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

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To my love, Farshid,
to our Ryan,

and to my parents

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General introduction

1

In the Netherlands approximately 14,000 women per year are diagnosed with invasive breast cancer. That means that about 1 in 8 women will develop breast cancer at some point in their lives. In the Netherlands, around 3,000 women die as a consequence of breast cancer annually (www.rivm.nl, accessed March 2017). According to the American cancer society in 2017 nearly 252,710 new cases of invasive breast cancer will be diagnosed in women and almost 40,610 women will die as a consequence of breast cancer (www.cancer.org, American Cancer Society: Cancer Facts and Figures 2017, accessed March 2017). Worldwide is breast cancer the most common cancer in women. Nearly 1.7 million new cases were diagnosed in 2012 (http://www.wcrf.org/int/cancer-facts-figures, World Cancer Research Fund International, Cancer facts & figures, Data on specific cancers, accessed April 2017). It is estimated that worldwide more than 508,000 women died in 2011 from breast cancer (Global health estimates, World health organisation 2013, www.who.int, accessed April 2017).

Breast cancer also occurs in men. In the Netherlands in 2015, 99 men were diagnosed with invasive breast cancer (http://www.cijfersoverkanker.nl, accessed May 2017). It is estimated that worldwide 2,470 new cases will be diagnosed in 2017 (www.cancer.org, American Cancer Society: Cancer Facts and Figures 2017, accessed March 2017).

BREAST CANCER RISK FACTORS

Genetic factors, as well as lifestyle factors are involved in the aetiology of breast cancer.

The major risk factor for breast cancer is advancing age. The breast cancer risk for a woman of 30 years old is 1 in 250 in the next 10 years, whereas the risk for a 70 years old woman is 1 in 27 (https://seer.cancer.gov/archive/csr/1975_2007, SEER Cancer Statistics Review, 1975-2007, accessed April 2017).

Lifestyle factors

Women who develop breast cancer are more likely to have higher endogenous or exogenous oestrogen and androgen levels (Pubmed health, https://www.ncbi.nlm.nih.gov/pubmedhealth, accessed June 2017). Women who experienced menarche before or at the age of 11 years have almost 20% higher risk of developing breast cancer compared to those who experienced menarche at age 14 years or older.[1] In the same way, late menopause is also a risk factor for breast cancer.[1] Moreover, Hormone therapy (HT) offered after menopause is shown to be associated with increased risk of breast cancer.[2, 3] Women with dense breasts have increased risk of breast cancer. How higher the degree of density, how higher the breast cancer risk. Women with slightly increased breast density have a relative risk (RR) of 1.79 compared with women who have the lowest breast density. The RR increases up to 4.64 for women with very dense breasts.[4] Other factors such as ionizing radiation and obesity are also shown to increase breast cancer risk. There is a relationship between exposure to ionizing radiation and breast cancer. Breast cancer risk is shown to increase with for example atomic bomb exposure or radiation therapy for example for

lymphoma.[5] Obesity is associated with increased breast cancer risk, particularly among postmenopausal women who do not use hormone therapy.[6] Also Alcohol consumption increases the risk of breast cancer.[7]

Factors which are proven to have an adequate evidence of decrease risk of breast cancer are: early pregnancy, breast feeding and exercise. Childbirth is followed by an increase in risk of breast cancer for several years. A long-term reduction in risk then follows which is greater for younger women.[8] Breast-feeding is associated with a lower risk of breast cancer,[9] and the RR decreases up to 4.3% for every 12 months of breast feeding.[10] Active exercise may reduce breast cancer risk, particularly in young women who have children,[11] in premenopausal women and those of normal or lower-than-normal body weight.[12]

Genetic factors

Breast cancer risk is shown to increase in women with a positive family history. If first-degree relatives are affected the breast cancer risk increases almost two folds.[13] Different models have been developed to calculate the breast cancer for different family members such as 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). Effect of heritable factors is estimated to be up to 27% in breast cancer.[14] This estimate is however, of limited value as it is greatly dependent on the assumed model.[15]

Based on the risk associated with different genetic factors and their allele frequency, different classes can be defined:

