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Short report

# Sensitivity and specificity of loss of heterozygosity analysis for the classification of rare germline variants in *BRCA1/2*: results of the observational AGO-TR1 study (NCT02222883)

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## ABSTRACT

Variant-specific loss of heterozygosity (LOH) analyses may be useful to classify *BRCA1/2* germline variants of unknown significance (VUS). The sensitivity and specificity of this approach, however, remains unknown. We performed comparative next-generation sequencing analyses of the *BRCA1/2* genes using blood-derived and tumour-derived DNA of 488 patients with ovarian cancer enrolled in the observational AGO-TR1 trial (NCT02222883). Overall, 94 pathogenic, 90 benign and 24 VUS were identified in the germline. A significantly increased variant fraction (VF) of a germline variant in the tumour indicates loss of the wild-type allele; a decreased VF indicates loss of the variant allele. We demonstrate that significantly increased VFs predict pathogenicity with high sensitivity (0.84, 95% CI 0.77 to 0.91), poor specificity (0.63, 95% CI 0.53 to 0.73) and poor positive predictive value (PPV; 0.71, 95% CI 0.62 to 0.79). Significantly decreased VFs predict benignity with low sensitivity (0.26, 95% CI 0.17 to 0.35), high specificity (1.0, 95% CI 0.96 to 1.00) and PPV (1.0, 95% CI 0.85 to 1.00). Variant classification based on significantly increased VFs results in an unacceptable proportion of false-positive results. A significantly decreased VF in the tumour may be exploited as a reliable predictor for benignity, with no false-negative result observed. When applying the latter approach, VUS identified in four patients can now be considered benign.

**Trial registration number** NCT02222883.

## INTRODUCTION

In cancer genetics, individual risk stratification and the choice of targeted therapies are increasingly dependent on the germline mutation status in disease-associated genes such as *BRCA1* (MIM: 113705) and *BRCA2* (MIM: 600185). Thus, the unambiguous classification of germline variants identified in a routine diagnostic setting is vitally important for the clinical management of the individuals seeking advice. Criteria for *BRCA1/2* germline variant classification were continuously

standardised, in particular through the work of the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA), the International Agency for Research on Cancer (IARC) and the American College of Medical Genetics and Genomics (ACMG).<sup>1–4</sup> While common *BRCA1/2* germline variants with a minor allele frequency (MAF)  $\geq 1\%$  in the general population are considered benign by default, the classification of rare *BRCA1/2* germline variants with a MAF  $< 1\%$  remains challenging, especially for those that cannot be predicted protein-truncating based on their mutation type. For intronic and missense variants, the multifactorial likelihood analysis demonstrated utility for quantitative assessment of variant pathogenicity, a model based on variant location, in silico prediction of variant effect, cosegregation, family cancer history, co-occurrence with a pathogenic variant in the same gene, tumour pathology and case-control information.<sup>5</sup> The multifactorial likelihood analysis, however, requires input data that may not be available for all rare *BRCA1/2* germline variants.

In tumours with a hereditary disease cause, it is generally suggested that the heterozygous germline inactivation of a predisposition gene may be accompanied by a somatic inactivation of the wild-type allele by another deleterious variant, loss of the wild-type allele or promoter methylation.<sup>5</sup> In 473 patients with ovarian cancer (OC (MIM: 167 000)) enrolled in the observational AGO-TR1 trial, we demonstrated that pathogenic germline variants in the *BRCA1/2* genes very rarely associate with deleterious somatic variants or promoter methylation.<sup>6</sup> In OC, more than 80% of the pathogenic *BRCA1/2* germline variants showed significantly increased proportion of reads that support the variant allele (variant allele fractions (VFs)) in the tumour-derived versus blood-derived DNA, indicating loss of the wild-type alleles.<sup>6–8</sup> Based on these findings, it was suggested that loss of heterozygosity (LOH) analyses might be useful to classify rare germline



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variants in the *BRCA1/2* genes.<sup>9 10</sup> For rare germline variants in (candidate) cancer predisposition genes showing significantly increased VFs in the tumour, a potential role in cancer susceptibility was frequently suggested, as for example in the analyses of 429 patients with OC included in The Cancer Genome Atlas (TCGA) project.<sup>7</sup> However, sensitivity along with specificity of LOH analyses for germline variant classification was not assessed so far. Thus, our study aims to quantify the sensitivity and the specificity of LOH analyses and their potential benefit for the classification of rare *BRCA1/2* germline variants in a well-characterised study sample of 488 patients with OC enrolled in the observational AGO-TR1 trial (NCT02222883).

