH
p.Ala3028Pro	1.18	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Gly2585Arg	1.24	PM1	-	-	-	BP4	PM2	-	-	PS3-published^	LPATH	LPATH
p.Leu2654Pro	1.31	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Phe2562Cys	1.32	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Tyr2726Cys	1.36	PM1	-	-	-	PP3	PM2	-	-	PS3-published^	LPATH	LPATH
p.Arg2784Trp	1.35	PM1	-	-	-	BP4	PM2	-	-	PS3-published^	LPATH	LPATH
p.Arg2824Thr	1.36	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH
p.Thr2722Ala	1.39	PM1	-	-	-	BP4	PM2	-	-	PS3	VUS	LPATH

PM1-protein structure; PM5-pathogenic effect for alteration of same residue; PM3-Fanconi anemia phenotype; BP2-healthy biallelic patients; PP1-cosegregation in family; BA1/BS1/PM2-Population Frequency Codes; BP5-Co-occurrence; BS3/PS3-published

*: previous classification using BS3/PS3 evidence from HDR assays published by the Couch laboratory; BS3/PS3-published

^: previously classified using BS3/PS3 evidence from functional studies by Couch and other laboratories (see Supplementary Table 2). Abbreviations: HDR-homology directed repair; CI-confidence interval; Multifac -Multi-factorial; LPATH-Likely Pathogenic; P-Pathogenic; LBEN-Likely Benign; BEN-Benign; VUS-Variant of Uncertain Significance.

Table 2:

HDR (functional score) of BRCA2 missense DBD variants and ACMG framework classification.

Variant (p.)	HDR Score	ACMG codes									Classification	
		PM1	PM5	PP1	PM3/BP2	PP3/BP4 (PROVEAN)	BA1/BS1/PM2 (Frequency)	BP5	PP_Multifac/ BP_Multifac	PS3/BS3	pre-HDR	post-HDR
p.Val2908Gly	2.53	PM1	-	-	-	BP4	PM2	-	-	BS3	LBEN	LBEN
p.Ser2522Phe	2.85	PM1	-	-	-	BP4	PM2	-	-	BS3	LBEN	LBEN
p.Leu2972Trp	2.78	PM1	-	-	-	BP4	-	-	-	BS3	VUS	LBEN
p.Gly2584Asp	2.79	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Arg2494Gln	2.79	PM1	-	-	-	BP4	-	-	-	BS3	VUS	LBEN
p.Arg2488Ser	2.96	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Pro2771Leu	2.81	PM1	-	-	-	PP3	PM2	-	-	BS3	VUS	VUS
p.Arg2488Gly	2.92	PM1	-	-	-	BP4	PM2	-	-	BS3	LBEN	LBEN
p.Val2969Met	3.01	PM1	-	-	BP2	BP4	-	-	-	BS3	LBEN	LBEN
p.Arg2787Cys	2.90	PM1	-	-	-	BP4	PM2	-	-	BS3-published[^]	LBEN	LBEN
p.Leu3011Pro	3.12	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Ser2807Leu	3.04	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Arg2973His	3.08	PM1	-	-	-	BP4	-	-	BP_Multifac	BS3	LBEN	LBEN
p.Pro2767Ser	3.05	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Tyr3035Ser	3.10	PM1	-	-	BP2	BP4	-	-	-	BS3	LBEN	LBEN
p.Gln3026Glu	3.06	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Gly2508Ser	3.22	PM1	-	-	BP2	BP4	BS1_Supporting	-	-	BS3	LBEN	LBEN
p.Val3081Ala	3.22	PM1	-	-	-	BP4	PM2	-	-	BS3-published[^]	LBEN	LBEN
p.Pro2608Ala	3.47	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Tyr3049Cys	3.32	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Phe2600Cys	3.33	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Ala2770Asp	3.51	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Cys2646Trp	3.39	PM1	-	-	-	PP3	PM2	-	-	BS3	VUS	VUS
p.Leu2768His	3.40	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Cys2765Gly	3.41	PM1	-	-	-	PP3	PM2	-	-	BS3	VUS	VUS
p.Cys3069Arg	3.52	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN

Variant (p.)	HDR Score	ACMG codes									Classification	
		PM1	PM5	PP1	PM3/BP2	PP3/BP4 (PROVEAN)	BA1/BS1/PM2 (Frequency)	BP5	PP_Multifac/ BP_Multifac	PS3/BS3	pre-HDR	post-HDR
p.Arg2520Pro	3.54	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Phe2794Leu	3.56	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Ile2495Thr	3.59	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Leu2587Phe	3.77	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Tyr3092Cys	3.62	PM1	-	-	-	PP3	-	-	BP_Multifac	BS3	LBEN	LBEN
p.Ser3123Gly	3.72	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Leu2929Trp	3.85	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Phe2873Cys	3.79	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Arg2991His	3.90	PM1	-	-	-	BP4	-	-	-	BS3-published^	LBEN	LBEN
p.Pro2639Ala	3.98	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Thr2662Met	3.89	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Arg2625Lys	3.94	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Glu2769Gln	3.97	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Arg2787His	3.97	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Thr2515Ile	4.22	PM1	-	-	-	BP4	-	-	BP_Multifac	BS3	LBEN	LBEN
p.Asp2679Gly	4.12	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Ile2672Val	4.37	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Pro3039Leu	4.13	PM1	-	-	-	BP4	-	-	-	BS3-published^	LBEN	LBEN
p.Tyr3035Cys	4.16	PM1	-	-	-	BP4	-	-	-	BS3	VUS	LBEN
p.Arg2520Gln	4.23	PM1	-	-	-	BP4	-	-	-	BS3-published^	LBEN	LBEN
p.Gly2901Asp	4.25	PM1	-	-	-	BP4	-	-	-	BS3-published*	LBEN	LBEN
p.Glu3071Asp	4.30	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Ile2490Thr	4.48	PM1	-	-	-	BP4	BA1	-	-	BS3-published^	BEN	BEN
p.Ser2810Gly	4.37	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Arg2888Cys	4.57	PM1	-	-	BP2	BP4	-	-	-	BS3-published*	LBEN	LBEN
p.Leu2774Arg	4.41	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN

