Hereditary breast cancer and the clinical significance of variants in the BRCA1 and BRCA2 genes

Moghadasi, S.

Variants of Uncertain Significance in *BRCA1* and *BRCA2;* assessment of in silico analysis and a proposal for communication in genetic counselling

S. Moghadasi, N. Hofland, J.N. Wouts, F.B.L. Hogervorst, J.T. Wijnen, M.P.G. Vreeswijk, C.J. van Asperen

J Med Genet. 2013 Feb;50(2):74-9.

2

ABSTRACT

Background

Nearly 15% of *BRCA1* and *BRCA2* DNA tests lead to the identification of Variants of Uncertain Significance (VUS). VUS are classified in the Netherlands according to the Bell system and it is current practice that class III VUS are communicated to counsellees, but not class II or lower VUS. Our aims were to investigate the utility of in silico characteristics in the classification of VUS and whether initial VUS classifications justify differences in communication protocols during counselling.

Methods

We classified 88 missense VUS in *BRCA1* and *BRCA2* on the basis of an in silico analysis and compared the classification of a subset of 60 VUS of which additional information including family, genetic and tumour data was available.

Results

VUS allocated to class III more frequently showed in silico indications of a deleterious effect than class II VUS. Of the 46 VUS assigned to class II by in silico analysis alone, nearly half were eventually recategorised as class I and 10% as class III when additional information was included.

Conclusions

As in silico analysis alone is not always sufficient to unambiguously assign VUS to either class II or class III, we would argue that the prospect of obtaining additional information from a family should be given more weight during the decision process preceding the communication of a VUS test result. Research initiatives such as the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA), which strive to combine diverse sources of information, will be valuable in aiding a definitive classification of a VUS.

INTRODUCTION

The ongoing development of sequence-based technologies in DNA diagnostic laboratories is resulting in the detection of an increasing number of variants of unknown clinical significance. These variants, referred to as Variants of Uncertain Significance (VUS), include missense changes, small in-frame deletions or insertions, non-synonymous nucleotide substitutions, as well as alterations in non-coding sequences or in untranslated regions.

Around 15% of DNA tests of the *BRCA1* and *BRCA2* genes result in the identification of VUS, and almost 1800 unique VUS are currently listed in the Breast Cancer Information Core database (http://research.nhgri.nih.gov/bic/) (accessed 4 Apr 2012).[1]

In the Netherlands, over 1800 families are now known to carry a *BRCA1* and *BRCA2* VUS (National working group for Breast Cancer DNA Diagnostics (LOB)). These families experience considerable psychological distress, due to the possibility that they may face a cancer risk as high as that for known pathogenic mutations, and due to the uncertainty surrounding this risk.[2, 3]

Interpretation of VUS with respect to predicted effect on protein function, and thus on the estimated cancer risk in the families, has become a major challenge when tailoring genetic counselling and disease prevention strategies. As genetic counsellors need to be able to communicate a meaningful VUS DNA test outcome and possible consequences in a careful and understandable way to the counsellees and their families, it is essential that specialists in DNA diagnostic laboratories give a clear and objective estimation of the probability of pathogenicity for each VUS.

A variety of methods have been developed to determine whether a given variant is pathogenic or is of little or no clinical significance.[4–6] Functional studies assess the impact of genetic variants on the activity of the protein in vitro. Some methods measure a direct association of the variant with disease, and include cosegregation of the variant with disease in a family,[7] family history,[8] co-occurrence of the variant with pathogenic *BRCA1* and *BRCA2* mutations on the second allele[9] and analysis of the tumour DNA (eg, loss of heterozygosity and array comparative genomic hybridisation analysis).[10] In silico approaches predict the consequences of DNA sequence changes in an indirect manner based on evolutionary nucleotide and amino acid conservation, the possible effect of amino acid substitutions on protein structure or the predicted effect on mRNA (messenger RNA) splicing.

In 2007, the Dutch and British societies for clinical molecular genetics proposed 'Good Practice Guidelines for the Interpretation and Reporting of Unclassified Variants in Clinical Molecular Genetics Laboratories'.[11] A four-class system was described, with increasing probability of pathogenicity (class I to IV). This was followed by a suggested classification into five groups (table 1), by Plon et al in 2008.[12]

The communication of a VUS to a counsellee often results in feelings of uncertainty, distress and a possible decision to undergo prophylactic surgery.[2, 3] As the prior probability that a VUS will be deleterious is less than 10%,[5] laboratory personnel in the Netherlands

Table 1. Four-class system according to Bell et al.[11], compared to the five-class system proposed by Plon et al. [12]

Class (Bell)	Description	Class (Plon)	Description	Probability of pathogenicity
I	Certainly not pathogenic	1	Not pathogenic or of no clinical significance	<0.001
		2	Likely not pathogenic or of little clinical significance	0.001-0.049
II	Unlikely to be pathogenic but cannot be formally proven	3	Uncertain	0.05-0.949
III	Likely to be pathogenic but cannot be formally proven			
IV	Certainly pathogenic	4	Likely pathogenic	0.95-0.99
		5	Definitely pathogenic	>0.99

show understandable reservations regarding the communication of the discovery of a VUS to the counsellor, as does the counsellor when communicating with the counsellee.

Each newly identified VUS is first categorised using in silico tools. Class II categorised VUS are communicated to the counsellors, but are not generally revealed to the counsellees. A class III VUS, which is more likely to be pathogenic, is communicated to the counsellees and if possible, additional studies are performed to obtain a more accurate assessment of pathogenicity (eg, cosegregation and RNA analysis). Risk estimates and surveillance policies for class II and class III VUS are generally based on family cancer history, and predictive DNA testing is not offered to the family members.[13] The distinction between class II and class III VUS is a frequent topic of debate in the Netherlands, and since allotment of a VUS to either class II or class III involves a distinct communication protocol during counselling, objective assessment of the VUS is crucial.

The aim of this study was to investigate whether VUS classified in class II and III by the LOB working group show significant differences in in silico characteristics, and thus whether current counselling protocols with respect to initial communication with the counsellees are justified.

MATERIALS AND METHODS

Family data and mutation analysis

High-risk breast and ovarian cancer families were tested for nucleotide variants in *BRCA1* and *BRCA2* when the prior probability of detecting a disease-causing mutation was about 10% or more,[14] or when breast cancer was diagnosed at a relatively young age (<36 years of age), irrespective of a family history of breast cancer.

Denaturing Gradient Gel Electrophoresis or High Resolution Melting Curve Analysis were used as mutation-scanning methods, followed by confirmation of aberrant samples by Sanger sequencing or direct Sanger sequencing and Multiplex Ligation-dependent Probe Amplification.

Selection of VUS

In the Netherlands, about 800 unique VUS have been identified in the *BRCA1* and *BRCA2* in a total of 1800 families. At Leiden University Medical Centre (LUMC) there are 216 families in whom 172 unique VUS have been identified between 2002 and 2010. Of these 172 variants, 88 were missense variants and our analysis was focused on those variants.

Classification of VUS

The four-class system developed by Bell is employed at the LUMC, as is the case for most Dutch and Belgian DNA diagnostic labs (table 1).[11] These laboratories are united in the LOB. Members of this group classify VUS identified in their centre using in silico data and literature searches and regularly enter VUS in a central database. Yearly meetings allow inconsistencies in classification between labs to be discussed and general agreement to be reached. VUS may eventually be reclassified based on additional data including family history, cosegregation with disease in a family, co-occurrence with a pathogenic mutation, tumour DNA analysis and functional studies. Among the 88 missense VUS which were identified at the LUMC, additional information was available for 60 VUS (see online supplementary table).

In silico analysis of the VUS was performed using Alamut mutation interpretation software (http://www.interactive-biosoftware. com/alamut.html) (accessed 4 Apr 2012). Alamut can predict the severity of amino acid substitutions by integrating nucleotide and amino acid conservation, by cross-species alignment using PhastCons scores, with other prediction methods including the Grantham score,[15] Sorting Intolerant From Tolerant (SIFT) (http:// blocks.fhcrc.org/sift/SIFT.html) (accessed 4 Apr 2012), and Align-Grantham Variation with Grantham Deviation (A-GVGD) (http://agvgd.iarc.fr) (accessed 4 Apr 2012). Alamut estimates nucleotide conservation by comparing the majority of available published sequences and the functional domains of *BRCA1* and *BRCA2*. PhastCons scores for nucleotide conservation were calculated by Alamut and VUS-PhastCons scores higher than 0.9 were considered to be strongly conserved, those with a score of 0.5–0.9 to be moderately conserved and a score of <0.5 was taken as an indication of weak conservation.[16] Amino acid conservation was based on cross-species alignments. Residues conserved in primates and other mammals were regarded as weakly conserved. Moderate conservation was assigned to amino acids conserved in birds, whereas amino acids conserved in tetraodon (puffer fish) were classified as strongly conserved (see online supplementary data).

