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

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Clinical significance of common Dutch *BRCA1* variants; application of the multifactorial likelihood model and correlation with functional data

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ABSTRACT

Variants of uncertain significance in known cancer susceptibility genes such as *BRCA1* and *BRCA2* are problematic for genetic counselling and clinical management of carriers and their families. The aim of this study was to assess pathogenicity of the most common *BRCA1* variants identified following patient referral to Clinical Genetics centres in the Netherlands. We applied an integrated approach using multifactorial likelihood analysis, including not only assessment of variant segregation in families and breast tumour histopathological features, but also array-comparative genomic hybridization as a new component of the model.

For 8 out of the 11 most common variants, results from previously published functional analyses were available. For 7 of these variants our results were consistent with the results from functional analysis.

The results from this study have direct implications for the classification of these VUS and thus for genetic counselling and medical management of families carrying these specific variants.

INTRODUCTION

Sequencing of the high-risk cancer predisposition genes *BRCA1* (MIM* 113705) and *BRCA2* (MIM* 600185) is increasingly offered to families with multiple breast and/or ovarian cancer cases when a genetic cause is suspected. In case a pathogenic variant in either of these genes is found, the best options for clinical management can be determined. However, the ongoing development of sequencing-based technologies in DNA diagnostic laboratories is resulting in the detection of an increasing number of variants in the *BRCA1* and *BRCA2* genes for which the clinical significance is unknown. These so called variants of uncertain significance (VUS) include missense changes, in-frame deletions or insertions, synonymous nucleotide substitutions, as well as alterations in non-coding sequences or in untranslated regions.

Breast and ovarian cancer risks for the counselees and their family members can be calculated based on age and number of affected individuals using algorithms and web based tools such as the Breast Cancer Risk Assessment Tool (Gail Model, https://www.cancer.gov/bcrisktool, accessed April 2017), IBIS Breast Cancer Risk Calculator Tool, http://www.ems-trials.org/riskevaluator, accessed April 2017), BRCAPRO (http://bcb.dfci.harvard.edu/bayesmendel/brcapro.php, accessed April 2017) and BOADICEA (http://ccge.medschl.cam.ac.uk/boadicea/, accessed April 2017). Based on the calculated risks, family members will then be given a specific surveillance advice or prophylactic surgery advice (www.oncoline.nl, accessed April 2017).

The development of a multifactorial likelihood model (MLM) for *BRCA1/2* variants was a major advance in the study of the VUS. The MLM combines complementary sources of data (i.e. in silico data,[1] family history,[2] cosegregation of the variant with disease in a family,[3] histopathological characteristics of the tumours[4, 5] and co-occurrence of the variant with a pathogenic *BRCA1* or *BRCA2* variant *in trans*)[6] to determine the probability that a given variant has a cancer risk equivalent to known high-risk pathogenic (predominantly truncating) variantsf.[7, 8]

The probability of pathogenicity based on each source of data, is calculated in the form of likelihood ratio (LR). LR is a measure of accuracy of a diagnostic test. The LR of a clinical finding is the probability of that finding when a condition is present divided by the probability of the same finding when the condition is absent.[9, 10] In order to improve the accuracy of classification, the MLM is constantly being updated by different research groups. These updates not only consist of revision of the existing likelihood ratios based on analysis of larger sample sets or new insights, but also of the incorporation of additional, new components representing independent data sources.

One of the most important limitations of the MLM is that there are often insufficient genetic and clinical data available for classification. A very robust component of the MLM is cosegregation of gene variants with disease, because it is not susceptible to uncertainties in variant frequencies or population stratification and is directly related to disease risk.[8] However, cosegregation data are in most cases not available, since according to

