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Clinical implications of VUS reclassification in a single-centre series from application of ACMG/AMP classification rules specified for *BRCA1/2*

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ABSTRACT

Background *BRCA1/2* testing is crucial to guide clinical decisions in patients with hereditary breast/ovarian cancer, but detection of variants of uncertain significance (VUSs) prevents proper management of carriers. The ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) *BRCA1/2* Variant Curation Expert Panel (VCEP) has recently developed *BRCA1/2* variant classification guidelines consistent with ClinGen processes, specified against the ACMG/AMP (American College of Medical Genetics and Genomics/Association for Molecular-Pathology) classification framework.

Methods The ClinGen-approved *BRCA1/2*-specified ACMG/AMP classification guidelines were applied to *BRCA1/2* VUSs identified from 2011 to 2022 in a series of patients, retrieving information from the VCEP documentation, public databases, literature and ENIGMA unpublished data. Then, we critically re-evaluated carrier families based on new results and checked consistency of updated classification with main sources for clinical interpretation of *BRCA1/2* variants.

Results Among 166 VUSs detected in 231 index cases, 135 (81.3%) found in 197 index cases were classified by applying *BRCA1/2*-specified ACMG/AMP criteria: 128 (94.8%) as Benign/Likely Benign and 7 (5.2%) as Pathogenic/Likely Pathogenic. The average time from the first report as 'VUS' to classification using this approach was 49.4 months. Considering that 15 of these variants found in 64 families had already been internally reclassified prior to this work, this study provided 121 new reclassifications among the 151 (80.1%) remaining VUSs, relevant to 133/167 (79.6%) families.

Conclusions These results demonstrated the effectiveness of new *BRCA1/2* ACMG/AMP classification guidelines for VUS classification within a clinical cohort, and their important clinical impact. Furthermore, they suggested a cadence of no more than 3 years for regular review of VUSs, which however requires time, expertise and resources.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The detection of variants of uncertain significance (VUSs) in *BRCA1/2* genes poses challenges in counselling and managing patients with cancer; to face this issue, the ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) consortium formed a ClinGen external expert panel to develop classification criteria for *BRCA1/2* gene variants.

WHAT THIS STUDY ADDS

⇒ This work represents one of the first practical applications of *BRCA1/2* specified ACMG/AMP classification guidelines recently developed by the ENIGMA Variant Curation Expert Panel and demonstrates their operability and effectiveness, yielding a reclassification rate of 81.3% in the series of VUSs reviewed using such criteria. Furthermore, it provides indications about the proper cadence of regular VUSs' review in a clinical context.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The application of these new criteria in the analysed series has a direct clinical impact for 197 families followed in our clinical centre, but its usefulness can be extended to all carriers of the same variants world-wide. Moreover, our findings may be useful to trigger re-evaluation of the importance of these criteria for future iterations of the *BRCA1/2*-specified ACMG/AMP classification criteria.

INTRODUCTION

The analysis of *BRCA1/2* genes to guide clinical decisions has become a well-established practice in clinical genetics and oncology. The detection of a constitutional deleterious variant in one of these genes, which are associated with hereditary breast and ovarian cancer, allows initiation of the most effective surgical and pharmacological management and follow-up plans in patients with cancer, and specific surveillance programmes with possible prophylactic surgery in unaffected pathogenic variant carrier patients, according to the main international guidelines.[1 2]

The classification of a germline variant into one of five classes ('Benign', 'Likely Benign', 'Variant of Uncertain Significance (VUS)', 'Likely Pathogenic' or 'Pathogenic') was introduced by the International Agency for Research on Cancer (IARC) as a means to better link variant classification to clinical

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recommendations.[3] It was considered that carriers of Pathogenic/Likely Pathogenic variants would benefit from specific clinical management, while carriers of Benign/Likely Benign variants and VUSs should be managed as if no (Likely) Pathogenic variant was identified (ie, predictive clinical genetic testing in relatives is discouraged), to minimise miscomprehension and wrong risk perception.[3–8]