In this study, all VUS were classified by the same molecular geneticist (JTW), based on the outcome of in silico analysis. Variants not tolerated by SIFT-analysis, with a relatively high Grantham score (>100) and a high A-GVGD score (C35–C65) were categorised in class III.[17] Variants were classified in class II when they showed 1) low Grantham score (<100), low A-GVGD score (C0–C25) and irrespective of the outcome of the SIFT-analysis or 2) the in silico programmes showed contrary outcomes, for example, low Grantham score combined with high A-GVGD score. No VUS were classified in class I or IV on the basis of the in silico data only.

Three different splice site prediction tools in Alamut were used for the analysis of variants. These Splice Site Prediction Programs are SpliceSiteFinder, MaxEntScan and GeneSplicer. When two out of three programmes show similar outcomes, this accurately predicts an effect on splicing.[18] For 12 variants in our study, a possible effect on RNA splicing was predicted and extra RNA analysis was performed for these variants when material was available (see online supplementary data). The in silico classification was then compared with the LOB-classification, which was based on the in silico outcome and on additional data including data derived from literature, cosegregation, array comparative genomic hybridisation, etc (see online supplementary table).

Statistical analyses Statistical analysis was performed with SPSS V. 20. Frequencies of each individual in silico parameter, within and between different classes of VUS, were compared using cross tabulation. In case of differences between groups, two-group analysis was performed using Pearson's $\chi 2$ test or occasionally Fisher's exact test, when the expected count was less than five. The outcome was considered statistically significant when the *P*-value was below 0.05.

RESULTS

In silico analysis of the variants

Grantham score

The Grantham score[15] examines the difference in the physicochemical nature of the amino acid substitutions. The score ranges between 0 and 215. A higher Grantham score is indicative of a greater difference in chemical properties between two amino acids (ie, polarity and molecular volume) and can indicate a stronger (negative) effect on protein structure and function. Grantham scores were determined for all 60 missense variants. The mean Grantham score was calculated and compared for each class of VUS classified by LOB. The mean Grantham scores for classes I, II, III and IV were 79, 78, 102 and 76, respectively (no significant differences between groups).

SIFT-analysis

The SIFT algorithm combines sequence homology and physical properties of amino acid substitutions to analyse whether or not amino acid substitutions are tolerated, in light of the predicted effect on the protein structure. The vast majority (92.3%) of class III VUS, as

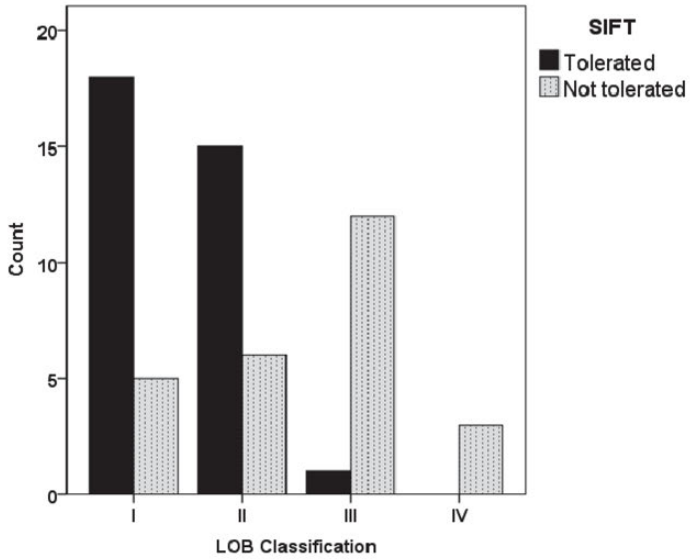

Figure 1. Sorting intolerant from tolerant (SIFT)-analysis of different Variants of Uncertain Significance classes classified by the National working group for Breast Cancer DNA Diagnostics (LOB). The bars represent the outcome of the SIFT-analysis depicted as tolerated or not tolerated.

classified by LOB, were predicted 'intolerant' by SIFT, in contrast to 28.6% of the class II VUS (figure 1). Unsurprisingly, the number of 'intolerant' VUS was significantly higher in class III when compared with class II (*P*=3.3e–4) or class I (*P*=5.2e–5). This result shows that LOB-classified class II and class III VUS can be broadly differentiated on the basis of SIFT analysis alone.

A-GVGD

Align-GVGD combines the biophysical characteristics of amino acids and multiple sequence alignments of proteins, weighing the cross species conservation of a particular amino acid and its specific physical characteristics, to predict where missense substitutions fall in a spectrum from enriched deleterious to enriched neutral.[19] A-GVGD scores amino acid substitutions on a 7-scale scoring system, from C0 to C65. An amino acid substitution with a C0 score is considered to be neutral, amino acids with C15 and C25 scores are considered intermediate, as changes to protein structure or function are uncertain, and C35 scores or higher are considered as likely deleterious.

The majority (88%) of VUS which are scored as neutral (C0) by A-GVGD are classified in class I and II by LOB (table 2). A significantly larger proportion of LOB-classified class III VUS score is more likely deleterious, with a score of C35 or higher, when compared with class II (*P*=6.2e–3) or class I (*P*=3.8e–2). These results indicate that LOB classified class II and class III VUS can be broadly differentiated on the basis of A-GVGD alone.

Table 2. A-GVGD analysis of different VUS classes, classified by LOB according to Bell et al.[11]

	LOB (in silico plus additional data)				
A-GVGD outcome	Class I	Class II	Class III	Class IV	Total
C0	21	19	5	-	45
C15-C25	-	2	4	1	7
C35-C65	2	-	4	2	8
Total	23	21	13	3	60

Nucleotide and amino acid conservation

The level of cross-species conservation was determined at the nucleotide and amino acid level for all missense variants. VUS were consistently scored as weakly, moderately or strongly conserved at nucleotide (figure 2A) and amino acid level (figure 2B), based on Alamut output. Statistically significant differences were apparent between class I and III (*P*=1.3e–4 and *P*=1.5e–4 for amino acid and nucleotide, respectively), and class II and III (*P*=3.4e–3 and *P*=8.9e–4 for amino acid and nucleotide, respectively), indicating that VUS at strongly conserved positions are significantly more frequently allocated to class III than to class I or II.

Classification

Of the 60 missense variants, 46 were classified in class II and 14 in class III based purely on in silico data. This classification was then compared with the LOB-classification for which

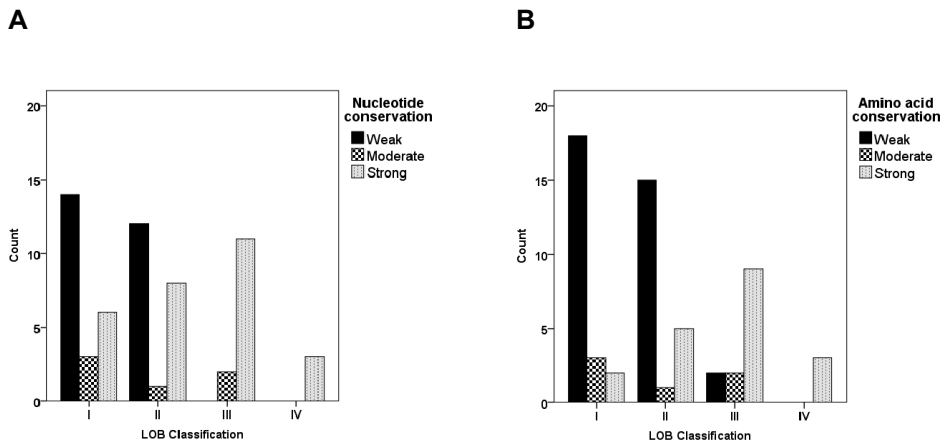

Figure 2. The nucleotide and amino acid conservation per Variants of Uncertain Significance class classified by the National working group for Breast Cancer DNA Diagnostics (LOB). (A) Nucleotide conservation based on Alamut output, which includes alignments of most published sequences and functional domains of *BRCA1* and *BRCA2*. (B) Amino acid conservation based on protein multialignment in Alamut.

additional data such as literature, cosegregation and co-occurrence were used (see online supplementary table).

Of the 46 VUS with an in silico categorisation in class II, 20 remained in class II, whereas more than half were recategorised, mostly in class I predominantly based on the presence in healthy controls or co-occurence. Five variants (11%) were categorised as class III. Of the VUS with a class III in silico categorisation, six were recategorised, of which three (21%) even being reassigned as pathogenic (class IV) (table 3, see online supplementary table). This analysis shows that the inclusion of additional information derived from peer review by the LOB can profoundly influence the classification outcome.