the Dutch national guidelines, other family members are not offered genetic screening in a diagnostic setting when a variant of uncertain significance is identified in a proband counselee. As for tumour histopathology, many researchers have studied different characteristics of breast and ovarian tumours.[4, 5, 11-15] Spurdle et al[4] have refined the LRs for histopathological characteristics of the tumours using the main commonly available data: Oestrogen, Progesterone and Her2-Neu receptor status and tumour grade. Their dataset included 4,477 *BRCA1* mutation carriers, 2,565 *BRCA2* mutation carriers, and 47,565 other breast cancer cases. However, especially in case of *BRCA2* tumours, these data do not contribute much to the final classification. That is because histopathological phenotype of *BRCA2*-related breast tumours do not much differ from non-*BRCA* tumours.[4] Additional characteristics to help distinguish between pathogenic and benign *BRCA1/2* variants are therefore needed and could be added to the multifactorial likelihood model. In 2009 Joosse et al[16] have introduced a method for classification of breast tumours by array-Comparative Genomic Hybridization (array-CGH). BRCA1 is involved in the DNA damage response pathway and loss of BRCA1 function will result in the accumulation of DNA damage and genomic instability. As a consequence, the *BRCA1*-mutated tumours develop a distinct pattern of genomic aberrations. Array-CGH can be used as an effective method to distinguish *BRCA1*-mutated from sporadic breast tumours.[16, 17]

In this study we applied a multifactorial likelihood approach to investigate the clinical significance of the most common VUS in the Netherlands, including variant segregation in the families and breast tumour histopathology. When available, the results of array-CGH on tumour tissue were included in the model as a new component. Furthermore, we compared the results from multifactorial likelihood analysis with the results from the functional analysis performed by Bouwman et al.[18] Our analysis adds more variants to the currently limited number of classified pathogenic missense variants in *BRCA1* that can be used as a calibration set for future studies incorporating functional assays into the multifactorial model.

MATERIALS AND METHODS

Ethics Statement

All probands were identified by genetic testing in one of 8 Clinical Genetics centres in the Netherlands (Amsterdam Medical Centre (AMC), Leiden University Medical Centre (LUMC), Maastricht University Medical Centre (MUMC), Dutch Cancer Institute (NKI), Radboud University Medical Centre (RadboudUMC), University Medical Centre Groningen (UMCG), University Medical Centre Utrecht (UMCU) and VU University Medical Centre (VUMC)). Pedigree data in combination with histopathological data (such as receptor status and grade, but also data on array-CGH) was collected. Approval from the Medical Research Ethics Committee was gained. All the research was performed in the Netherlands.

Array-comparative genomic hybridization

Array-CGH analysis was performed according to previously published methods[16, 17] at the Dutch Cancer Institute (NKI). Array-CGH has not been previously included in the existing likelihood ratio models.[8, 15]In this study, we calculated the LRs for array-CGH as previously described by Spurdle et al[4] as L[*BRCA1*-like|*BRCA1* tumors]/L[*BRCA1*-like|Sporadic tumors]. For example, if *m* tumours have a *BRCA1*-like array-CGH pattern out of a total of *M BRCA1* mutation carriers, and *s* sporadic breast tumours out of a total of *S* show the same *BRCA1*-like pattern, the LR is calculated by $(m/M)/(s/S)$. An approximate variance of log(LR) is calculated as $\mathrm{Var}(\ln(LR)=[1/m - 1/M + 1/s - 1/S])$. Assuming a normal distribution, 95% confidence intervals (95%CI) are given by $\exp[\ln(LR) +/- 1.96\sqrt{(\mathrm{Var}(\ln(LR)))}]$.[4] Using this technique[16, 17]188 tumours were tested. In this set, 53 out of 73 *BRCA1*-related tumours (73%) showed a *BRCA1*-like profile, while also 22 out of 115 sporadic tumours (19%) showed a *BRCA1*-like profile. We calculated the LRs which correspond to these array-CGH results. It led to a positive LR of 3.80 (95%CI: [2.54-5.67]) in favour of pathogenicity and a negative LR of 0.34 against pathogenicity (95%CI: [0.23-0.50]) (unpublished data).