## METHODS

### Study sample

A total of 523 consecutive patients with invasive epithelial OC were enrolled. All patients were older than 18 years at study inclusion and provided written informed consent prior to enrolment. Venous blood samples were available from all 523 patients and formalin-fixed paraffin-embedded (FFPE) tumour samples were available from 496 patients. Genomic DNA was isolated from blood samples and from FFPE tumour samples as described previously.<sup>6</sup> Briefly, for the isolation of DNA from FFPE tumour samples, H&E-stained 3 µm tissue sections were centrally investigated (Institute of Pathology, University Hospital Bonn, Bonn, Germany); that is, tumour areas containing >80% tumour nuclei were chosen for DNA isolation (see online supplemental materials and methods for details).

### Next-generation sequencing (NGS)

Targeted NGS of blood and tumour samples of 496 patients was performed using a customised gene panel covering the coding regions and exon-flanking sequences ( $\pm 15$  nt) of the *BRCA1* (NM\_007294.3) and *BRCA2* (NM\_000059.3) genes.<sup>6</sup> The hybridisation capture-based NGS method (Agilent SureSelect XT protocol optimised for 200 ng of genomic DNA) was suitable for the analysis of DNA derived from either blood or FFPE tumour samples. Sequencing was performed on a HiSeq4000 device (Illumina, San Diego, California, USA). NGS analyses with a mean read coverage of at least 100× were considered successful. NGS data derived from both blood and corresponding FFPE tumour samples of 488 individuals achieved this threshold. The clinical characteristics of the 488 individuals were described in the online supplemental table 1. For the 488 individuals included, the mean read coverage was 455× (range 171×–882×) for NGS of blood-derived DNA and 570× (range 110×–1802×) for tumour-derived DNA. Bioinformatic analyses, including variant calling, were carried out using the VARBANK V.2.10–2.24 pipeline of the Cologne Center for Genomics and the DDM1 platform (Sophia Genetics, Saint-Sulpice, Switzerland).

### Germline variant classification

We employed criteria based on the ENIGMA and ACMG Guidelines for variant classification.<sup>4</sup> Rare variants were defined as variants with a MAF <1% in large outbred control reference groups. Common variants with a MAF above this threshold were generally considered benign and excluded from this investigation. All rare variants in splice regions and non-synonymous single-nucleotide/indel variants were included in this investigation. CNVs were not considered. To determine MAFs, we used Exome Aggregation Consortium (ExAC)<sup>11</sup> data of individuals of European, non-Finnish ancestry, excluding samples from TCGA. All rare *BRCA1/2* germline variants were classified using

a five-tier variant classification system as proposed by the IARC Unclassified Genetic Variants Working Group,<sup>12</sup> namely, pathogenic=class 5, likely pathogenic=class 4, variant of uncertain significance (VUS)=class 3, likely benign=class 2 and benign=class 1. For reasons of clarity, class 4/5 are referred to as pathogenic variants and class 1/2 variants as benign variants in the following.

### LOH analysis

VFs were derived from VARBANK VCF files by division of the number of reads showing the variant allele and the observed read depth. Fold changes, that is, the ratio of tumour and blood VFs, were computed for each rare germline variant. Fisher's exact test was applied to assess the significance level of deviating proportions of reads showing a variant allele between blood and tumour sample, with p values <0.05 after correction for multiple testing using the Benjamini-Hochberg approach<sup>13</sup> considered significant. A significantly increased VF of a variant in the tumour suggests loss of the wild-type allele. A significantly decreased VF of a variant in the tumour suggests loss of the variant allele. Statistical analyses were performed using SPSS Statistics V.25 and the epiR-Package under R V.3.6.2.