DISCUSSION

When a VUS is identified in either the *BRCA1* or *BRCA2* gene, a molecular geneticist provides an initial indication of pathogenicity, an opinion primarily based on in silico analysis. In a majority of the cases where no additional information is available, initial classification of the VUS will depend solely on these data and will guide the genetic counsellor in deciding whether or not and how to communicate information about the VUS to the counsellee.

This study demonstrates that missense variants in *BRCA1* and *BRCA2*, assigned to class II and III, show statistically significant differences in most VUS-related in silico characteristics. As expected, class III VUS more frequently showed in silico parameter outcomes indicating a deleterious effect on protein function, when compared with class II VUS. However, of the class II VUS classified using in silico data, nearly half (45%) were eventually recategorised in class I and 11% in class III and of the VUS classified in class III using in silico data, even 21% were recategorised as pathogenic when additional information was included for classification (table 3, see online supplementary table). In light of these data, we conclude that in silico analysis alone is not sufficient to unambiguously assign VUS to Bell's class II or class III.

The five-group classification system developed by Plon et al[12] is based on the degree of likelihood of pathogenicity and each class is associated with specific recommendations

Table 3. Classification (Bell et al.[11]) based on purely in silico data compared to the classification by LOB.

		LOB (*in silico* plus additional data)				
Classification		I	II	III	IV	Total
In silico	I	-	-	-	-	-
	II	21	20	5	-	46
	III	2	1	8	3	14
	IV	-	-	-	-	-
	Total	23	21	13	3	60

for clinical management of at-risk relatives. The majority of the VUS, however, receive a classification of class III in this system (0.05– 0.95 probability of being pathogenic; similar to class II and class III variants of Bell's classification (table 1)), indicating that this system is also unable to offer the improved subclassification so urgently needed by clinicians.

Of the 60 missense variants included in this study, some showed a discrepancy between the LOB classification and the most recent international publications. In a recent publication by Lindor et al[20] for example, the *BRCA2* variant c.4585G>A; p.Gly1529Arg is categorised as class I, based on an article by Easton et al.[21] However, this variant is registered in the LOB database as a class III variant, because the biological effect of this mutation has clearly been shown by Tal et al.[22] Although Dutch molecular geneticists generally use the Bell classification system and Lindor et al[20] have used the Plon classification (table 1), the discrepancy in the classification of these variants remains striking and shows that considerable effort and regular meetings at national and international levels are still required to reach a uniform and updated consensus.

The functional effect of most of the VUS on ovarian cancer risk has been less extensively studied, when compared with breast cancer. Pal et al[23] reported detection of VUS in about 8% of invasive carcinomas. Akbari et al[24] assembled a historical cohort of 4030 female first-degree relatives of 1345 unselected patients with ovarian cancer, who had been screened for *BRCA1* and *BRCA2* mutations. They showed that cumulative risk of cancer among relatives of patients carrying a VUS was similar to the risk of cancer for relatives of non-carriers. This result is, however, based on different VUS studied collectively. In contrast, a recent study by Spurdle et al[25] showed a higher cumulative risk for ovarian cancer in the carriers of the *BRCA1* c.5096G>A; p.Arg1699Gln variant, compared with the non-carriers. Although the separate estimation of breast and ovarian cancer risk is somewhat difficult, it could be that there is a difference in ovarian cancer compared with breast cancer risk associated with missense variants. Therefore, a study of a large number of such variants would be necessary to address this possibility—with important clinical implications.

Given the increasing number of families that are confronted with VUS test outcomes and the division in expert opinion regarding classification explained above, a well-defined VUS classification system would help to facilitate standardised counselling of VUS and provide uniform recommendations regarding communication and risk estimates for each class of VUS. From this study, it can be concluded that important clinical decisions regarding the interpretation of variants cannot be made based on the in silico outcomes only. The accuracy (about 80%)[17] and the magnitude of the Odds Ratio (OR) are insufficient for the classification of variants without the use of additional information.[5] The addition of other data, such as cosegregation and RNA analysis, to the existing in silico data will lead to an increase in the sensitivity and specificity of the classification method. The development of a multifactorial likelihood model for *BRCA1* and *BRCA2* variants was a major advance in the study of these variants, allowing the assessment of a range of features for a variant (eg, cosegregation, co-occurrence), in addition to in silico characteristics. This model

establishes a likelihood ratio for pathogenicity versus non-pathogenicity.[5] The most accurate classification of variants would be achieved if a combination of cosegregation data and functional study results could be used. However, as complete cosegregation data on individual variants is often not available and functional analysis is labour intensive and usually conflicting, in silico analysis remains the most important tool for the classification of the variants. For a more secure classification, the collection of additional material and information in multiple families per variant is therefore essential. Once sufficient families are included, one could even determine whether a variant confers intermediate breast and ovarian cancer risk, as shown by Spurdle et al.[25]

As previously mentioned, clinical genetics departments in the Netherlands generally only communicate discovery of class III VUS to the counsellee. In light of the fact that VUS may be recategorised when additional information becomes available (table 3), one could argue that a result of current communication guidelines is that clinically unimportant and potentially pathogenic variants will go unrecognised and remain categorised as class II VUS. Communication of a VUS test result provides the opportunity to discuss collection of additional information and material with the counsellee.

The classification of a VUS is dynamic and although we have shown that in silico categorisation is fairly robust we also clearly showed that additional information is central to an accurate appraisal. We would now argue that the prospect of obtaining additional information from a family, and biological material for additional analyses, should be given appropriate weight in the decision process preceding the communication of a VUS test result. Research initiatives such as the Evidence-based Network for the Interpretation of Germline Mutant Alleles consortium (http://www.enigmaconsortium.org/) (accessed 4 Apr 2012) which strive to combine diverse sources of information will be valuable in aiding a definitive classification of a VUS.

ACKNOWLEDGEMENTS

We would like to thank the Dutch and Belgian LOB members Efraim H. Rosenberg (The Netherlands Cancer Institute, Amsterdam, the Netherlands), Arjen Mensenkamp, Danielle Bodmer, Marcel Nelen (Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands), Ans van den Ouweland, Rick van Minkelen (Erasmus Medical Centre, Rotterdam, the Netherlands), Nienke van der Stoep (Leiden University Medical Centre, Leiden, the Netherlands), Rob B. van der Luijt (University Medical Centre Utrecht, Utrecht, the Netherlands), Johan J.P. Gille (VU University Medical Centre, Amsterdam, the Netherlands), Marinus J. Blok, Kees van Roozendaal (University Hospital Maastricht, Maastricht, the Netherlands), Annemarie H. van der Hout (University Medical Centre Groningen University, the Netherlands), Kathleen Claes (Ghent University Hospital, Ghent, Belgium), Katrien Storm, Sandra Willocx (Antwerp University Hospital, Antwerpen, Belgium), Geneviève Michils (University of Leuven, Leuven, Belgium) and Erik Teugels (Vrije Universiteit Brussel, Brussels, Belgium) for contribution of data to the LOB database, used in the classification of the variants.