Multifactorial Likelihood Analysis

For this study we initially selected 22 *BRCA1* variants which were identified at least two times in the Netherlands and were classified as class II or III according to Bell's classification system[19] (It is important to note that at the time of selection of the variants for data collection, the 5-tier IARC classification system[20] was not yet applied in the Netherlands). Out of these 22, we had sufficient information from various sources on 11 variants. Variants for which we had no cosegregation data were excluded from the study. In addition, families were excluded when there was another pathogenic variant segregating in the family. We assumed that the results from array-CGH were not independent from histopathological data, therefore when for one tumour both data was available we have used only one of these two sources of data in the calculation. The one which was more in concordance with the other LRs for that variant.

LR for cosegregation was calculated in families in which more than one person was genotyped using the cosegregation model developed by Mohammadi et al.[21]

Overall likelihood of pathogenicity was calculated based on LR of cosegregation and LRs based on tumour pathology (Oestrogen, Progesterone and Her2-Neu receptor status and grade),[4] and when available, array-CGH data[16, 22] as previously described.[8, 23]In summary, to determine the "overall likelihood ratio" for pathogenicity *versus* non-pathogenicity of a particular VUS, all the available LRs for the VUS, under the assumption of independence, are multiplied. These LRs may be composed of multiple families, tumours, etc. Then "prior probability" is estimated based on evolutionary conservation and biophysical characteristics (*in silico* data). The "overall likelihood ratio" estimates in combination with *in silico* data are used to calculate the "posterior probability" of a VUS being pathogenic, through first determining the "Posterior Odds of pathogenicity"

by using the formula: **Posterior Odds = Likelihood ratio × [prior probability/(1-prior probability)].** In the final step, the posterior probability of pathogenicity is calculated using Bayes theorem: **Posterior Probability = Posterior Odds /(Posterior Odds + 1).**[23] The scale of posterior probability is between 0 and 1.00 and is often expressed as a percentage.[23] For some variants we combined our overall likelihood ratios with overall likelihood data from other studies by multiplication to generate updated likelihood ratios. This could be done because the datasets were independent.[23] The posterior probability is translated to the IARC classification system as outlined in Plon et al[20] to categorize each variant into a specific class; namely: not pathogenic or of no clinical significance (class 1, posterior probability: <0.001), likely not pathogenic or of little clinical significance (class 2, posterior probability: 0.001- 0.049), uncertain (class 3, posterior probability: 0.05–0.949), likely pathogenic (class 4, posterior probability: 0.95–0.99) and pathogenic (class 5, posterior probability: >0.99). The classification system assigns recommendations related to surveillance and patient and family management guidelines.[20]

We compared the results from MLM with information available on public databases such as ClinVar,[24] *BRCA* exchange[25] and functional analysis.[18] ClinVar is a freely accessible, public archive of reports of the relationships among human variations and phenotypes, with supporting evidence[24] and "the *BRCA* Exchange aims to advance our understanding of the genetic basis of breast cancer, ovarian cancer and other diseases by pooling data on *BRCA1/2* genetic variants and corresponding clinical data from around the world".[25]

Frequency data

The identification of VUS in control populations can be an effective tool to classify it as a functionally neutral variant. The presence of a variant in more than 1% (MAF ≥ 0.01) of a healthy population strongly argues against its pathogenicity.[26, 27] In this study, when available, we added frequency of variant occurrence in NHLBI Exome Sequencing Project (ESP) (http://evs.gs.washington.edu/EVS/, accessed May 2017) and ExAC database (http://exac.broadinstitute.org/, accessed May 2017) in table 1 as additional evidence for classification.

Functional tests

BRCA1-deficient tumours are shown to be highly sensitive to platinum chemotherapy both *in vitro* and *in vivo*.[28-30] Platinum chemotherapy generates inter-strand cross-links (ICL) which can only be properly repaired by homologous recombination (HR)-based DNA repair. In the absence of HR, cells are therefore, sensitive to agents which generate ICLs. Bouwman et al studied the proliferation response and cisplatin cytotoxicity of the cells in which endogenous mouse *Brca* allele was inactivated[18, 31] and showed that cisplatin sensitivity was a reliable method to distinguish variants affecting HR function of *BRCA1* from those that did not.