High risk

When a genetic factor confers a relative risk higher than 4 times, it is called a high risk gene.[16] Pathogenic variants in the two tumour suppressor genes, *BRCA1* (MIM* 113705), identified in 1994 and *BRCA2* (MIM* 600185), identified in 1995, are known to be associated with high risk of breast and ovarian cancer. Pathogenic variants are variants such as frameshifts or nonsense variants which lead to loss of function of the proteins and are therefore disease-causing. BRCA1 and BRCA2 proteins are known to help repair damaged DNA and are, therefore, important in maintaining the genomic stability. Mutation in these genes which results in production of a non-functional protein or when no protein can be made will eventually lead to accumulation of DNA damage which cannot be properly repaired. As a result, cells are more likely to develop additional genetic alterations that can lead to cancer.[17] Pathogenic variants in *BRCA1* and *BRCA2* are proven to increase the cumulative risk of female breast cancer up to 88% and ovarian cancer up to 68% (depending on the method used for risk calculation and selection criteria)[16, 18, 19] and

they have been associated with increased risks of several other cancers. Increased risk of prostate and pancreatic cancer in *BRCA2* carriers is strongly confirmed.[18, 20-24] There is also evidence for an increased risk of gall bladder, bile duct, stomach cancer and also malignant melanoma, however this evidence is limited.[20-23, 25] It is shown that *BRCA1* carriers have an elevated risks of pancreatic, prostate, testicular and uterine cancer.[23, 26-28] The prostate and pancreatic cancer risks are however, lower than in *BRCA2* carriers. Moreover, male *BRCA2* carriers, and to a lesser extent *BRCA1* carriers, are at an increased risk of developing breast cancer.[23, 29, 30]

Pathogenic variants in *BRCA1* and *BRCA2* together account for about 15 to 20 percent of hereditary breast cancers (www.cancer.gov, accessed March 2017).[31, 32]

Other rare but high penetrant genes for breast cancer include *TP53* (MIM***** 191170),[33, 34] *PTEN* (MIM+ 601728),[35, 36] *STK11* (MIM***** 602216)[37, 38] and *CDH1* (MIM***** 192090)[39] each giving rise to a different clinical syndrome. Together with *BRCA1* and *BRCA2*, it is estimated that these six high-risk genes account for around 25% of hereditary breast cancer cases.[15, 40]

Moderate risk

When the relative risk of breast cancer is increased between 2 to 4-5 times, we speak of a moderate risk. Pathogenic variants in genes such as *PALB2*[41] (MIM* 610355), *ATM*[42] (MIM* 607585) and *CHEK2*[43] (MIM+ 604373) are also shown to increase breast cancer risk (Figure 1) in this range and are therefore known as moderate risk genes. There are several other genes such as *XRCC2* (MIM* 600375), *RAD51C* (MIM* 602774) and *BARD1* (MIM* 601593) in which variants have been shown to be associated with breast cancer susceptibility, but their allele frequency and/or breast cancer risk estimates have not yet been robustly established.

Recently some specific variants in high risk genes such as c.5096G>A, p.Arg1699Gln in *BRCA1* are shown to confer a lower risk compared with the average truncating variants in these genes as explained in the previous section. Their risk is in the same range as the moderate risk genes and are defined as intermediate risk variants.[44-46] Figure 1 shows roughly the genetic landscape of breast cancer with common susceptibility SNPs (single nucleotide polymorphisms) low right on the graph and the moderate-high risk rare variants on the left side of the graph.

Low risk

Genome-wide association studies (GWAS) have resulted in identification of several common, low-risk susceptibility variants (SNPs) associated with breast cancer risk. In the past few years, Breast Cancer Association Consortium (BCAC) as part of the Collaborative Oncological Gene-Environment Study (COGS) identified new risk-associated variants in a large-scale replication study. SNPs were genotyped in over 40,000 breast cancer cases and 40,000 control women, using a custom array (iCOGS, http://ccge.medschl.cam.ac.uk/files/2014/03/iCOGS_detailed_lists_ALL1.pdf, accessed April 2017). This study

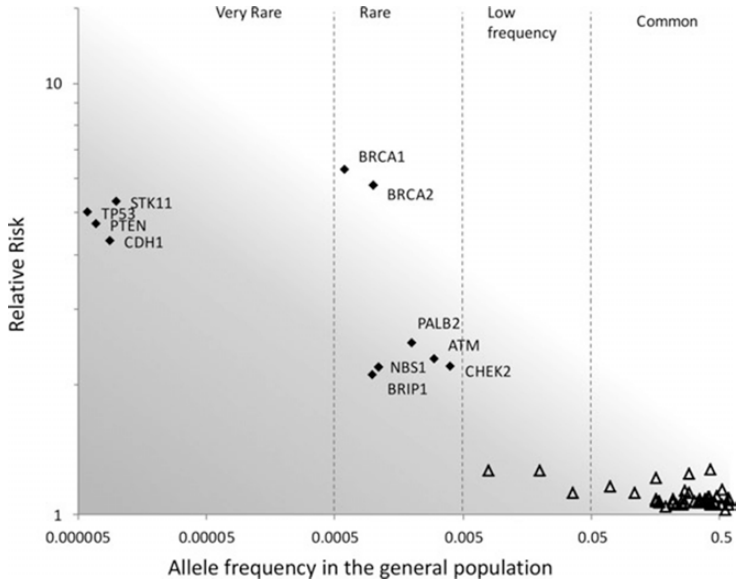

Figure 1. The genetic landscape of breast cancer. This figure shows the allele frequencies in the general population and relative risks for the known breast cancer risk genes and single nucleotide polymorphisms (SNPs). Reprinted from Clinical Genetics, 84, Hilbers FS, Vreeswijk MP, van Asperen C J, Devilee P, The impact of next generation sequencing on the analysis of breast cancer susceptibility: a role for extremely rare genetic variation?, 407-14, Copyright (2013), with permission from John Wiley & Sons.[47]