REFERENCES

1. Spurdle AB, Healey S, Devereau A, Hogervorst FBL, Monteiro ANA, Nathanson KL, Radice P, Stoppa-Lyonnet D, Tavtigian S, Wappenschmidt B, Couch FJ, Goldgar DE, on behalf of E. ENIGMA—Evidence-based network for the interpretation of germline mutant alleles: an international initiative to evaluate risk and clinical significance associated with sequence variation in BRCA1 and BRCA2 genes. Hum Mutat 2011;33:2–7.
2. Vos J, Gómez-García E, Oosterwijk JC, Menko FH, Stoel RD, van Asperen CJ, Jansen AM, Stiggelbout AM, Tibben A. Opening the psychological black box in genetic counseling. The psychological impact of DNA testing is predicted by the counselees' perception, the medical impact by the pathogenic or uninformative BRCA1/2-result. Psychooncology 2010;21:29–42.
3. Vos J, Otten W, van Asperen C, Jansen A, Menko F, Tibben A. The counsellees' view of an unclassified variant in BRCA1/2: recall, interpretation, and impact on life. Psychooncology 2008;17:822–30.
4. Spurdle AB. Clinical relevance of rare germline sequence variants in cancer genes: evolution and application of classification models. Curr Opin Genet Dev 2010;20:315–23.
5. Goldgar DE, Easton DF, Deffenbaugh AM, Monteiro ANA, Tavtigian SV, Couch FJ. Integrated Evaluation of DNA Sequence Variants of Unknown Clinical Significance: application to BRCA1 and BRCA2. Am J Hum Genet 2004;75:535–44.
6. Goldgar DE, Easton DF, Byrnes GB, Spurdle AB, Iversen ES, Greenblatt MS. Genetic evidence and integration of various data sources for classifying uncertain variants into a single model. Hum Mutat 2008;29:1265–72.
7. Mohammadi L, Vreeswijk M, Oldenburg R, van den Ouweland A, Oosterwijk J, van der Hout A, Hoogerbrugge N, Ligtenberg M, Ausems M, van der Luijt R, Dommering C, Gille J, Verhoef S, Hogervorst F, van Os T, Gomez Garcia E, Blok M, Wijnen J, Helmer Q, Devilee P, van Asperen C, van Houwelingen H. A simple method for co-segregation analysis to evaluate the pathogenicity of unclassified variants; BRCA1 and BRCA2 as an example. BMC Cancer 2009;9:211.
8. Gomez Garcia E, Oosterwijk J, Timmermans M, van Asperen C, Hogervorst F, Hoogerbrugge N, Oldenburg R, Verhoef S, Dommering C, Ausems M, van Os T, van der Hout A, Ligtenberg M, van den Ouweland A, van der Luijt R, Wijnen J, Gille J, Lindsey P, Devilee P, Blok M, Vreeswijk M. A method to assess the clinical significance of unclassified variants in the BRCA1 and BRCA2 genes based on cancer family history. Breast Cancer Res 2009;11:R8/
9. Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, de Die-Smulders C, Persky N, Grompe M, Joenje H, Pals G, Ikeda H, Fox EA, D'Andrea AD. Biallelic Inactivation of BRCA2 in Fanconi Anemia. Science 2002;297:606–9.
10. Joosse S, van Beers E, Tielen I, Horlings H, Peterse J, Hoogerbrugge N, Ligtenberg M, Wessels L, Axwijk P, Verhoef S, Hogervorst F, Nederlof P. Prediction of BRCA1-association in hereditary non-BRCA1/2 breast carcinomas with array-CGH. Breast Cancer Res Treat 2009;116:479–89.
11. Bell J, Bodmer D, Sistermans E, Ramsden SC. Practice guidelines for the Interpretation and Reporting of Unclassified Variants (UVs) in Clinical Molecular Genetics.
12. Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, Hogervorst FBL, Hoogerbrugge N, Spurdle AB, Tavtigian SV. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer

susceptibility genetic test results. Hum Mutat 2008;29:1282–91.

13. Vink GR, van Asperen CJ, Devilee P, Breuning MH, Bakker E. Unclassified variants in disease-causing genes: nonuniformity of genetic testing and counselling, a proposal for guidelines. Eur J Hum Genet 2005;13:525–7.
14. Frank TS, Deffenbaugh AM, Reid JE, Hulick M, Ward BE, Lingenfelter B, Gumpper KL, Scholl T, Tavtigian SV, Pruss DR, Critchfield GC. Clinical Characteristics of Individuals with Germline Mutations in BRCA1 and BRCA2: analysis of 10,000 Individuals. J Clin Oncol 2002;20:1480–90.
15. Grantham R. Amino acid difference formula to help explain protein evolution. Science 1974;185:862–4.
16. Liu X, Jian X, Boerwinkle E. dbNSFP: a lightweight database of human nonsynonymous SNPs and their functional predictions. Hum Mutat 2011;32:894–9.
17. Tavtigian SV, Greenblatt MS, Lesueur F, Byrnes GB. In silico analysis of missense substitutions using sequence-alignment based methods. Hum Mutat 2008;29:1327–36.
18. Vreeswijk MPG, Kraan JN, van der Klift HM, Vink GR, Cornelisse CJ, Wijnen JT, Bakker E, van Asperen CJ, Devilee P. Intronic variants in BRCA1 and BRCA2 that affect RNA splicing can be reliably selected by splice-site prediction programs. Hum Mutat 2009;30:107–14.
19. Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, de Silva D, Zharkikh A, Thomas A. Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. J Med Genet 2006;43:295–305.
20. Lindor NM, Guidugli L, Wang X, Vallée MP, Monteiro ANA, Tavtigian S, Goldgar DE, Couch FJ. A review of a multifactorial probability-based model for classification of BRCA1 and BRCA2 variants of uncertain significance (VUS). Hum Mutat 2012;33:8–21.
21. Easton DF, Deffenbaugh AM, Pruss D, Frye C, Wenstrup RJ, Allen-Brady K, Tavtigian SV, Monteiro ANA, Iversen ES, Couch FJ, Goldgar DE. A systematic genetic assessment of 1,433 sequence variants of unknown clinical significance in the BRCA1 and BRCA2 breast cancer-predisposition genes. American J HumGenet 2007;81:873–83.
22. Tal A, Arbel-Goren R, Stavans J. Cancer-Associated Mutations in BRC Domains of BRCA2 Affect Homologous Recombination Induced by Rad51. J Mol Biol 2009;393:1007–12.
23. Pal T, Permuth-Wey J, Betts JA, Krischer JP, Fiorica J, Arango H, LaPolla J, Hoffman M, Martino MA, Wakeley K, Wilbanks G, Nicosia S, Cantor A, Sutphen R. BRCA1 and BRCA2 mutations account for a large proportion of ovarian carcinoma cases. Cancer 2005;104:2807–16.
24. Akbari MR, Zhang S, Fan I, Royer R, Li S, Risch H, McLaughlin J, Rosen B, Sun P, Narod SA. Clinical impact of unclassified variants of the BRCA1 and BRCA2 genes. J Med Genet 2011;48:783–6.
25. Spurdle AB, Whiley PJ, Thompson B, Feng B, Healey S, Brown MA, Pettigrew C, Van Asperen CJ, Ausems MGEM, Kattentidt-Mouravieva AA, van den Ouweland AMW, Lindblom A, Pigg MH, Schmutzler RK, Engel C, Meindl A, Caputo S, Sinilnikova OM, Lidereau R, Couch FJ, Guidugli L, Thomassen M, Eccles DM, Tucker K, Benitez J, Domchek SM, Toland AE, Van Rensburg EJ, Wappenschmidt B, Borg A, Vreeswijk MPG, Goldgar DE, kConFab, Belgium Uv Consortium D, German Consortium of Hereditary Breast and Ovarian C, French Cgc, Hansen TvO. BRCA1 R1699Q variant displaying ambiguous functional abrogation confers intermediate breast and ovarian cancer risk. J Med Genet 2012;49:525–32.

SUPPLEMENTARY TABLE

Supplementary Table. Summary of 60 *BRCA1* and *BRCA2* missense variants

	In silico prediction programs					Splice site prediction programs		
Variant BRCA1	SIFT	Grantham	AGVGD	Amino acid conservation	Nucleotide conservation	SSF	MES	GS
c.441G>C p.Leu147Phe	N	22	C0	Strong	Strong	-	+	-
c.494T>C p.Leu165Pro	N	98	C25	Weak	Strong	-	-	-
c.536A>G p.Tyr179Cys	N	194	C35	Weak	Strong	++	-	-
c.557C>A p.Ser186Tyr	N	144	C15	Strong	Strong	-	+	-
c.1865C>T p.Ala622Val	Y	64	C0	Strong	Weak	-	-	-
c.3418A>G p.Ser1140Gly	Y	56	C0	Weak	Weak	+	+	-
c.3640G>A p.Glu1214Lys	N	56	C0	Strong	Strong	-	-	-
c.4691T>C p.Leu1564Pro	Y	98	C0	Weak	Weak	-	-	-
c.4840C>T p.Pro1614Ser	Y	74	C0	Weak	Weak	-	-	-
c.4951T>C p.Ser1651Pro	Y	74	C0	Strong	Moderate	-	-	-
c.4956G>A p.Met1652Ile	Y	10	C0	Weak	Strong	-	-	-
c.5095C>T p.Arg1699Trp	N	101	C65	Strong	Strong	-	-	-
c.5096G>A p.Arg1699Gln	N	43	C35	Strong	Strong	++	+	-
c.5158A>G p.Thr1720Ala	Y	58	C0	Weak	Strong	++	+	-
c.5300G>C p.Cys1767Ser	N	112	C0	Strong	Strong	++	-	-
c.5309G>T p.Gly1770Val	N	109	C0	Strong	Strong	-	-	-