Given the increasingly frequent detection of VUSs consequent to the increase in the number and sensitivity of the analyses performed, the interpretation and handling of VUSs is a major issue in modern clinical genetics; it is estimated that about 10–20% of patients undergoing *BRCA* genetic screening will carry a VUS.[9]

To face this issue, the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) consortium (https://enigmaconsortium.org), considered the main reference for interpretation of *BRCA1/2* variants in Italy,[10] formed a ClinGen external expert panel to develop classification criteria for *BRCA1/2* gene variants in 2017 (https://enigmaconsortium.org/wp-content/uploads/2020/08/ENIGMA_Rules_2017-06-29-v2_5_1.pdf). The criteria were mainly based on multifactorial likelihood modelling,[11] together with additional qualitative criteria used in common genetic practice at the time, such as the type of sequence alteration (ie, if nonsense or frameshift variants), the frequency of the variant in general population, the eventual deletion/duplication of one or more exons, etc. Alongside, the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) developed generic guidelines for structured interpretation of sequence variations as pertinent to Mendelian disease,[12] recommendations which have been adopted increasingly outside of the USA. To align with global trends, the former external expert panel restructured as a ClinGen internal *BRCA1/2* Variant Curation Expert Panel (VCEP) to develop ClinGen-approved (https://www.clinicalgenome.org) ACMG/AMP classification guidelines specified for *BRCA1* and *BRCA2* (Classification Criteria V1.0.0 2023-09-08—https://cspec.genome.network/cspec/ui/svi/affiliation/50087). These criteria, approved in August 2023, allow for use of evidence types not formally recognised in the baseline ACMG/AMP criteria,[12] with application of likelihood ratio-based weights as per previous Bayesian modelling of the ACMG/AMP criteria.[13]

In this study, we aimed to reanalyse all the *BRCA1/2* VUSs detected over the past 11 years in our laboratory, using the new ACMG/AMP classification guidelines specified for *BRCA1* and *BRCA2* by the ENIGMA *BRCA1/2* VCEP, to test their efficacy and evaluate the clinical implications for patients.

MATERIALS AND METHODS

Study design

We retrospectively reviewed all the *BRCA1/2* variants that, from 1 October 2011 to 31 December 2022, were identified in our laboratory and reported as VUS in the first proband found to be a carrier. The dataset included variants already reported in a previous study,[14] as well as variants detected after publication of that paper.

For each variant, the classification as VUS was based on the guidelines in use at the time of the first report. Up to 2017, all the variants were evaluated through the retrieval of information available in the following public databases: UMD (http://www.umd.be), BRCA Exchange (https://brcaexchange.org), ARUP Scientific Resource for Research and Education: BRCA Database (https://arup.utah.edu/database/BRCA/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), LOVD IARC (https://databases.lovd.nl/shared/genes/BRCA1) and LOVD3 (https://www.lovd.nl/3.0/home). From 2017, the ENIGMA *BRCA1/2* Gene Variant Classification Criteria (https://enigmaconsortium.org/wp-content/uploads/2020/08/ENIGMA_Rules_2017-06-29-v2_5_1.pdf) were followed, according to Italian Scientific Societies consensus opinion.[10]

Variant classification following new ACMG/AMP classification rules specified for *BRCA1/2*

We reassessed all the VUSs by applying the ClinGen-approved ACMG/AMP classification guidelines specified for *BRCA1* and *BRCA2* by the ENIGMA *BRCA1/2* VCEP, released publicly in 2023 (Classification Criteria V1.0.0 2023-09-08—https://cspec.genome.network/cspec/ui/svi/affiliation/50087).