We have chosen the cisplatin assay because most of the variants in our analysis were already tested using this assay. We compared the results from this assay with the multifactorial likelihood analysis from our study.

RESULTS

Results from classification of variants based on the multifactorial likelihood model compared to classification by others are shown in table 1. Detailed clinical and genetic data which are used in the multifactorial likelihood model are shown in supplementary table 1.

For 11 of the initially selected 22 *BRCA1* variant, clinical and genetic data were available. Functional data was available for 8 out of these 11 variants. Five out of these 11 variants had a discrepancy between results from ClinVar,[24] *BRCA* Exchange[25] and functional data.[18]

The posterior probability of pathogenicity of these 11 variants was calculated on the basis of cosegregation, histopathological data and family history when available. Detailed information for these 11 variants will be discussed below.

BRCA1 c.53T>C p.Met18Thr

This variant has been identified in 18 families in the Netherlands LOVD (Leiden Open Variation Database).[32] In this study we had access to data from cosegregation in 5 families carrying this variant. In 4 out of 5 families LR was in favour of pathogenicity (3.42, 0.004, 24.10, 1.55 and 6.46). Histopathological data from three tumours and array-CGH resulted from another tumour were combined. This led the variant to be assigned to class 4 (likely pathogenic).[20] We combined the overall likelihood ratio as published in Lindor et al[33] (overall LR=31.61) with the overall likelihood ratio from this study, as they are resulting from two independent datasets. The combination of these data led to classification of this variants to class 5 with a posterior probability of >0.999.[20] Functional analysis by Bouwman et al[18] classified this variant as deleterious.

BRCA1 c.199G>T, p.Asp67Tyr

This variant has been identified 13 times in the Netherlands[32]. It was classified in ClinVar as benign.[24] Functional tests classified this variant as neutral[18] which is in accordance with the results from our multifactorial likelihood model, which based on cosegregation data from one family and two tumours, assigns this variant to class 2 (likely benign).[20]

BRCA1 c.2566T>C, p.Tyr856His

This variant has been identified 4 times in the Netherlands[32]. Also this variant was classified in ClinVar as Benign.[24] Functional tests classified this variant as Neutral[18]. In our database we had cosegregation data from one family and histopathological data from one tumour. Multifactorial likelihood analysis of this variant from this study led to classification of this variant as likely benign (class 2).[20]

Table 1. Classification of the variant based on the multifactorial information compared to different sources of information

Variant		A-GVGD (Prior probability) [1, 2]	Posterior probability (number of families)	IARC class	Functional test results By Bouwman et al. [18]
c.53T>C	p.Met18Thr	C45 (0.66)	0.9992 (n=5)	5	Deleterious
c.199G>T	p.Asp67Tyr	C0 (0.03)	0.0026 (n=3)	2	Neutral
c.2566T>C	p.Tyr856His	C0 (0.02)	0.0036 (n=2)	2	Neutral
c.3302G>A	p.Ser1101Asn	C0 (0.02)	0.0243 (n=1)	2	Neutral