### Web resources

OMIM: <http://www.omim.org/>

ClinVar: <https://www.ncbi.nlm.nih.gov/clinvar/>

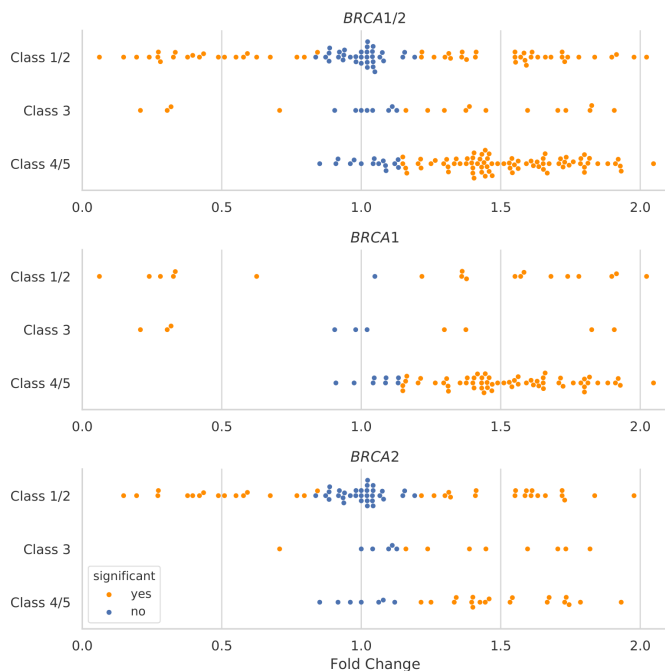
ENIGMA: <https://enigmaconsortium.org/>

## RESULTS

Germline analysis revealed 208 rare variants in 181 of the 488 patients (37.1%). One hundred and fifty-seven patients carried one (32.2%), 21 carried two (4.3%) and 3 patients carried three rare germline variants (0.6%) (online supplemental figure 1A). Of the 208 rare variants, 94 were pathogenic (class 4/5), 90 were benign (class 1/2) and 24 were of unknown significance (VUS, class 3). The combined *BRCA1/2* genotypes of the 181 patients with rare variants are illustrated in online supplemental figure 1B). All rare variants were listed in the online supplemental table 2).

All rare germline variants were also detected in the corresponding tumour samples. Of the 94 pathogenic germline variants, 79 (84.0%) showed significantly increased VF in the tumour suggesting loss of the wild-type alleles, with fold changes ranging from 1.15 to 2.05 (figure 1, online supplemental table 2). The VF differences of the remaining 15 class 4/5 variants (16%) were statistically not significant with fold changes ranging from 0.85 to 1.13. Of note, none of the class 4/5 variants showed a significantly decreased VF in the tumour. Of the 90 class 1/2 variants, 33 (36.7%) showed significantly increased VFs in the tumour with fold changes ranging from 1.22 to 2.02, 34 showed non-significant differences (37.8%, fold changes ranging from 0.87 to 1.16) and for the remaining 23 variants, VFs were significantly decreased in tumour samples (25.6%, fold changes ranging from 0.06 to 0.84) (figure 1, online supplemental table 2).

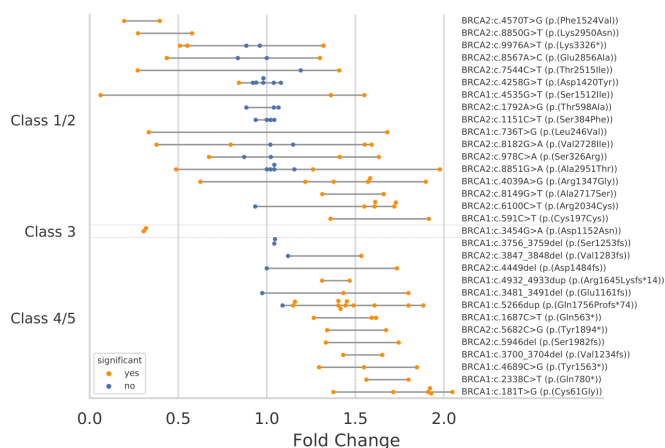
Variant classification based on significantly increased VFs shows a high sensitivity of 0.84 (95% CI 0.77 to 0.91), but a poor specificity of 0.63 (95% CI 0.53 to 0.73) and a poor positive predictive value (PPV) of 0.71 (95% CI 0.62 to 0.79). For this approach, the positive likelihood ratio (LR+) for pathogenicity is 2.29 (95% CI 1.72 to 3.05). Briefly, variant classification based on significantly increased VFs is hampered by the random distribution of VFs observed for benign variants. At least in a routine diagnostic setting, classification of rare *BRCA1/2*