Briefly, these guidelines use the classic ACMG/AMP criteria with some specifications to adapt them to the characteristics of pathogenic variants in the *BRCA1* and *BRCA2* genes. To each variant, 10 criteria towards pathogenicity (PVS1, PS1, PS3, PS4, PM2, PM3, PM5, PP1, PP3 and PP4) and 9 towards benignity (BA1, BS1, BS2, BS3, BS4, BP1, BP4, BP5 and BP7) can be applied. While some criteria are applied at a fixed weight, others may vary in weight (Supporting, Moderate, Strong or Very Strong) depending on the strength of the evidence on which the application is based. For any criterion, the weight can also be translated into a numerical score based on recommendations of Tavtigian *et al*[15] (Supporting =±1, Moderate =±2, Strong =±4 and Very Strong =±8),[15] where the criteria towards pathogenicity have a positive score, while those towards benignity have a negative score. Once all the applicable criteria with their weights have been assigned to each variant, the variant can be classified into one of the five classes (Pathogenic, Likely Pathogenic, VUS, Likely Benign or Benign) through the combination of the various criteria assigned (standard method), following the predefined combinations indicated in the original published guidelines, with minor modifications in combinations as recommended by Bayesian modelling of the ACMG/AMP guidelines.[13] If both pathogenic and benign criteria are assigned to a variant, the classification is not be assigned using the standard method of combining criteria, but rather by adding the points provided by each criterion (point-based method), as follows: ≤ −7=Benign, −6 to −2=Likely Benign, −1 to 5=VUS, 6–9=Likely Pathogenic, ≥ 10 = Pathogenic.

The information necessary for the assignment of the various criteria was searched for in documentation related to the specifications (including tables and appendices), in public databases, in the literature and, in some instances, in unpublished data collated by members of the ENIGMA consortium. Specifically:

- Data for the assignment of PVS1, PS3, PM5_PTC and BS3 criteria were searched in the tables of the specifications, as recommended (Classification Criteria V1.0.0 2023-09-08).
- Data necessary for the assignment of PS1 and PS4 criteria, as well as BayesDel scores for missense variant impact for bioinformatic predictions criteria, were accessed via the BRCA Exchange database (https://brcaexchange.org).
- For bioinformatic prediction data useful for the assignment of PP3, BP1, BP4 and BP7 criteria, we ran all the variants in the SpliceAI tool splicing predictor[16] and followed the flow chart described in the specifications.
- Frequency data, for the assignment of PMS2, BA1 and BS1 criteria, were taken from the gnomAD v2.1 non-cancer exomes and gnomAD v3.1 non-cancer genomes datasets (https://gnomad.broadinstitute.org).

- For the assignment of PM3 and BS2 criteria, the reported occurrence of the variants included in this work in trans with a *BRCA1* pathogenic variant in patients with or without Fanconi Anaemia phenotype was searched for in the literature and in ENIGMA unpublished data.
- Multifactorial data for the assignment of PP1, PP4, BS4 and PP5 were accessed from an ENIGMA-maintained resource representing an updated version of data from Parsons *et al*.[17]

All the databases and sources were last accessed on 30 April 2023.

After assessment of all the criteria, we classified the variants as 'Benign', 'Likely Benign', 'VUS', 'Likely Pathogenic' and 'Pathogenic' by applying the *BRCA1/2* VCEP rules, following the standard method with limited code combinations permissible,[12 13] and also the point-based method, including for any variants where both benign and pathogenic criteria were met.

Prior to this study, some of the included variants had already been internally reclassified in the light of new evidence emerging in the literature (see the Results section), but we reviewed them anyway with the new criteria to evaluate consistency in classification with the previous reclassification.

Clinical contextualisation

After assignment of the ACMG/AMP-based variant classification, we critically re-evaluated the carrier patients and families in the light of the new results, calculating the potential number of individuals who could clinically benefit from this new information, and the time elapsed from first reporting of each variant as a VUS to eventual reclassification.

Then, we rechecked the eventual classification in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) in order to highlight any classification discrepancies between the main sources for clinical interpretation of *BRCA1/2* variants and those assigned following application of the *BRCA1/2*-specified ACMG/AMP rules.