Clinvar#					
Class 1 or 2	Class 3	Class 4 or 5	Final classification in Clinvar	BRCA exchange	Allele frequency
NA	– BIC$ (1999)	– Ambry genetics (2015) – GeneDx (2014) – SCRP *(2011)	Conflicting interpretations of pathogenicity	Not yet reviewed	Unknown
– Invitae (2016) – GeneDx (2016) – ENIGMA (2015) – Ambry Genetics (2014) – Counsyl (2014)	– Children's hospital of Eastern Ontario (2015) – University of Washington Medical Centre (2014) – SCRP (2007) – BIC (2002)	NA	Benign	Benign/ little clinical significance	RS ID: 80357102 GO-ESP: 0.000154 ExAC: 0.00008
– Invitae (2017) – Baylor Miraca genetics laboratories (2017) – University of Michigan (2016) – Illumina (2016) – ENIGMA (2015) – Fulgent genetics (2015) – GeneDx (2014) – Ambry genetics (2014) – Counsyl (2014) – University of Washington Medical centre (2014) – SCRP (2011)	– BIC (2006)	NA	Benign	Benign/ little clinical significance	RS ID: 80356892 GO-ESP: 0.00008 ExAC: 0.00152
– ENIGMA (2015) – Vantari genetics (2015) – Invitae (2017) – Ambry genetics (2014) – GeneDx(2016) – Children's hospital of Eastern Ontario (2015) – Counsyl (2014) – SCRP (2008)	– BIC (2002)		Benign	Benign /little clinical significance	RS ID: 41293447 GO-ESP: 0.00015 ExAC: 0.00016

Table 1. (continued)

Variant		A-GVGD (Prior probability) [1, 2]	Posterior probability (number of families)	IARC class	Functional test results By Bouwman et al. [18]
c.5057A>G	p.His1686Arg	C25 (0.29)	0.7481 (n=1)	3	Deleterious
c.5066T>A	p.Met1689Lys	C35 (0.66)	0.8928 (n=1)	3	NA
c.5072C>T	p.Thr1691Ile	C65 (0.81)	0.8232 (n=2)	3	Variant of Uncertain Significance
c.5216A>T	p.Asp1739Val	C65 (0.81)	0.9726 (n=2)	4	Deleterious
c.1846_1848delTCT	p.Ser616del	0.02 Outside functional domains	0.0031 (n=1)	2	NA
c.3891_3893delTTC	p.Ser1297del	0.02 Outside functional domains	0.0157 (n=1)	2	Neutral
c.4186-1511_c.4986+939del14098		0.35 In frame deletion in BRCT domain	0.9603 (n=7)	4	NA

Clinvar: https://www.ncbi.nlm.nih.gov/clinvar/, accessed June 2017
$ (BIC) : Breast cancer information core
*(SCRP): Sharing Clinical Reports Project

BRCA1 c.3302G>A, p.Ser1101Asn

According to data in the LOVD database, this variant has been identified 5 times in the Netherlands.[32] According to ClinVar[24] and *BRCA* Exchange[25] it is benign or likely not pathogenic. In our database we had cosegregation data from one family and histopathological data from one tumour. The posterior probability based on multifactorial model for this variant is 0.0036, thus it will be assigned as class 2 (likely benign). Functional tests previously classified this variant as neutral.[18]

Clinvar					
Class 1 or 2	Class 3	Class 4 or 5	Final classification in Clinvar	BRCA exchange	Allele frequency
NA	– GeneDx (2016)	– Invitae (2017) – Medical University Innsbruck (2015)	Conflicting interpretations of pathogenicity	Not yet reviewed	Unknown
NA	– BIC (2004)	NA	Uncertain significance	Not yet reviewed	Unknown
	– GeneDx(2016) – SCRP (2007) – BIC (2004)	– Invitae (2017)	Conflicting interpretations of pathogenicity	Not yet reviewed	Unknown
NA	NA	NA	NA	Not yet reviewed	Unknown
NA	– GeneDx (2015)	NA	Uncertain significance	Not yet reviewed	RS ID: 80358329 Go-ESP: 0.0016 ExAC: 0.00032
NA	– GeneDx (2015)	NA	Uncertain significance	Not yet reviewed	Unknown
NA	NA	NA	NA	NA	Unknown

BRCA1 c.5057A>G, p.His1686Arg

This variant has been identified in 3 families in the Netherlands.[32] For this variant there was discrepancy between classification according to ClinVar data[24] and functional analysis. ClinVar assigned it as conflicting interpretations of pathogenicity varying between VUS, likely pathogenic and pathogenic.[24] However, functional analysis[18] classified this variant as deleterious. Based on cosegregation data from one family and data from one triple negative tumour, for which in both cases LR was in favour of pathogenicity, this variant reached a posterior probability of 0.75 and remained classified as a variant of uncertain significance.[20]