**Figure 1** Fold change of VF between blood-derived and tumour-derived DNA observed for 208 class 4/5, class 3 and class 1/2 germline variants in the *BRCA1/2* genes observed in the overall study sample. Significant differences in VFs between blood-derived and tumour-derived DNA were indicated by orange dots. Non-significant differences in VFs between blood-derived and tumour-derived DNA were indicated by blue dots. VFs, variant fraction.

germline variants may not be based on significantly increased VFs due to an unacceptable proportion of false-positive results.

As an alternative approach, a significantly decreased VF of a variant in the tumour, suggesting loss of the variant allele, may be useful to classify a rare *BRCA1/2* germline variant as benign. Significantly decreased VFs were specific for benign variants and were not observed for pathogenic germline variants (figure 1).



**Figure 2** Fold change of VF observed for 31 recurrent class 4/5, class 3 and class 1/2 germline variants in the *BRCA1/2* genes observed in the overall study sample. Significant differences in VFs between blood-derived and tumour-derived DNA were indicated by orange dots. Non-significant differences in VFs between blood-derived and tumour-derived DNA were indicated by blue dots. VFs, variant fractions.

Of the benign variants observed in 90 patients, 17 were recurrent and found at least twice in the sample set. For most of the recurrent benign variants, we found a high variability of fold changes, occasionally ranging from a significant decrease to a significant increase (figure 2). Classification of benign *BRCA1/2* germline variants based on significantly decreased VFs results in a low sensitivity of 0.26 (95% CI 0.17 to 0.35) but a high specificity of 1.0 (95% CI 0.96 to 1.00) and a high PPV of 1.0 (95% CI 0.85 to 1.00). For this approach, the LR+ for benignity was 49.07 (95% CI 3.02 to 795.93) after Haldane-Anscombe correction (online supplemental table 3). A significantly decreased VF of a variant in the tumour may be exploited as a reliable predictor for benignity, with no false-negative result observed. This also holds true when analysis were performed for both genes separately (figure 1, online supplemental table 3). When applying this approach to the 24 VUS identified in our study sample, three distinct VUS found in four patients, that is, *BRCA1* p.(Val525Ile), *BRCA1* p.(Asp1152Asn) and *BRCA2* p.(Lys2498del), may be considered benign (figure 2).

**DISCUSSION**

It was controversially discussed whether the results of LOH analyses may be useful for the classification of rare *BRCA1/2* germline variants.<sup>7–10 14–18</sup> Information from LOH analyses has not been implemented in the current ENIGMA variant classification system<sup>3 19</sup> based on the previously published data<sup>16</sup> suggesting that LOH analyses are not sufficiently reliable. Using paired analyses of blood-derived and tumour-derived DNA, we demonstrated that rare germline variants in the *BRCA1/2* genes might be classified benign based on significantly decreased VFs in the tumour. This approach reached a specificity of 1.0 (95% CI 0.96 to 1.00), a PPV of 1.0 (95% CI 0.85 to 1.00) and a LR+ of 49.07 (95% CI 3.02 to 795.93). Given the fact that changes in VFs of benign variants occur randomly (figure 2), this approach shows a limited sensitivity of only 0.26 (95% CI 0.17 to 0.36). As of March 2020, more than 6.100 distinct *BRCA1/2* germline VUS were listed in the ClinVar database, indicating the need for additional sources for the classification of *BRCA1/2* germline variants. We suggest that large-scale comparative germline/tumour NGS analyses with sufficient read depths may significantly reduce the number of VUS, especially for VUS for which data regarding cosegregation, family cancer history, co-occurrence with a pathogenic variant in the same gene and case-control information are not available.<sup>3</sup>

**Limitations of the study**

In the overall study sample of patients with OC enrolled in the observational AGO-TR1 study, pathogenic germline mutations in non-*BRCA1/2* OC predisposition genes such as *RAD51C/D* and *BRIP1* were observed. However, the prevalence of pathogenic germline mutations in these genes was too low to perform meaningful calculations. Larger studies are required to quantify the sensitivity and the specificity of LOH analyses for the classification of rare germline mutations in additional OC predisposition genes. Moreover, this investigation was focused on patients with OC and FFPE samples with a high tumour content. It remains elusive to which extent our approach may be transferred to breast tumour analyses that are usually associated with lower *BRCA1/2* LOH rates<sup>20</sup> and probably lower tumour contents in FFPE samples.