Other evidence for LOB classification					*In silico* classification	LOB classification	Breast Cancer Information Core Database	References
Co-segregation	Co-occurrence	In healthy controls	Effect on RNA splicing	Array-CGH				
	+		-		2	2		
-					3	3		
-	+				3	1	Unknown	[1-9]
					3	3	Unknown	[9-11]
					2	2	Unknown	[10]
					2	2	Unknown	[9, 11, 12]
					2	2	Unknown	[10, 13]
	+				2	1	No	[5, 9, 11, 14, 15]
	+				2	1	No	[5, 10, 14]
Unclear			-		2	3		[16]
	+	+ >1%			2	1	Unknown	[3, 7, 9, 17-31]
+					3	4	Yes	[7, 10, 17, 20, 27-29, 31-40]
					3	3	Unknown	[17, 27-29, 31, 34, 36, 37, 40-47]
					2	2	Unknown	[3, 10, 11, 17, 20, 27, 28, 31, 48]
				Not BRCA1-like	2	3		
+					2	3		

Supplementary Table. (continued)

	In silico prediction programs					Splice site prediction programs		
Variant BRCA1	SIFT	Grantham	AGVGD	Amino acid conservation	Nucleotide conservation	SSF	MES	GS
c.5585A>T p.His1862Leu	Y	99	C0	Weak	Weak	-	-	-
c.125A>G p.Tyr42Cys	Y	194	C0	Moderate	Strong	-	-	-
c.322A>C p.Asn108His	Y	68	C0	Weak	Moderate	++	-	-
c.502C>A p.Pro168Thr	N	38	C0	Strong	Strong	-	+	-
c.526A>T p.Thr176Ser	Y	58	C0	Moderate	Strong	++	+	-
c.978C>A p.Ser326Arg	Y	110	C0	Weak	Weak	-	-	-
c.1151C>T p.Ser384Phe	Y	155	C0	Weak	Weak	-	+	-
c.1262A>G p.Gln421Arg	N	43	C0	Weak	Weak	-	+	-
c.1514T>C p.Ile505Thr	Y	89	C0	Weak	Weak	-	-	-
c.1786G>C p.Asp596His	N	81	C0	Weak	Moderate	-	-	-
c.1889C>T p.Thr630Ile	Y	89	C0	Weak	Weak	-	-	-
c.2138A>T p.Gln713Leu	Y	113	C0	Weak	Weak	++	++	-
c.2680G>A p.Val894Ile	Y	29	C0	Weak	Weak	++	+	-
c.2803G>A p.Asp935Asn	Y	23	C0	Moderate	Light	-	++	-
c.2971A>G p.Asn991Asp	Y	23	C0	Weak	Weak	-	-	-
c.3055C>G p.Leu1019Val	Y	32	C0	Weak	Strong	++	+	-
c.4241C>T p.Thr1414Met	Y	81	C0	Weak	Weak	-	-	-

Other evidence for LOB classification					*In silico* classification	LOB classification	Breast Cancer Information Core Database	References
Co-segregation	Co-occurrence	In healthy controls	Effect on RNA splicing	Array-CGH				
				BRCA1 and 2-like	2	2	Unknown	
	+				2	1	Unknown	[18, 24, 26, 34, 41, 45, 49-57]
					2	2	Unknown	[3, 58]
					2	2	Unknown	[10, 59]
-					2	2		
					2	1		[52, 53]
					2	1	No	[3, 26, 45, 53, 60]
-					2	2		
	+				2	1		[52]
					2	1	No	[61, 62]
					2	2		[10]
	+				2	2	Unknown	[52]
-					2	2	Unknown	[10]
-					2	1	No	[8, 26, 52, 58]
					2	1	Unknown	[3, 19, 55, 58, 63, 64]
					2	2	Unknown	[10, 52, 65]
					2	1	No	[58, 66]

Supplementary Table. (continued)

	In silico prediction programs					Splice site prediction programs		
Variant BRCA1	SIFT	Grantham	AGVGD	Amino acid conservation	Nucleotide conservation	SSF	MES	GS
c.4301A>T p.Lys1434Ile	N	102	C15	Weak	Strong	-	-	-
c.4585G>A p.Gly1529Arg	N	125	C65	Strong	Strong	-	-	-
c.5704G>A p.Asp1902Asn	Y	23	C0	Weak	Weak	-	+	-
c.5737T>C p.Cys1913Arg	Y	180	C0	Weak	Weak	-	-	-
c.6100C>T p.Arg2034Cys	Y	180	C0	Weak	Weak	-	+	-
c.6317T>C p.Leu2106Pro	Y	98	C0	Weak	Weak	-	+	-
c.6706G>A p.Glu2236Lys	N	56	C0	Strong	Strong	-	-	-
c.6935A>T p.Asp2312Val	N	152	C15	Moderate	Strong	++	+	-
c.7150C>A p.Gln2384Lys	Y	53	C0	Weak	Weak	-	-	-
c.7397C>T p.Ala2466Val	Y	64	C0	Weak	Weak	-	+	-
c.7954G>A p.Val2652Met	N	21	C15	Strong	Strong	-	+	-
c.7976G>A p.Arg2659Lys	N	26	C25	Strong	Strong	++	+	-
c.7978T>G p.Tyr2660Asp	N	160	C65	Strong	Strong	++	+	-
c.8149G>T p.Ala2717Ser	Y	99	C0	Weak	Moderate	-	-	-
c.8182G>A p.Val2728Ile	Y	29	C0	Weak	Weak	-	-	-
c.8187G>T p.Lys2729Asn	No	94	C0	Weak	Moderate	++	+	-
c.8525G>A p.Arg2842His	N	29	C25	Strong	Strong	++	-	-
c.8662C>T p.Arg2888Cys	Y	180	C0	Weak	Weak	-	-	-

Other evidence for LOB classification					*In silico* classification	LOB classification	Breast Cancer Information Core Database	References
Co-segregation	Co-occurrence	In healthy controls	Effect on RNA splicing	Array-CGH				
					2	2		[53]
					3	3		[10, 67-69]
		+ >1%			2	1	No	[58, 61, 66]
					2	2	Unknown	[70]
					2	1	Unknown	[3, 12, 24, 26, 30, 53, 58, 71]
					2	2	Unknown	[53]
+			-		3	3	Unknown	
			-		3	3	Unknown	[10, 72]
		+ >1%			2	1	Unknown	[10]
		+ >1%			2	1	Unknown	[58, 63]
-					2	3		
			+		3	4	Unknown	[10, 49, 50]
			-		3	3	Unknown	[34, 44, 73]
		+ >1%			2	1	No	[3, 8, 26, 52, 70, 73, 74]
		+ >1%			2	1	No	[3, 30, 55, 73]
					2	3	Unknown	[10, 49, 73, 75]
	+				3	2	Unknown	[10, 72]
					2	2	Unknown	[10, 34, 73]

Supplementary Table. (continued)

	In silico prediction programs					Splice site prediction programs		
Variant BRCA1	SIFT	Grantham	AGVGD	Amino acid conservation	Nucleotide conservation	SSF	MES	GS
c.8830A>T p.Ile2944Phe	N	21	C0	Moderate	Strong	++	-	-
c.8850G>T p.Lys2950Asn	N	94	C35	Strong	Strong	-	-	-
c.8851G>A p.Ala2951Thr	N	58	C0	Strong	Strong	++	-	-
c.9104A>C p.Tyr3035Ser	N	144	C55	Moderate	Strong	-	-	-
c.9154C>T p.Arg3052Trp	N	101	C65	Strong	Strong	-	-	-
c.9161C>T p.Pro3054Leu	Y	98	C0	Weak	Weak	-	-	-
c.9235G>A p.Val3079Ile	Y	29	C0	Weak	Moderate	-	-	-
c.9634G>C p.Gly3212Arg	Y	125	C0	Weak	Weak	++	++	-
c.10234A>G p.Ile3412Val	Y	29	C0	Weak	Weak	++	-	-

SIFT tolerated: Y=Yes, N=No
Splice Site Prediction Programs: SpliceSiteFinder (SSF), MaxEntScan (MES) and GeneSplicer (GS). Strong effect is depicted here as ++, small effect as + and no effect as -.
Co-segregation is based on the results of analysis in at least one family.
Array-CGH data has been obtained from tumour tissue of individual who is a carrier of the variant and has been counselled in the LUMC. The analysis is performed in the Netherlands Cancer Institute at the department of Pathology under the supervision of Dr. P.M. Nederlof.
Variants in which addition of extra information changed their *in silico* classification are shown in bold.