BRCA1 c.5066T>A, p.Met1689Lys

For this variant in ClinVar there was only one entry from Breast cancer information core (BIC) from 2004.[24, 34] According to data in the LOVD database, this variant has been identified only 2 times in the Netherlands.[32] There were no data available from functional analysis.[18]In our dataset cosegregation data from one family and one tumour were in favour of pathogenicity. For another tumour from this family array-CGH was available. The results from this test however, showed a sporadic-like profile. Discrepancy between results from these tests led to uncertain classification of this variant (posterior probability 0.892).[20]

BRCA1 c.5072C>T, p.Thr1691Ile

Five Dutch families are listed in the LOVD database carrying this variant.[32] For c.5072C>T, p.Thr1691Ile, classification based on ClinVar data[24] and functional analysis[18] did not result in a clear classification and the variants remained assigned as a VUS. For this variant we had cosegregation data from two families both in favour of pathogenicity. However, likelihood ratios for the histopathological characteristics of the three tumours were all against pathogenicity. Therefore, in spite of a high prior probability of pathogenicity (C65, prior probability=0.81),[1] the contradictory evidence resulted in uncertainty in classification of this variant and it remained a variant of uncertain significance (posterior probability: 0.823).[20]

BRCA1 c.5216A>T, p.Asp1739Val

This variant has been identified 4 times in the Netherlands.[32] For this variant there is no information on ClinVar[24] or *BRCA* Exchange[25]. We had access to cosegregation data from one family and three tumours. Combination of these data led to classification of this variant as likely pathogenic (class 4).[20] Functional analysis by Bouwman et al[18] previously classified this variant as deleterious.

BRCA1 c.1846_1848delTCT, p.Ser616del

For c.1846_1848delTCT, p.Ser616del with conflicting interpretations of pathogenicity in ClinVar[24] there was no functional data.[18] Classification in ClinVar varied from class 1 (benign) to class 3 (VUS).[20, 24] Our cosegregation data from one family and histopathological data from one tumour classified this variant as likely benign (class 2).

BRCA1, c.3891_3893delTTC, p.Ser1297del

Is another variant with discrepancy in different classification sources varying between class 1 and 3.[20] This variant has been found in 7 families in the Netherlands.[32] It is not located in a functional domain of the BRCA1, so according to the data in table 5 by Easton et al[2] this variant has a prior probability of 0.02 (95% CI: 0.00-0.04) to be pathogenic. For this variant we had data from two families. In one family the index was affected with contralateral breast cancer at the age of 39 years. Unfortunately, no other individual was genotyped in

this family. For the other family cosegregation was available. Furthermore, we had data on one breast tumour. These data together resulted in classification of the variant as likely benign (class 2). This variant was classified by Bouwman et al[18] as neutral.

BRCA1 c.4186-1511_c.4986+939del14098

The c.4186-1511_c.4986+939del14098 deletion is found in 7 families in the Netherlands and is not previously reported in international *BRCA1* and *BRCA2*-related databases. The deletion removes residues p.1396-p.1662 encoded by exon 13 to 16 and gives rise to an in frame deletion resulting in the absence of 267 amino acids, deleting part of the first BRCT domain of the protein. As this variant is located in a functional domain in BRCA1, its prior probability of pathogenicity is estimated to be 0.35 (95% CI: 0.26-0.45).[2] In our study, combination of cosegregation data, histopathological characteristics of the tumour, together with array-CGH resulted in classification of this variant as likely pathogenic (class 4).[20]

DISCUSSION

The use of the multifactorial likelihood model (MLM) is limited by the availability of data. The frequency of many variants is low and very often there is no cosegregation information available. To address this problem, we collected nationwide data from different Dutch Clinical Genetics centres. Furthermore, to tackle the problem of lack of data, we incorporated the results from array-CGH as a new component of the multifactorial likelihood model.