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#### REFERENCES

- Maxwell KN, Hart SN, Vijai J, Schrader KA, Slavin TP, Thomas T, Wubbenhorst B, Ravichandran V, Moore RM, Hu C, Guidugli L, Wenz B, Domchek SM, Robson ME, Szabo C, Neuhausen SL, Weitzel JN, Offit K, Couch FJ, Nathanson KL. Evaluation of ACMG-Guideline-Based variant classification of cancer susceptibility and Non-Cancer-Associated genes in families affected by breast cancer. *Am J Hum Genet* 2016;98:801–17.
- Nykamp K, Anderson M, Powers M, Garcia J, Herrera B, Ho Y-Y, Kobayashi Y, Patil N, Thusborg J, Westbrook M, Topper S, Invitae Clinical Genomics Group. Sherloc: a comprehensive refinement of the ACMG-AMP variant classification criteria. *Genet Med* 2017;19:1105–17.
- Parsons MT, Tudini E, Li H, Hahnen E, Wappenschmidt B, Feliubadaló L, Aalfs CM, Agata S, Aittomäki K, Alducci E, Alonso-Cerezo MC, Arnold N, Auber B, Austin R, Azzollini J, Balmaña J, Barbieri E, Bartram CR, Blanco A, Blümcke B, Bonache S, Bonanni B, Borg Åke, Bortesi B, Brunet J, Bruzone C, Bucksch K, Cagnoli G, Caldes T, Caliebe A, Caligo MA, Calvello M, Capone GL, Caputo SM, Carnevali I, Carrasco E, Caux-Moncoutier V, Cavalli P, Cini G, Clarke EM, Concolino P, Cops EJ, Cortesi L, Couch FJ, Darder E, de la Hoya M, Dean M, Debatin I, Del Valle J, Delnatte C, Derive N, Diez O, Ditsch N, Domchek SM, Dutranon V, Eccles DM, Ehrencrona H, Enders U, Evans DG, Farra C, Faust U, Felbor U, Feroce I, Fine M, Foulkes WD, Galvao HCR, Gambino G, Gehrig A, Gensini F, Gerdes A-M, Germani A, Giesecke J, Gismondi V, Gómez C, Gómez García EB, González S, Grau E, Grill S, Gross E, Guerrieri-Gonzaga A, Guillaud-Bataille M, Gutiérrez-Enríquez S, Haaf T, Hackmann K, Hansen TV, Harris M, Hauke J, Heinrich T, Hellebrand H, Herold KN, Honisch E, Horvath J, Houdayer C, Hübbel V, Iglesias S, Izquierdo A, James PA, Janssen LAM, Jeschke U, Kaulfuß S, Keupp K, Kiechle M, Kölbl A, Krieger S, Kruse TA, Kvist A, Lalloo F, Larsen M, Lattimore VL, Lauthrup C, Ledig S, Leinert E, Lewis AL, Lim J, Loeffler M, López-Fernández A, Lucci-Cardisco E, Maass N, Manoukian S, Marabelli M, Matricardi L, Meindl A, Michelli RD, Moghadas S, Moles-Fernández A, Montagna M, Montalban G, Monteiro AN, Montes E, Mori L, Moserle L, Müller CR, Mundhenke C, Naldi N, Nathanson KL, Navarro M, Nevanlinna H, Nichols CB, Niederacher D, Nielsen