REFERENCES

1. Augello C, Bruno L, Bazan V, Calò V, Agnese V, Corsale S, Cascio S, Gargano G, Terrasi M, Barbera F, Fricano S, Adamo B, Valerio MR, Colucci G, Sumarcz E, Russo A. Y179C, F486L and N550H are BRCA1 variants that may be associated with breast cancer in a Sicilian family: results of a 5-year GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study. *Ann Oncol* 2006;**17**(suppl 7):vii30-vii3.
2. Caligo MA, Bonatti F, Guidugli L, Aretini P, Galli A. A yeast recombination assay to characterize human BRCA1 missense variants of unknown pathological significance. *Hum Mutat* 2009;**30**(1):123-33.
3. Díez O, Osorio A, Durán M, Martinez-Ferrandis JI, Hoya Mdl, Salazar R, Vega A, Campos B, Rodríguez-López R, Velasco E, Chaves J, Díaz-Rubio E, Jesús Cruz J, Torres M, Esteban E, Cervantes A, Alonso C, San Román

Other evidence for LOB classification					*In silico* classification	LOB classification	Breast Cancer Information Core Database	References
Co-segregation	Co-occurrence	In healthy controls	Effect on RNA splicing	Array-CGH				
		+ >1%			2	1	Unknown	[12, 58, 73, 76]
	+				3	1	Unknown	[3, 8, 10, 24, 52, 73, 77]
-	+	+ >1%			2	1	No	[3, 18, 30, 51, 58, 73, 78]
					3	3	Unknown	[73]
					3	4		[34, 44, 49, 55, 73, 79]
					2	2		[73]
		+			2	1	Unknown	[10, 73]
	+				2	2	Unknown	
-					2	1	Unknown	

JM, González-Sarmiento R, Miner C, Carracedo A, Eugenia Armengod M, Caldés T, Benítez J, Baiget M. Analysis of BRCA1 and BRCA2 genes in Spanish breast/ovarian cancer patients: A high proportion of mutations unique to Spain and evidence of founder effects. *Hum Mutat* 2003;**22**(4):301-12.

4. Guidugli L, Rugani C, Lombardi G, Aretini P, Galli A, Caligo M. A recombination-based method to characterize human BRCA1 missense variants. *Breast Cancer Res Treat* 2011;**125**(1):265-72.

5. Judkins T, Hendrickson BC, Deffenbaugh AM, Eliason K, Leclair B, Norton MJ, Ward BE, Pruss D, Scholl T. Application of Embryonic Lethal or Other Obvious Phenotypes to Characterize the Clinical Significance of Genetic Variants Found in Trans with Known Deleterious Mutations. *Cancer Res* 2005;**65**(21):10096-103.

6. Judkins T, Hendrickson BC, Deffenbaugh AM, Scholl T. Single nucleotide polymorphisms in clinical genetic testing: the characterization of the clinical significance of genetic variants and their

application in clinical research for BRCA1. *Mutat Res* 2005;**573**(1–2):168-79.

7. Osorio A, Milne RL, Honrado E, Barroso A, Diez O, Salazar R, de la Hoya M, Vega A, Benitez J. Classification of missense variants of unknown significance in BRCA1 based on clinical and tumor information. *Hum Mutat 2007 May;28(5):477-85* 2007.
8. 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. *J Clin Oncol* 2008;**26**(10):1657-63.
9. Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, de Silva D, Zharkikh A, Thomas A. Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. *J Med Genet* 2006;**43**(4):295-305.
10. Easton DF, Deffenbaugh AM, Pruss D, Frye C, Wenstrup RJ, Allen-Brady K, Tavtigian SV, Monteiro ANA, Iversen ES, Couch FJ, Goldgar DE. A Systematic Genetic Assessment of 1,433 Sequence Variants of Unknown Clinical Significance in the BRCA1 and BRCA2 Breast Cancer-Predisposition Genes. *Am J Hum Genet* 2007;**81**(5):873-83.
11. McKean-Cowdin R, Spencer Feigelson H, Xia LY, Pearce CL, Thomas DC, Stram DO, Henderson BE. BRCA1 variants in a family study of African-American and Latina women. *Hum Genet* 2005;**116**(6):497-506.
12. Johnson N, Fletcher O, Palles C, Rudd M, Webb E, Sellick G, dos Santos Silva I, McCormack V, Gibson L, Fraser A, Leonard A, Gilham C, Tavtigian SV, Ashworth A, Houlston R, Peto J. Counting potentially functional variants in BRCA1, BRCA2 and ATM predicts breast cancer susceptibility. *Hum Mol Genet* 2007;**16**(9):1051-7.
13. Burk-Herrick A, Scally M, Amrine-Madsen H, Stanhope M, Springer M. Natural selection and mammalian BRCA1 sequences: elucidating functionally important sites relevant to breast cancer susceptibility in humans. *Mamm Genome* 2006;**17**(3):257-70.
14. Carvalho MA, Marsillac SM, Karchin R, Manoukian S, Grist S, Swaby RF, Urmenyi TP, Rondinelli E, Silva R, Gayol L, Baumbach L, Sutphen R, Pickard-Brzosowicz JL, Nathanson KL, Sali A, Goldgar D, Couch FJ, Radice P, Monteiro ANA. Determination of Cancer Risk Associated with Germ Line BRCA1 Missense Variants by Functional Analysis. *Cancer Res* 2007;**67**(4):1494-501.
15. Hayes F, Cayanan C, Barillà D, Monteiro ANA. Functional Assay for BRCA1: Mutagenesis of the COOH-Terminal Region Reveals Critical Residues for Transcription Activation. *Cancer Res* 2000;**60**(9):2411-8.
16. Karchin R, Monteiro ANA, Tavtigian SV, Carvalho MA, Sali A. Functional Impact of Missense Variants in BRCA1 Predicted by Supervised Learning. *PLoS Comput Biol* 2007;**3**(2):e26.
17. Glover J. Insights into the Molecular Basis of Human Hereditary Breast Cancer from Studies of the BRCA1 BRCT Domain. *Fam Cancer* 2006;**5**(1):89-93.
18. Spitzer E, Abbaszadegan MR, Schmidt F, Hauser A, Buwitt U, Lauter F-R, Pötschick K, Krocker J, Elling D, Grosse R. Detection of BRCA1 and BRCA2 mutations in breast cancer families by a comprehensive two-stage screening procedure. *Int J Cancer* 2000;**85**(4):474-81.
19. Tommasi S, Pilato B, Pinto R, Monaco A, Bruno M, Campana M, Digennaro M, Schittulli F, Lacalamita R, Paradiso A. Molecular and in silico analysis of BRCA1 and BRCA2 variants. *Mutat Res* 2008;**644**(1–2):64-70.
20. Mirkovic N, Marti-Renom MA, Weber BL, Sali A, Monteiro ANA. Structure-Based Assessment of Missense

Mutations in Human BRCA1. *Cancer Res* 2004;**64**(11):3790-7.

21. Arnold N, Peper H, Bandick K, Kreikemeier M, Karow D, Teegen B, Jonat W. Establishing a control population to screen for the occurrence of nineteen unclassified variants in the BRCA1 gene by denaturing high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002;**782**(1–2):99-104.
22. Gough CA, Gojobori T, Imanishi T. Cancer-related mutations in BRCA1-BRCT cause long-range structural changes in protein–protein binding sites: A molecular dynamics study. *Proteins* 2007;**66**(1):69-86.
23. Greenman J, Mohammed S, Ellis D, Watts S, Scott G, Izatt L, Barnes D, Solomon E, Hodgson S, Mathew C. Identification of missense and truncating mutations in the BRCA1 gene in sporadic and familial breast and ovarian cancer. *Genes Chromosomes Cancer* 1998;**21**(3):244-9.
24. Beristain E, Guerra I, Vidaurrazaga N, Burgos-Bretones J, Tejada M. LOH analysis should not be used as a tool to assess whether UVs of BRCA1/2 are pathogenic or not. *Fam Cancer* 2010;**9**(3):289-90.
25. Carvalho M, Pino MA, Karchin R, Beddor J, Godinho-Netto M, Mesquita RD, Rodarte RS, Vaz DC, Monteiro VA, Manoukian S, Colombo M, Ripamonti CB, Rosenquist R, Suthers G, Borg A, Radice P, Grist SA, Monteiro ANA, Billack B. Analysis of a set of missense, frameshift, and in-frame deletion variants of BRCA1. *Mutat Res* 2009;**660**(1–2):1-11.
26. Salazar R, Cruz-Hernandez JJ, Sanchez-Valdivieso E, Rodriguez CA, Gomez-Bernal A, Barco E, Fonseca E, Portugal T, Gonzalez-Sarmiento R. BRCA1–2 mutations in breast cancer: Identification of nine new variants of BRCA1–2 genes in a population from central Western Spain. *Cancer Lett* 2006;**233**(1):172-7.
27. Williams RS, Lee MS, Hau DD, Glover JNM. Structural basis of phosphopeptide recognition by the BRCT domain of BRCA1. *Nat Struct Mol Biol* 2004;**11**(6):519-25.
28. Lee MS, Green R, Marsillac SM, Coquelle N, Williams RS, Yeung T, Foo D, Hau DD, Hui B, Monteiro ANA, Glover JNM. Comprehensive Analysis of Missense Variations in the BRCT Domain of BRCA1 by Structural and Functional Assays. *Cancer Res* 2010;**70**(12):4880-90.
29. Rowling PJE, Cook R, Itzhaki LS. Toward Classification of BRCA1 Missense Variants Using a Biophysical Approach. *J Biol Chem* 2010;**285**(26):20080-7.
30. Deffenbaugh AM, Frank TS, Hoffman M, Cannon-Albright L, Neuhausen SL. Characterization of Common BRCA1 and BRCA2 Variants. *Genet Test* 2002;**6**(2):119-21.
31. Williams RS, Chasman DI, Hau DD, Hui B, Lau AY, Glover JNM. Detection of Protein Folding Defects Caused by BRCA1-BRCT Truncation and Missense Mutations. *J Biol Chem* 2003;**278**(52):53007-16.
32. Nikolopoulos G, Pyrpassopoulos S, Thanassoulas A, Klimentzou P, Zikos C, Vlassi M, Vorgias CE, Yannoukakos D, Nounesis G. Thermal unfolding of human BRCA1 BRCT-domain variants. *Biochim Biophys Acta* 2007;**1774**(6):772-80.
33. Shiozaki EN, Gu L, Yan N, Shi Y. Structure of the BRCT Repeats of BRCA1 Bound to a BACH1 Phosphopeptide: Implications for Signaling. *Mol Cell* 2004;**14**(3):405-12.
34. Gomez Garcia E, Oosterwijk J, Timmermans M, van Asperen C, Hogervorst F, Hoogerbrugge N, Oldenburg R, Verhoef S, Dommering C, Ausems M, van Os T, van der Hout A, Ligtenberg M, van den Ouweland A, van der Luijt R, Wijnen J, Gille J, Lindsey P, Devilee P, Blok M, Vreeswijk M. A method to assess the clinical significance of unclassified variants in the BRCA1 and BRCA2 genes based on cancer family history. *Breast Cancer Res* 2009;**11**(1):R8.
35. Worley T, Vallon-Christersson J, Billack B, Borg Å, Monteiro AA. A Naturally