RESULTS

Dataset description

From 1 October 2011 to 31 December 2022, 3232 index cases who fulfilled criteria for *BRCA* testing according to the regional protocol[18] underwent gene testing at our laboratory: 231 of these (7.2%) were found to carry at least 1 of 166 different variants first reported as VUS (55 in *BRCA1* and 111 in *BRCA2*). Among these, 11 patients were carriers of multiple VUSs (2 *BRCA2* VUSs, 1 patient; 2 *BRCA1* VUSs, 1 patient; 1 *BRCA1* and 1 *BRCA2* VUS, 7 patients; 2 *BRCA2* and 1 *BRCA1* VUS, 1 patient; 1 *BRCA2* and 2 *BRCA1* VUSs, 1 patient), and 10 of a VUS in association with a pathogenic variant in the same or the other *BRCA* gene (same gene in cis in one case; same gene in trans in three cases, one of which also carried a pathogenic variant on the other gene; same gene unknown phase in five cases; other gene in one case). Each VUS was present in 1.46 families on average, 1.27 after excluding the *BRCA1:*c.5017_5019del (p.His1673del) founder variant (present in 32 families and subject of our parallel studies)[19] (Innella *et al* unpublished data) and 142 (85.5%) were found in single families.

Regarding the type of variant, 141 (85.0%) were missense, 13 (7.8%) intronic, 6 (3.6%) indel/in-frame deletions, 5 (3.0%) synonymous and 1 nonsense (0.6%). Among exonic variants, 35/153 (22.9%) were located within a predicted functional domain of the respective gene.

On 30 April 2023, 147 (88.6%) of these variants were present in ClinVar, with 21 (12.7%) reported as 'Benign', 'Likely Benign', 'Likely Pathogenic' or 'Pathogenic' without conflicting interpretations. Prior to this work, 15 of these variants present in 64 families, including the *BRCA1* c.5017_5019del (p.His1673del), had already been internally reclassified in the light of new evidence emerging in the literature, and the clinical report of carriers had been updated.

All features of the variants under study are reported in supplemental table 1 and summarised in table 1.

Table 1 Main features of variants under study

Feature		*BRCA1* NM_007294.4	*BRCA2* NM_000059.4	Total
Variants (n)		55	111	166
Index cases (n)		101*	141*	231*
Type of variant, n (%)	Missense	42 (76.4)	99 (90.0)	141 (85.0)
	Intronic	7 (12.7)	6 (5.4)	13 (7.8)
	Indel/In-frame deletions	3 (5.5)	3 (2.7)	6 (3.6)
	Synonymous	3 (5.5)	2 (1.8)	5 (3.0)
	Nonsense	0 (0.0)	1 (0.9)	1 (0.6)
ClinVar†, n (%)	Benign/Likely Benign	4 (7.3)	13 (11.7)	17 (10.2)
	Pathogenic/ Likely Pathogenic	2 (3.6)	2 (1.8)	4 (2.4)
	VUS/CI/np	39 (79.9)	87 (78.4)	126 (75.9)
	Absent	8 (14.6)	9 (8.1)	19 (11.5)

*Some patients were identified to have variants in both genes; these numbers include 32 index cases carrying *BRCA1:*c.5017_5019del (p.His1673del) founder variant.
†Last accessed: 30 April 2023.
CI, conflicting interpretations of pathogenicity; np, not provided; VUS, variant of uncertain significance.

Reclassification of VUSs following new ACMG/AMP classification rules specified for *BRCA1/2*

Overall, 135/166 variants (81.3%) were classified following the *BRCA1/2*-specified ACMG/AMP classification guidelines: 128 (94.8%) as Benign or Likely Benign and 7 (5.2%) as Pathogenic or Likely Pathogenic. Of those, 79 out of 135 (58.5%) were classified using the standard method with limited code combinations, while the other 56 (41.5%) with the point-based method for the meeting of both Benign and Pathogenic criteria. Classification results are summarised in table 2, family trees of families carrying variants reclassified as Pathogenic/Likely Pathogenic are shown in figure 1, and the assignment of each criterion informing classification for each variant is detailed in Supplementary Table 1.