HR, Ong K-R, Pachter N, Palmero El, Papi L, Pedersen IS, Peissel B, Perez-Segura P, Pfeifer K, Pineda M, Pohl-Rescigno E, Poplawski NK, Porfirio B, Quante AS, Ramser J, Reis RM, Revillion F, Rhiem K, Riboli B, Ritter J, Rivera D, Rofes P, Rump A, Salinas M, Sánchez de Abajo AM, Schmidt G, Schoenwiese U, Seggewiß J, Solanes A, Steinemann D, Stiller M, Stoppa-Lyonnet D, Sullivan KJ, Susman R, Sutter C, Tavtigian SV, Teo SH, Teulé A, Thomassen M, Tibiletti MG, Tischkowitz M, Tognazzo S, Toland AE, Tornero E, Törngren T, Torres-Esquius S, Toss A, Trainer AH, Tucker KM, van Asperen CJ, van Mackelenbergh MT, Varesco L, Vargas-Parra G, Varon R, Vega A, Velasco Ángela, Vesper A-S, Viel A, Vreeswijk MPG, Wagner SA, Waha A, Walker LC, Walters RJ, Wang-Gohrke S, Weber BHF, Weichert W, Wieland K, Wiesmüller L, Witzel I, Wöckel A, Woodward ER, Zachariae S, Zampiga V, Zeder-Göb C, Lázaro C, De Nicolo A, Radice P, Engel C, Schmutzler RK, Goldgar DE, Spurdle AB, KConFab Investigators. Large scale multifactorial likelihood quantitative analysis of *BRCA1* and *BRCA2* variants: An ENIGMA resource to support clinical variant classification. *Hum Mutat* 2019;40:1557–78.
- Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of medical genetics and genomics and the association for molecular pathology. *Genet Med* 2015;17:405–23.
- Knudson AG. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 1971;68:820–3.
- Hauke J, Hahnen E, Schneider S, Reuss A, Richters L, Kommos S, Heimbach A, Marmé F, Schmidt S, Prieske K, Gevensleben H, Burges A, Borde J, De Gregorio N, Nürnberg P, El-Balat A, Thiele H, Hilpert F, Altmüller J, Meier W, Dietrich D, Kimmig R, Schoemig-Markiefka B, Kast K, Braicu E, Baumann K, Jackisch C, Park-Simon T-W, Ernst C, Hunker L, Pfisterer J, Schnelzer A, du Bois A, Schmutzler RK, Harter P. Deleterious somatic variants in 473 consecutive individuals with ovarian cancer: results of the observational AGO-TR1 study (NCT02222883). *J Med Genet* 2019;56:574–80.
- Kanchi KL, Johnson KJ, Lu C, McLellan MD, Leiserson MDM, Wendt MC, Zhang Q, Koboldt DC, Xie M, Kandoth C, McMichael JF, Wyczalkowski MA, Larson DE, Schmidt HK, Miller CA, Fulton RS, Spellman PT, Mardis ER, Druley TE, Graubert TA, Goodfellow PJ, Raphael BJ, Wilson RK, Ding L. Integrated analysis of germline and somatic variants in ovarian cancer. *Nat Commun* 2014;5:3156.