Occurring Allele of BRCA1 Coding for a Temperature-Sensitive Mutant Protein. *Cancer Biol Ther* 2002;**1**(5):473-7.

36. Clapperton JA, Manke IA, Lowery DM, Ho T, Haire LF, Yaffe MB, Smerdon SJ. Structure and mechanism of BRCA1 BRCT domain recognition of phosphorylated BACH1 with implications for cancer. *Nat Struct Mol Biol* 2004;**11**(6):512-8.
37. Vallon-Christersson J, Cayanan C, Haraldsson K, Loman N, Bergthorsson Jon T, Brøndum-Nielsen K, Gerdes A-M, Møller P, Kristoffersson U, Olsson H, Borg Å, Monteiro ANA. Functional analysis of BRCA1 C-terminal missense mutations identified in breast and ovarian cancer families. *Hum Mol Genet* 2001;**10**(4):353-60.
38. Williams RS, Green R, Glover JNM. Crystal structure of the BRCT repeat region from the breast cancer-associated protein BRCA1. *Nat Struct Mol Biol* 2001;**8**(10):838-42.
39. Varma AK, Brown RS, Birrane G, Ladias JAA. Structural Basis for Cell Cycle Checkpoint Control by the BRCA1−CtIP Complex. *Biochemistry* 2005;**44**(33):10941-6.
40. Spurdle AB, Whiley PJ, Thompson B, Feng B, Healey S, Brown MA, Pettigrew C, kConFab, Van Asperen CJ, Ausems MGEM, Kattentidt-Mouravieva AA, van den Ouweland AMW, Belgium Uv Consortium D, Lindblom A, Pigg MH, Schmutzler RK, Engel C, Meindl A, German Consortium of Hereditary Breast and Ovarian C, Caputo S, Sinilnikova OM, Lidereau R, French Cgc, Couch FJ, Guidugli L, Hansen TvO, Thomassen M, Eccles DM, Tucker K, Benitez J, Domchek SM, Toland AE, Van Rensburg EJ, Wappenschmidt B, Borg A, Vreeswijk MPG, Goldgar DE. BRCA1 R1699Q variant displaying ambiguous functional abrogation confers intermediate breast and ovarian cancer risk. *J Med Genet* 2012;**49**(8):525-32.
41. Goldgar DE, Easton DF, Deffenbaugh AM, Monteiro ANA, Tavtigian SV, Couch FJ. Integrated Evaluation of DNA Sequence Variants of Unknown Clinical Significance: Application to BRCA1 and BRCA2. *Am J Hum Genet* 2004;**75**(4):535-44.
42. Pettigrew C, Wayte N, Lovelock P, Tavtigian S, Chenevix-Trench G, Spurdle A, Brown M. Evolutionary conservation analysis increases the colocalization of predicted exonic splicing enhancers in the BRCA1 gene with missense sequence changes and in-frame deletions, but not polymorphisms. *Breast Cancer Res* 2005;**7**(6):R929 - R39.
43. Lovelock P, Spurdle A, Mok M, Farrugia D, Lakhani S, Healey S, Arnold S, Buchanan D, Investigators k, Couch F, Henderson B, Goldgar D, Tavtigian S, Chenevix-Trench G, Brown M. Identification of BRCA1 missense substitutions that confer partial functional activity: potential moderate risk variants? *Breast Cancer Res* 2007;**9**(6):R82.
44. Mohammadi L, Vreeswijk M, Oldenburg R, van den Ouweland A, Oosterwijk J, van der Hout A, Hoogerbrugge N, Ligtenberg M, Ausems M, van der Luijt R, Dommering C, Gille J, Verhoef S, Hogervorst F, van Os T, Gómez García E, Blok M, Wijnen J, Helmer Q, Devilee P, van Asperen C, van Houwelingen H. A simple method for co-segregation analysis to evaluate the pathogenicity of unclassified variants; BRCA and BRCA2 as an example. *BMC Cancer* 2009;**9**(1):1-11.
45. 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, Investigators k, Easton D, Tavtigian SV, Goldgar D, Spurdle AB. Genetic and Histopathologic Evaluation of BRCA1 and BRCA2 DNA Sequence Variants of Unknown Clinical Significance. *Cancer Res* 2006;**66**(4):2019-27.
46. Loman N, Johannsson O, Kristoffersson U, Olsson H, Borg Å. Family History of Breast

and Ovarian Cancers and BRCA1 and BRCA2 Mutations in a Population-Based Series of Early-Onset Breast Cancer. *J Natl Cancer Inst* 2001;**93**(16):1215-23.