Clinical implications

The 135 variants that were reclassified following application of the *BRCA1/2*-specified criteria were relevant to 197 index cases. The average time that elapsed from the report as VUS in the first patient to reclassification based on this study was 50.5 months (64.6 for variants reclassified as Pathogenic/Likely Pathogenic, 48.6 for those reclassified as Benign/Likely Benign)—see table 2. Considering the date of internal reclassification for 15 variants, the average time that elapsed from the first report as VUS to the first reclassification was 48.1 months.

Twenty-one out of 166 variants were already reported as Benign, Likely Benign, Likely Pathogenic or Pathogenic without conflicting interpretations in ClinVar, and the

Table 2 Classification of variants under study according to *BRCA1/2*-specified ACMG/AMP classification guidelines

ACMG/AMP classification	Variants, n (%)	Proportion of variants classified with the standard method*	Index cases (n)	Average time from first report (as VUS) to reclassification (months)
Benign	35 (21.1)	31/34 (91.2%)	54	48.6
Likely Benign	93 (56.0)	41/94 (43.6%)	106	
VUS	31 (18.7)	18/31 (58.1%)	37	/
Likely Pathogenic	3 (1.8)	3/3 (100.0%)	3	64.6
Pathogenic	4 (2.4)	4/4 (100.0%)	41†	
Total reclassified	135 (81.3)	79/135 (58.5%)	197‡	49.4

*As per new ACMG/AMP classification guidelines specified for *BRCA1* and *BRCA2*.
†Including 32 carriers of the *BRCA1*:c.5017_5019del (p.His1673del) founder variant.
‡Some patients carry more than one variant.
ACMG/AMP, American College of Medical Genetics and Genomics/Association for Molecular Pathology; VUS, variant of uncertain significance .

consistency with classification assigned in this work was 95.2%. For the 15 variants previously internally reclassified, 11 had been reclassified based on ClinVar reports, and the remaining based on other sources, as summarised in table 3; application of ACMG/AMP criteria downgraded classification for one variant previous reclassified as Pathogenic based on a ClinVar report. In addition, application of the ACMG/AMP criteria provided new reclassifications for 121/151 (80.1%) additional VUSs, relevant to 133/167 (79.6%) additional index cases.

DISCUSSION

Given the important clinical and family implications of the detection of *BRCA1/2* pathogenic variants in patients with cancer,[19] *BRCA1/2* testing is increasingly required in daily clinical practice, leading to an ever more frequent detection of VUSs in these genes, with consequent challenges for counselling and managing patients.[3 4 7] Therefore, one of the major efforts of the international clinical genetics community is developing methods and guidelines to classify these variants.

In this work, we tested the effectiveness of ACMG/AMP classification guidelines specified for *BRCA1* and *BRCA2* recently developed by the ENIGMA *BRCA1/2* VCEP, by applying them to a single institute series of VUSs.

From 2011 to 2022, 166 different *BRCA1/2* variants found in 227 index cases were reported as VUS in our laboratory. Prior to this work, 9.0% of these variants had been reclassified to a tier outside of VUS through ad hoc application of previous methods/guidelines. The application of *BRCA1/2* specified ACMG/AMP criteria performed here resulted in reclassification of 81.3% of variants initially reported as VUS: the vast majority (94.8%) as Benign/Likely Benign, and only 5.2% as Pathogenic/Likely Pathogenic, in line with data from the literature.[8 20–22] Of the 15 variants that had already been reclassified before this study, classification was the same or consistent within a confidence band for 14 (93.2%). A single variant, *BRCA2* c.476-3C>A, had been