- 8 Kechin AA, Boyarskikh UA, Ermolenko NA, Tyulyandina AS, Lazareva DG, Avdalyan AM, Tyulyandin SA, Kushlinskii NE, Filipenko ML. Loss of heterozygosity in BRCA1 and BRCA2 genes in patients with ovarian cancer and probability of its use for clinical classification of variations. *Bull Exp Biol Med* 2018;165:94–100.
- 9 Osorio A, de la Hoya M, Rodríguez-López R, Martínez-Ramírez A, Cazorla A, Granizo JJ, Esteller M, Rivas C, Caldés T, Benítez J. Loss of heterozygosity analysis at the BRCA loci in tumor samples from patients with familial breast cancer. *Int J Cancer* 2002;99:305–9.
- 10 Osorio A, Milne RL, Honrado E, Barroso A, Diez O, Salazar R, de la Hoya M, Vega A, Benítez J. Classification of missense variants of unknown significance in BRCA1 based on clinical and tumor information. *Hum Mutat* 2007;28:477–85.
- 11 Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, Tukiainen T, Birnbaum DP, Kosmicki JA, Duncan LE, Estrada K, Zhao F, Zou J, Pierce-Hoffman E, Berghout J, Cooper DN, DeFlaux N, DePristo M, Do R, Flannick J, Fromer M, Gauthier L, Goldstein J, Gupta N, Howrigan D, Kiezun A, Kurki MI, Moonshine AL, Natarajan P, Orozco L, Peloso GM, Poplin R, Rivas MA, Ruano-Rubio V, Rose SA, Ruderfer DM, Shakir K, Stenson PD, Stevens C, Thomas BP, Tiao G, Tusie-Luna MT, Weisburd B, Won H-H, Yu D, Altshuler DM, Ardissino D, Boehnke M, Danesh J, Donnelly S, Elosua R, Florez JC, Gabriel SB, Getz G, Glatt SJ, Hultman CM, Kathiresan S, Laakso M, McCarroll S, McCarthy MI, McGovern D, McPherson R, Neale BM, Palotie A, Purcell SM, Saleheen D, Scharf JM, Sklar P, Sullivan PF, Tuomilehto J, Tsuang MT, Watkins HC, Wilson JG, Daly MJ, MacArthur DG, Exome Aggregation Consortium. Analysis of protein-coding genetic variation in 60,706 humans. *Nature* 2016;536:285–91.
- 12 Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, Hogervorst FBL, Hoogerbrugge N, Spurdle AB, Tavtigian SV, IARC Unclassified Genetic Variants Working Group. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. *Hum Mutat* 2008;29:1282–91.
- 13 Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J of the Soc Series B* 1995;57:289–300.
- 14 Walsh MF, Ritter DI, Kesserwan C, Sonkin D, Chakravarty D, Chao E, Ghosh R, Kemel Y, Wu G, Lee K, Kulkarni S, Hedges D, Mandelker D, Ceyhan-Birsoy O, Luo M, Drazer M, Zhang L, Offit K, Plon SE. Integrating somatic variant data and biomarkers for germline variant classification in cancer predisposition genes. *Hum Mutat* 2018;39:1542–52.
- 15 Ellard S, Baple EL, Owens M, Eccles DM, Abbs S, Deans ZC, Newman WG, McMullan DJ. *ACGS best practice guidelines for variant classification 2017: ACGS guidelines*, 2017.
- 16 Chenevix-Trench G, Healey S, Lakhani S, Waring P, Cummings M, Brinkworth R, Deffenbaugh AM, Burbidge LA, Pruss D, Judkins T, Scholl T, Bekessy A, Marsh A, Lovelock P, Wong M, Tesoriero A, Renard H, Southey M, Hopper JL, Yannoukakos K, Brown M, Easton D, Tavtigian SV, Goldgar D, Spurdle AB, kConFab Investigators. Genetic and histopathologic evaluation of BRCA1 and BRCA2 DNA sequence variants of unknown clinical significance. *Cancer Res* 2006;66:2019–27.
- 17 Arason A, Agnarsson BA, Johannesdottir G, Johannsson OT, Hilmarsdottir B, Reynisdottir I, Barkardottir RB. The BRCA1 c.4096+3A>G variant displays classical characteristics of pathogenic BRCA1 mutations in hereditary breast and ovarian cancers, but Still allows homozygous viability. *Genes* 2019;10:882.
- 18 Spearman AD, Sweet K, Zhou X-P, McLennan J, Couch FJ, Toland AE. Clinically applicable models to characterize BRCA1 and BRCA2 variants of uncertain significance. *J Clin Oncol* 2008;26:5393–400.
- 19 Spurdle AB, Lakhani SR, Healey S, Parry S, Da Silva LM, Brinkworth R, Hopper JL, Brown MA, Babikyan D, Chenevix-Trench G, Tavtigian SV, Goldgar DE. Clinical classification of BRCA1 and BRCA2 DNA sequence variants: the value of cytokeratin profiles and evolutionary analysis—a report from the kConFab investigators. *JCO* 2008;26:1657–63.
- 20 Maxwell KN, Wubbenhorst B, Wenz BM, De Sloover D, Pluta J, Emery L, Barrett A, Kraya AA, Anastopoulos IN, Yu S, Jiang Y, Chen H, Zhang NR, Hackman N, D'Andrea K, Daber R, Morrisette JJD, Mitra N, Feldman M, Domchek SM, Nathanson KL. Brca locus-specific loss of heterozygosity in germline BRCA1 and BRCA2 carriers. *Nat Commun* 2017;8:319.