47. Abkevich V, Zharkikh A, Deffenbaugh AM, Frank D, Chen Y, Shattuck D, Skolnick MH, Gutin A, Tavtigian SV. Analysis of missense variation in human BRCA1 in the context of interspecific sequence variation. *J Med Genet* 2004;**41**(7):492-507.
48. Phelan CM, Dapic V, Tice B, Favis R, Kwan E, Barany F, Manoukian S, Radice P, van der Luijt RB, van Nesselrooij BPM, Chenevix-Trench G, Caldes T, de La Hoya M, Lindquist S, Tavtigian SV, Goldgar D, Borg Ã, Narod SA, Monteiro ANA. Classification of BRCA1 missense variants of unknown clinical significance. *J Med Genet* 2005;**42**(2):138-46.
49. Farrugia DJ, Agarwal MK, Pankratz VS, Deffenbaugh AM, Pruss D, Frye C, Wadum L, Johnson K, Mentlick J, Tavtigian SV, Goldgar DE, Couch FJ. Functional Assays for Classification of BRCA2 Variants of Uncertain Significance. *Cancer res* 2008;**68**(9):3523-31.
50. Wu K, Hinson SR, Ohashi A, Farrugia D, Wendt P, Tavtigian SV, Deffenbaugh A, Goldgar D, Couch FJ. Functional Evaluation and Cancer Risk Assessment of BRCA2 Unclassified Variants. *Cancer Res* 2005;**65**(2):417-26.
51. Bergthorsson JT, Ejlertsen B, Olsen JH, Borg A, Nielsen KV, Barkardottir RB, Klausen S, Mouridsen HT, Winther K, Fenger K, Niebuhr A, Harboe TL, Niebuhr E. BRCA1 andBRCA2 mutation status and cancer family history of Danish women affected with multifocal or bilateral breast cancer at a young age. *J Med Genet* 2001;**38**(6):361-8.
52. Edwards SM, Kote-Jarai Z, Meitz J, Hamoudi R, Hope Q, Osin P, Jackson R, Southgate C, Singh R, Falconer A, Dearnaley DP, Ardern-Jones A, Murkin A, Dowe A, Kelly J, Williams S, Oram R, Stevens M, Teare DM, Bruce Ponder AJ, Gayther SA, Easton DF, Eeles RA. Two Percent of Men with Early-Onset Prostate Cancer Harbor Germline Mutations in the BRCA2 Gene. *Am J Hum Genet* 2003;**72**(1):1-12.
53. 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**(33):5393-400.
54. Milner J, Ponder B, Hughes-Davies L, Seltmann M, Kouzarides T. Transcriptional activation functions in BRCA2. *Nature* 1997;**386**(6627):772-3.
55. Kuznetsov SG, Liu P, Sharan SK. Mouse embryonic stem cell-based functional assay to evaluate mutations in BRCA2. *Nat Med* 2008;**14**(8):875-81.
56. Wong JMS, Ionescu D, Ingles CJ. Interaction between BRCA2 and replication protein A is compromised by a cancer-predisposing mutation in BRCA2. *Oncogene* 0000;**22**(1):28-33.
57. Turner BC, Harrold E, Matloff E, Smith T, Gumbs AA, Beinfield M, Ward B, Skolnick M, Glazer PM, Thomas A, Haffty BG. BRCA1/BRCA2 Germline Mutations in Locally Recurrent Breast Cancer Patients After Lumpectomy and Radiation Therapy: Implications for Breast-Conserving Management in Patients With BRCA1/BRCA2 Mutations. *J Clin Oncol* 1999;**17**(10):3017-24.
58. Wagner TMU, Hirtenlehner K, Shen P, Moeslinger R, Muhr D, Fleischmann E, Concin H, Doeller W, Haid A, Lang AH, Mayer P, Petru E, Ropp E, Langbauer G, Kubista E, Scheiner O, Underhill P, Mountain J, Stierer M, Zielinski C, Oefner P. Global Sequence Diversity of BRCA2: Analysis of 71 Breast Cancer Families and 95 Control Individuals of Worldwide Populations. *Hum Mol Genet* 1999;**8**(3):413-23.
59. Pettigrew C, Wayte N, Wronski A, Lovelock P, Spurdle A, Brown M. Colocalisation of predicted exonic splicing enhancers in BRCA2 with reported sequence variants. *Breast Cancer Res Treat* 2008;**110**(2):227-34.

60. Wappenschmidt B, Fimmers R, Rhiem K, Brosig M, Wardelmann E, Meindl A, Arnold N, Mallmann P, Schmutzler RK. Strong evidence that the common variant S384F in BRCA2 has no pathogenic relevance in hereditary breast cancer. *Breast Cancer Res* 2005;**7**(5):R775 - R9.

61. Zhou X, Iversen ESJr, Parmigiani G. Classification of Missense Mutations of Disease Genes. *Am Stat Assoc* 2005;**100**:51–60.

62. Mullineaux LG, Castellano TM, Shaw J, Axell L, Wood ME, Diab S, Klein C, Sitarik M, Deffenbaugh AM, Graw SL. Identification of germline 185delAG BRCA1 mutations in non-Jewish Americans of Spanish ancestry from the San Luis Valley, Colorado. *Cancer* 2003;**98**(3):597-602.

63. Freedman ML, Penney KL, Stram DO, Le Marchand L, Hirschhorn JN, Kolonel LN, Altshuler D, Henderson BE, Haiman CA. Common variation in BRCA2 and breast cancer risk: a haplotype-based analysis in the Multiethnic Cohort. *Hum Mol Genet* 2004;**13**(20):2431-41.

64. Hadjisavvas A, Charalambous E, Adamou A, Christodoulou CG, Kyriacou K. BRCA2 germline mutations in Cypriot patients with familial breast/ovarian cancer. *Hum Mutat* 2003;**21**(2):171-.

65. Balia C, Galli A, Caligo M. Effect of the overexpression of BRCA2 unclassified missense variants on spontaneous homologous recombination in human cells. *Breast Cancer Res Treat* 2011;**129**(3):1001-9.

66. Fackenthal JD, Sveen L, Gao Q, Kohlmeir EK, Adebamowo C, Ogundiran TO, Adenipekun AA, Oyesegun R, Campbell O, Rotimi C, Akang EEU, Das S, Olopade OI. Complete allelic analysis of BRCA1 and BRCA2 variants in young Nigerian breast cancer patients. *J Med Genet* 2005;**42**(3):276-81.

67. Tal A, Arbel-Goren R, Stavans J. Cancer-Associated Mutations in BRC Domains of BRCA2 Affect Homologous Recombination Induced by Rad51. *Journal of Molecular Biology* 2009;**393**(5):1007-12.

68. Chen C-F, Chen P-L, Zhong Q, Sharp ZD, Lee W-H. Expression of BRC Repeats in Breast Cancer Cells Disrupts the BRCA2-Rad51 Complex and Leads to Radiation Hypersensitivity and Loss of G2/M Checkpoint Control. *J Biol Chem* 1999;**274**(46):32931-5.

69. Pellegrini L, Yu DS, Lo T, Anand S, Lee M, Blundell TL, Venkitaraman AR. Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. *Nature* 2002;**420**(6913):287-93.

70. The Single Nucleotide Polymorphism database (dbSNP)

71. Caputo S, Benboudjema L, Sinilnikova O, Rouleau E, Béroud C, Lidereau R, Consortium tFBG. Description and analysis of genetic variants in French hereditary breast and ovarian cancer families recorded in the UMD-BRCA1/BRCA2 databases. *Nucleic Acids Res* 2012;**40**(D1):D992-D1002.

72. Li L, Biswas K, Habib LA, Kuznetsov SG, Hamel N, Kirchhoff T, Wong N, Armel S, Chong G, Narod SA, Claes K, Offit K, Robson ME, Stauffer S, Sharan SK, Foulkes WD. Functional redundancy of exon 12 of BRCA2 revealed by a comprehensive analysis of the c.6853A>G (p.I2285V) variant. *Hum Mut* 2009;**30**(11):1543-50.

73. Karchin R, Agarwal M, Sali A, Couch F, Beattie MS. Classifying Variants of Undetermined Significance in BRCA2 with Protein Likelihood Ratios. *Cancer Inform* 2008;**6**(CIN-4-Beattie-et-al):0.

74. Meindl A, German Consortium for Hereditary Breast and Ovarian C. Comprehensive analysis of 989 patients with breast or ovarian cancer provides BRCA1 and BRCA2 mutation profiles and frequencies for the German population. *Int J Cancer* 2002;**97**(4):472-80.

75. Biswas K, Das R, Alter BP, Kuznetsov SG, Stauffer S, North SL, Burkett S, Brody LC, Meyer S, Byrd RA, Sharan

SK. A comprehensive functional characterization of BRCA2 variants associated with Fanconi anemia using mouse ES cell-based assay. *Blood* 2011;**118**(9):2430-42.

76. Pal T, Permuth-Wey J, Holtje T, Sutphen R. BRCA1 and BRCA2 Mutations in a Study of African American Breast Cancer Patients. *Cancer Epidemiol Biomarkers Preven* 2004;**13**(11):1794-9.
77. Gayther SA, de Foy KAF, Harrington P, Pharoah P, Dunsmuir WD, Edwards SM, Gillett C, Ardern-Jones A, Dearnaley DP, Easton DF, Ford D, Shearer RJ, Kirby RS, Dowe AL, Kelly J, Stratton MR, Ponder BAJ, Barnes D, Eeles RA, Collaborators TCRCBPGUKFPCS. The Frequency of Germ-line Mutations in the Breast Cancer Predisposition Genes BRCA1 AND BRCA2 in Familial Prostate Cancer. *Cancer Res* 2000;**60**(16):4513-8.
78. Hammet F, George J, Tesoriero A, Jenkins M, Schroen C, Smith L, Grabosch-Meehan A, Dite G, McCredie M, Giles G, Tavtigian S, Hopper J, Southey M. Is BRCA2 c.9079 G > A a predisposing variant for early onset breast cancer? *Breast Cancer Res Treat* 2008;**109**(1):177-9.
79. Walker LC, Whiley PJ, Couch FJ, Farrugia DJ, Healey S, Eccles DM, Lin F, Butler SA, Goff SA, Thompson BA, Lakhani SR, Da Silva LM, Tavtigian SV, Goldgar DE, Brown MA, Spurdle AB. Detection of splicing aberrations caused by BRCA1 and BRCA2 sequence variants encoding missense substitutions: implications for prediction of pathogenicity. *Hum Mutat* 2010;**31**(6):E1484-E505.