For this study we focused on collecting data on variants which were previously classified as variants of uncertain significance based on Bell's classification system.[19] We chose variants that were ascertained in more than one family in the Netherlands. For 11 out of the 22 *BRCA1* variants on our list there was enough information which could be used for the purpose of classification. In this study five variants were classified as (likely benign), three were (likely) pathogenic and three remained as variant of uncertain significance.

In general, there was a good correlation between the results from this study and the available data from public databases and functional analysis results by Bouwman et al.[18] For two variants there was already a classification available on BRCA Exchange.[25] Both variants had a comparable classification. Four out of 6 variants which had a ClinVar classification,[24] had comparable results in our study. The other variants were classified as VUS in ClinVar,[24] whereas in our study they were classified as likely benign. Comparing functional analysis with our study, 7 out of 8 variants for the results matched. However, some variants need additional discussion:

For *BRCA1* c.5057A>G, p.His1686Arg Bouwman et al concluded that this variant is deleterious based on their functional analysis[18]. A different missense substitution at the same codon (p.His1686Gln) has been determined to be (likely) pathogenic.[18, 35, 36] This suggests that the histidine residue is critical for BRCA1 protein function and that other

missense substitutions at this position may also be pathogenic. Based on our results, using MLM, this variant remained classified as variant of uncertain significance. However, also in our study all the available data were in favour of pathogenicity (LR>1).

For *BRCA1* c.1846_1848delTCT, p.Ser616del there was a discrepancy between cosegregation data and tumour histopathological characteristics regarding their pathogenicity however, we had data only from one family. Data from more families carrying this variant is needed to be able to classify this variant with more certainty.

Lack of sufficient data for most of the VUS has led many researchers to focus more on the use of functional tests, at this moment mostly on *BRCA1* and *BRCA2*. During the ENIGMA Consortium Meeting on 15-17 January 2017 in Limassol, Cyprus (ENIGMA: Evidence-based Network for the Interpretation of Germline Mutant Alleles)[37] it was agreed that functional data can be used in clinical classification, provided that it is not the sole data to base a classification on. The main argument against using results from functional test as the only source of data for variant classification is that as functional assays do not measure cancer risk directly, they need to be calibrated for sensitivity and specificity against variants of known clinical significance in *BRCA1/2* genes which are located in domains relevant to the functional assays being tested.[38, 39] In case of some specific types of variants (e.g. missense variants) this can be particularly challenging, simply because the number of variants reliably classified to be used as a validation set is limited. For translating functional effects to cancer risk, the use of functional test results in the multifactorial likelihood model is necessary. A model is already developed to estimate the LRs for *BRCA2* VUS which were analysed with the Homology-directed repair (HDR) assay.[40, 41] The model derives a probability of pathogenicity for each variant using estimates of the mean and the variances of the distribution of the HDR results for the known pathogenic and the non-pathogenic variants. LRs could be included in the multifactorial likelihood model, next to data from other sources such as family history and cosegregation which could eventually give posterior probability of pathogenicity.[42] Iversen et al developed a computational approach for determining the disease relevance of VUS in *BRCA1* from data derived from an in vitro functional assay. This approach is based on a Bayesian hierarchical model that accounts for sources of experimental heterogeneity.[43] Using this approach they showed that functional assays provide a robust tool for the clinical classification of VUS.[44] Furthermore, as the *BRCA1* and *BRCA2* have different functions and not all their functions might be relevant for tumour suppression, absence of a functional effect does not translate directly to low cancer predisposition. In order to tackle this problem, a panel of different assays representative for different functions of the gene should be used to evaluate variants in order to minimize the risk that a specific functional effect of the protein will be overlooked.[45] Moreover, highly quantitative assays are needed to discriminate between variants that totally inactivate or only partially inactivate protein function as the intermediate risk variants such as the *BRCA1* c.5096G>A p.Arg1699Gln (R1699Q).[46] Nonetheless, in time, functional assay data with clinical/genetic data will be used for the evaluation of pathogenicity of VUS and in this way will be a valuable

and indispensable tool for the assessment of the clinical relevance of variants of uncertain significance.[45]

CONCLUSION

Using a multifactorial likelihood model, we could classify 8 out of 11 most common Dutch *BRCA1* variants. Results from this study have direct implications for genetic counselling and medical management of families that carry these specific variants. However, as many individual variants are unique in the population and because often there is not enough genetic information for classification purposes, intensive international collaborations such as ENIGMA[37] are pivotal to get access to more data in order to reliably determine the probability of pathogenicity of these variants.