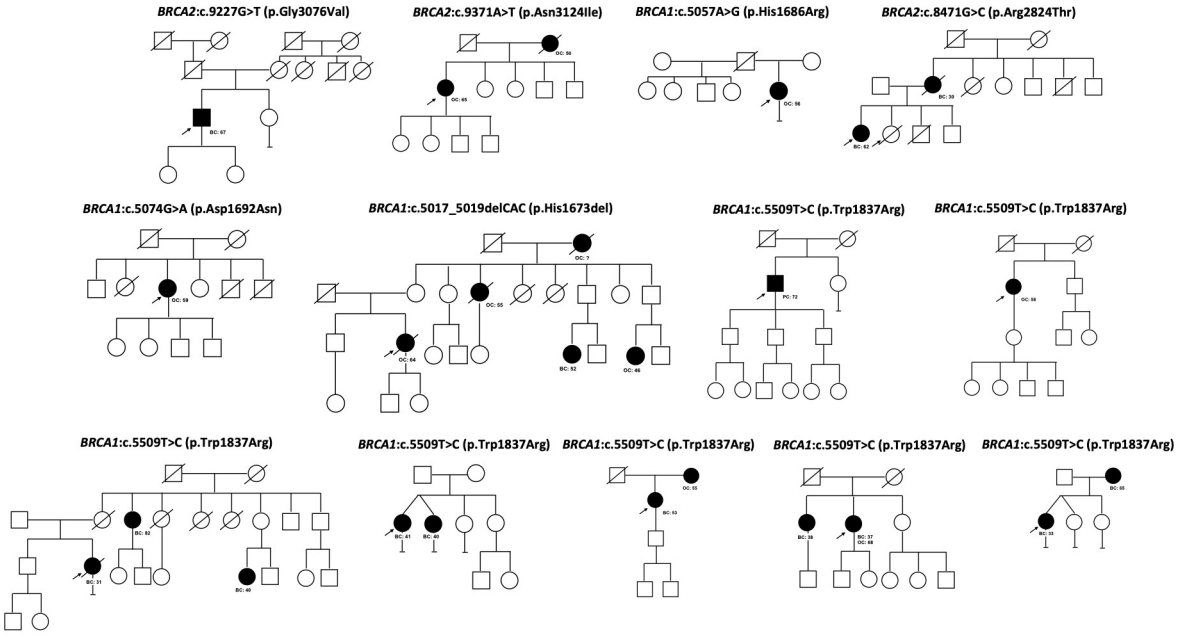

Figure 1 Family trees of families carrying variants reclassified as Pathogenic/Likely Pathogenic. Arrows indicate probands (who are the only ones tested in the family at the time of classification of the respective variants as VUS). Only *BRCA*-related tumours (BC=breast cancer, OC=ovarian cancer, PC=pancreatic cancer) are highlighted (in black). The *BRCA1*:c.5017_5019del (p.His1673del) variant is frequent in our area, so it is present in several of our families and is the subject of our parallel studies[35]; therefore, in this figure we have shown only one tree of a representative family in which it is present.

Table 3 Variants reported as classified by at least one source* prior to this study