Variant (p.)	HDR Score	ACMG codes									Classification	
		PM1	PM5	PP1	PM3/BP2	PP3/BP4 (PROVEAN)	BA1/BS1/PM2 (Frequency)	BP5	PP_Multifac/ BP_Multifac	PS3/BS3	pre-HDR	post-HDR
p.Gly2544Ser	4.43	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Ser2695Leu	4.45	PM1	-	-	BP2	BP4	PM2	-	-	BS3	LBEN	LBEN
p.Leu2865Val	4.45	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Val2728Ala	4.49	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Asp3170Gly	4.73	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Pro2735Arg	4.51	PM1	-	-	-	PP3	PM2	-	-	BS3	VUS	VUS
p.Lys2849Glu	4.56	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Pro2827Ser	4.61	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Asp2665Gly	4.74	PM1	-	-	-	BP4	-	-	-	BS3-published^	LBEN	LBEN
p.Ser2533Cys	4.64	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Gln2561Arg	4.66	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Gly3086Ala	4.73	PM1	-	-	-	BP4	-	-	-	BS3	VUS	LBEN
p.Pro2785Leu	4.79	PM1	-	-	-	PP3	PM2	-	-	BS3	VUS	VUS
p.Ser2704Phe	4.81	PM1	-	-	-	BP4	PM2	-	-	BS3-published*	LBEN	LBEN
p.Gln2858Arg	4.81	PM1	-	-	-	BP4	PM2	BP5	-	BS3	LBEN	LBEN
p.Glu2981Lys	4.86	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Val3079Ile	5.16	PM1	-	-	BP2	BP4	BS1_Supporting	-	-	BS3-published*	LBEN	LBEN
p.Glu3152Lys	4.90	PM1	-	-	-	BP4	-	BP5	-	BS3	LBEN	LBEN
p.Tyr3098His	5.31	PM1	-	-	-	BP4	BS1_Supporting	-	-	BS3-published*	LBEN	LBEN
p.Arg2678Gly	5.12	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Asp2913Glu	4.92	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Ser2806Leu	4.94	PM1	-	-	-	BP4	BS1_Supporting	-	-	BS3	LBEN	LBEN
p.Ala3029Val	4.95	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Ala2756Gly	5.18	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Ser3070Phe	5.00	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Arg2502Cys	5.04	PM1	-	-	BP2	BP4	BS1_Supporting	BP5	-	BS3	LBEN	LBEN
p.Ser2483Asn	5.09	PM1	-	-	BP2	BP4	PM2	-	-	BS3	LBEN	LBEN

Variant (p.)	HDR Score	ACMG codes									Classification	
		PM1	PM5	PP1	PM3/BP2	PP3/BP4 (PROVEAN)	BA1/BS1/PM2 (Frequency)	BP5	PP_Multifac/ BP_Multifac	PS3/BS3	pre-HDR	post-HDR
p.Asp2965His	5.33	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN
p.Asp2712Asn	5.19	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Gln2858Lys	5.23	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Cys3069Gly	5.37	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Ser2697Asn	5.39	PM1	-	-	BP2	BP4	-	-	-	BS3	LBEN	LBEN
p.Lys2950Asn	5.41	PM1	-	-	-	BP4	BS1_Supporting	BP5_Strong	-	BS3	BEN	BEN
p.Ile3107Thr	5.61	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Glu2571Gly	5.50	PM1	-	-	-	BP4	-	-	-	BS3-published^	LBEN	LBEN
p.Asp3112Asn	5.67	PM1	-	-	-	BP4	PM2	-	-	BS3	VUS	LBEN
p.Met2676Thr	5.66	PM1	-	-	-	BP4	PM2	-	-	BS3-published^	LBEN	LBEN

PM1-protein structure; PM5-pathogenic effect for alteration of same residue; PM3-Fanconi anemia phenotype; BP2-healthy biallelic patients; PP1-cosegregation in family; BA1/BS1/PM2-Population Frequency Codes; BP5-Co-occurrence; BS3-published

*: previous classification using BS3/PS3 evidence from HDR assays published by the Couch laboratory; BS3-published

^: previously classified using BS3/PS3 evidence from functional studies by Couch and other laboratories (see Supplementary Table 2). Abbreviations: HDR-homology directed repair; CI-confidence interval; Multifac-Multi-factorial; LPATH-Likely Pathogenic; P-Pathogenic; LBEN-Likely Benign; BEN-Benign; VUS-Variant of Uncertain Significance.