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SUPPLEMENTARY DATA

Table S1. Classification of the variant based on the multifactorial information

Variant		A-GVGD (Prior probability#) [1, 2]	Likelihood ratios	
			Segregation	Tumour pathology*
c.53T>C	p.Met18Thr	C45 (0.66)	3.30 Fam 1: 3.42 Fam 2: 40x10^-3 Fam 3: 24.10 Fam 4: 1.55 Fam 5: 6.46	12.48 Er- G3 B40: 3.16 Er-B53, B55: 3.31 TN- B38: 3.73
c.199G>T	p.Asp67Tyr	C0 (0.03)	0.52	0.16 NTN B39: 0.4 NTN B46: 0.4
c.2566T>C	p.Tyr856His	C0 (0.02)	1.32	NTN B45: 0.4
c.3302G>A	p.Ser1101Asn	C0 (0.02)	3.05	NTN B33: 0.4
c.5057A>G	p.His1686Arg	C25 (0.29)	1.95	TN B47: 3.73
c.5066T>A	p.Met1689Lys	C35 (0.66)	3.99	Er-Gr3 B41: 3.16
c.5072C>T	p.Thr1691Ile	C65 (0.81)	3.97 Fam 1: 3.96 Fam 2: 1.001	0.27 Er+ G3 B52: 0.9 Er+ B53 G2: 0.36 Er+ G3 B57: 0.9
c.5216A>T	p.Asp1739Val	C65 (0.81)	1.87	4.45 TN B44: 3.73 TN B41: 3.73 Er+ B47: 0.32
c.1846_1848delTCT	p.Ser616del	0.02 Outside functional domains	1.92	Er+ G1 B36:0.08
c.3891_3893delTTC	p.Ser1297del	0.02 Outside functional domains	1.95	NTN B39:0.4

LR Family history [3]	Array-CGH	Co-occurrence	Overall likelihood[$]	Overall odds by Lindor et al. [3]	Posterior probability[&]	IARC class
1.41	3.80 BRCA1-like		19.87	31.61	0.9991	5
			0.08		0.0026	2
	0.34 Sporadic-like		0.18		0.0036	2
			1.22		0.0243	2
			7.27		0.7481	3
	0.34 Sporadic- like		4.29		0.8928	3
			1.09		0.8232	3
			8.33		0.9726	4
			0.15		0.0031	2
			0.78		0.0156	2

Table S1. (continued)

Variant	A-GVGD (Prior probability#) [1, 2]	Likelihood ratios	
		Segregation	Tumour pathology*
c.4186-1511_c.4986+939del14098	0.35 In frame deletion	5.60 Fam 1: 8.95 Fam 2: 3.79 Fam 3: 0.01 Fam 4: 0.59 Fam 5: 1.04 Fam 6: 1.94 Fam 7: 10.47	2.11088 G3 B39: 1.67 NTN B32 : 0.4 Er– Gr3 B39: 3.16

Abbreviations:
Er: Oestrogen receptor, negative or positive
TN: triple negative, NTN: Not triple-negative
G1: Grade 1, G2: Grade 2, G3: Grade 3
Bxx: Breast cancer at age xx

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LR Family history [3]	Array-CGH	Co-occurrence	Overall likelihood[$]	Overall odds by Lindor et al. [3]	Posterior probability[&]	IARC class
	3.80 BRCA1-like		44.89		0.96027	4