Variant†	Previous internal reclassification‡	Source for previous reclassification‡	Clinical significance (ClinVar)§	ACMG/AMP classification¶
BRCA2:c.476-3C>A	P	ClinVar (historical record of pathogenic submission from CIMBA, now removed)	VUS	VUS
BRCA2:c.476-3dup			B	VUS
BRCA2:c.1786G>C (p.Asp596His)	B	ClinVar (ENIGMA submission)	B	B
BRCA2:c.1810A>G (p.Lys604Glu)	B	ClinVar (ENIGMA submission)	B	B
BRCA2:c.2475T>C (p.Asn825=)			LB	LB
BRCA2:c.2755G>A (p.Glu919Lys)	B	ClinVar (ENIGMA submission)	B	B
BRCA2:c.5635G>A (p.Glu1879Lys)	B	Zuntini *et al*[14]	CI	B
BRCA2:c.5671G>A (p.Ala1891Thr)			LB	LB
BRCA2:c.6290C>T (p.Thr2097Met)	B	ClinVar (ENIGMA submission)	B	B
BRCA2:c.7057G>C (p.Gly2353Arg)	B	ClinVar (ENIGMA submission)	B	B
BRCA2:c.7534C>T (p.Leu2512Phe)	B	ClinVar (ENIGMA submission)	B	B
BRCA2:c.8972G>A (p.Arg2991His)			B/LB	B
BRCA2:c.9227G>T (p.Gly3076Val)	LP	ClinVar	P/LP	P
BRCA2:c.9371A>T (p.Asn3124Ile)	P	ClinVar (ENIGMA submission)	P	P
BRCA2:c.9501+3A>T	B	ClinVar (ENIGMA submission)	B	B
BRCA2:c.9502-12T>G	B	ClinVar (ENIGMA submission)	B	B
BRCA2:c.9875C>T (p.Pro3292Leu)			B	B
BRCA1:c.652T>G (p.Leu218Val)			B	LB
BRCA1:c.901A>C (p.Lys301Gln)			LB	LB
BRCA1:c.2589T>G (p.Val863=)			LB	LB
BRCA1:c.3783A>T (p.Leu1261Phe)			LB	LB
BRCA1: c.5017_5019del (p.His1673del)	LP	Zuntini *et al*[35] and Innella *et al* unpublished data	CI	P
BRCA1:c.5057A>G (p.His1686Arg)			P/LP	LP
BRCA1:c.5074G>A (p.Asp1692Asn)	LP	Current literature, internal data	CI	LP
BRCA1:c.5509T>C (p.Trp1837Arg)	P	ClinVar (ENIGMA submission)	P	P
Consistency with ACMG/AMP class **	**93.3%**		**95.2%**	

In GREEN previous classification consistent with ACMG/AMP classification, in RED discrepant classification; B/LB and P/LP were considered together, VUS and CI variants were not considered.
*Including sources considered for eventual reclassification prior to this work and ClinVar.
†*BRCA1* transcript: NM_007294.4; *BRCA2* transcript: NM_000059.4.
‡By our laboratory and subsequently reported to the patients as non-VUS.
§Last accessed: 30 April 2023.
¶This work.
**ACMG.AMP class as assigned from this work; consistency reviewed only for variants classified as B/LB/LP/P previously (internal or in ClinVar) sources.
B, Benign; CI, conflicting interpretation of pathogenicity; CIMBA, Consortium of Investigators of Modifiers of *BRCA*1/2; LB, Likely Benign; LP, Likely Pathogenic; P, Pathogenic; VUS, variant of uncertain significance.

previously managed as Likely Pathogenic in our clinic based on a previous interpretation submitted to ClinVar by the Consortium of Investigators of Modifiers of *BRCA1/2* (CIMBA) and subsequent discussion within the Italian cancer genetics community; this variant was classified as VUS based on our application of ACMG/AMP criteria, consistent with the current ClinVar classification (which now excludes the submission from CIMBA); further investigations of this variant (ie, segregation analysis) are warranted to collect evidence to assess its clinical significance. Of the remaining variants, 121/151 (80.1%) variants present in 133 index cases were reclassified for the first time since initial report, providing new information of relevance for patient and family management.

Although the variants studied here will require formal evaluation by the ENIGMA *BRCA1/2* VCEP to be conclusively reclassified following strict ClinGen-approved VCEP processes, these results show how application of these new classification criteria in a single clinical centre has potential to modify the clinical management of a large proportion of families—197/231 (85.3%) in this series. Given the potentially much higher number of patients who could benefit from this information (relatives of existing probands, and new probands), the results emphasise the significant clinical impact of this reclassification effort. This clinical impact is expected to be greater for smaller proportion of variants upgraded to Pathogenic/Likely Pathogenic, for which carriers can benefit from specific surveillance programmes and the extension of predictive testing to family members,[1 2] as highlighted in family trees shown in figure 1. However, the downgrade of a VUS to Benign/Likely Benign is also very important, because it could reduce anxiety for patients and their relatives and avoid the execution of inappropriate genetic tests and/or surgical procedures.[23–25]

Regarding the agreement of the classification assigned in this work with classification reported in ClinVar (95.2%), *BRCA2*:c.476-3dup variant is reported as Benign in ClinVar based on the interpretation provided by a single submitter (with

neither references nor functional evidence), while with *BRCA1/2*-specified ACMG/AMP criteria one supporting criterion towards benignity (BP4) was applicable and this variant was consequently classified as VUS.

Also, there are other variants in this series that deserve specific consideration:

- *BRCA1*:c.301+6T>C: found in one patient, evidence towards and against pathogenicity was initially identified; functional assay data from a high throughput experiment demonstrating impact for this variant have since been revoked due to discovery of inadvertent introduction of a spliceogenic 'silent' variant as part of the original study design[26]; repeat functional analysis has shown this variant to have no impact on function (https://www.cangene-canvaruk.org/_files/ugd/ed948a_0399a952a1dc4767bed4364a04f6408b.pdf). After excluding the initial functional data report, this variant was classified as Benign using the point-based method, a classification consistent with previous studies.[27 28]
- *BRCA1*:c.4096+1G>A: found in three families, it was once considered as Likely Pathogenic, but later downgraded to VUS by the ENIGMA *BRCA1/2* VCEP since it did not exhibit the clinical characteristics of a standard high-risk pathogenic *BRCA1* variant (ENIGMA unpublished data); according to *BRCA1/2*-specified ACMG/AMP criteria, it remained VUS precisely because it presented contradictory evidence, confirming how non-standard variants make formal classification complicated. Large-scale penetrance studies are likely to provide the most definitive evidence of pathogenicity for suspected reduced penetrance variants like this.
- *BRCA2*:c.8471G>C: found through whole exome sequencing in trans with a pathogenic frameshift variant (c.6468_6469del;p.Gln2157IlefsTer18) in two sisters with a peculiar phenotype (microcephaly, primary amenorrhea, multiple intestinal polyps and multiple tumours),[29] but the patients did not present with the classic Fanconi Anaemia phenotype in childhood as expected for biallelic *BRCA2* pathogenic variants[30]; the fact that the variant was classified as Likely Pathogenic according to *BRCA1/2*-specified ACMG/AMP criteria suggests that it may be a hypomorphic variant that allows residual function of the protein.

Further, this work highlighted which ACMG/AMP criteria are most frequently responsible for contradictory evidence, leading to application of point-based method instead of the standard one for variant classification. The criterion most frequently contradictory to remaining evidence types was PM2 (applied only at supporting level, as per the specifications), met in 52/56 (92.9%) cases classified as Benign or Likely Benign with the point-based method; only two of these variants had additional conflicting evidence towards pathogenicity (PP3 in both cases). For 39 of these variants, there was only one additional criterion met—BP1 applied at strong level, reflecting the convincing evidence against pathogenicity for missense variants located outside of known functional domains by clinical calibration studies.[31] These findings may be useful to trigger re-evaluation of the importance of these criteria for future iterations of the *BRCA1/2*-specified ACMG/AMP classification criteria.

Given the evolution of VUS classification methods and accumulation of new evidence, periodic re-evaluation of VUSs is considered an important practice and is generally recommended, but there is no clear indication on how often to carry it out.[10 32–34] The reclassifications provided by our study emphasise the importance of regular re-evaluation of VUSs by diagnostic laboratories. We have calculated that in our centre the average time from the first detection of a VUS to its first reclassification was 46.7 months, with most VUS undergoing assessment as part of this large-scale research effort. On this basis, we demonstrate that VUS reassessment after 3 years can be beneficial, although ideally should be performed more regularly. Such reclassification effort is a huge workload for the clinical geneticist and/or the diagnostic molecular scientist, with cost to clinically directed activities because it requires much time and a specific expertise. It will thus be important for clinical centres and testing laboratories to provide evidence of the relevance of this activity to obtain adequate recognition and support.

In conclusion, this work demonstrated that the new *BRCA1/2*-specified ACMG/AMP classification guidelines are strongly effective for the classification of VUSs, with potential to significantly impact clinical assessment for a large number of individuals. Furthermore, present results demonstrate need for regular reinterpretation of VUSs, including dedicated support for such activities.

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