increased the number of SNPs associated with breast cancer from 27 to more than 70 (Figure 2).[41, 48] Recent literature indicates that single nucleotide polymorphisms are important determinants of personal cancer risk in women carrying a pathogenic variant in *BRCA1* and *BRCA2*[49] but also in moderate risk genes.[16, 50, 51] The term "low risk" is used for variants conferring a risk that is less than moderate (RR<2). It is important to be careful using this term in the medical practice as these variants do not lower the risk. The carriers of such variants still have an increased risk of breast cancer.[16]

As the knowledge about breast cancer risk factors is increasing, especially in genetics, guidelines are being defined based on the stratification of patients according to their cancer risk. The breast cancer risk can be calculated using algorithms and web based tools such as 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).

These risk levels are used to provide guidance to identify women who can benefit from surveillance using regular mammography and/or magnetic resonance imaging (MRI) or risk

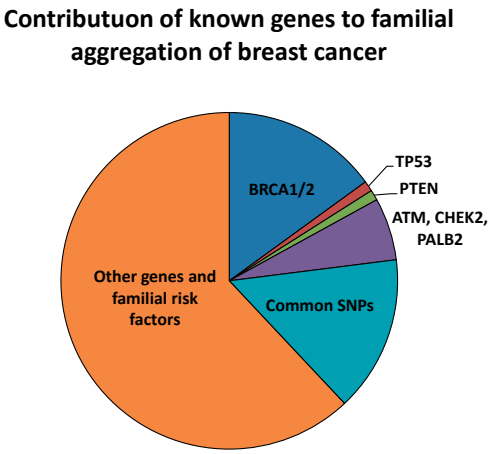

Figure 2. This figure shows the fraction of cases caused by genes known to contain pathogenic variants that predispose to breast cancer and by common genetic risk factors (common SNPs), adapted from Couch FJ. et al[52] and http://discoverysedge.mayo.edu/2015/10/07/breast-cancer-predicting-individual-risk, accessed on April 2017).

reducing measures depending on the national guidelines of the country (www.oncoline.nl, accessed April 2017), (Familial breast cancer: classification, care and managing breast cancer and related risks in people with a family history of breast cancer, www.nice.org.uk/guidance, accessed May 2017), (BRCA1 and BRCA2: Cancer Risk and Genetic Testing, www.cancer.gov, accessed May 2017).

GENETIC COUNSELING

Since identification of *BRCA1* in 1994 and *BRCA2* in 1995 genetic counselling of breast cancer patients gradually started with the aim to identify the individuals who are at high risk of cancer. Identification of the carriers of the pathogenic variants has several benefits:

1. Intensive surveillance and risk reducing surgeries

As the carriers have a high risk on breast and ovarian cancer, they are offered intensive screening programs and/or prophylactic surgeries starting from 25 years as described in the local guidelines (www.oncoline.nl, accessed April 2017), (Familial breast cancer: classification, care and managing breast cancer and related risks in people with a family history of breast cancer, www.nice.org.uk/guidance, accessed May 2017), (BRCA1 and BRCA2: Cancer Risk and Genetic Testing, www.cancer.gov, accessed May 2017).

2. Personalised therapy

The affected individuals who are proven to be carrier of a pathogenic variant in *BRCA1* or *BRCA2* can benefit from personalized treatments with platinum salts (carboplatin and

cisplatin) or poly ADP-ribose polymerase (PARP)-inhibitors. Treatment with platinum has resulted in better progression-free survival and overall survival in patients carrying *BRCA1/2* pathogenic variants due to homologous recombination (HR) deficiency in their tumours. The damaged ability of *BRCA*-deficient tumour cells to repair platinum-induced double-stand breaks (DSBs), results in their increased sensitivity to chemotherapy.[53, 54] In the same way, inhibition of PARP enzymes by PARP-inhibitors in HR-deficient *BRCA1* and *BRCA2* cells leads to DSBs which are subjected to error-prone repair by non-homologous end joining (NHEJ). PARP enzymes repair single-stranded DNA breaks mainly through the base excision repair pathway.[55] The absence of precise DNA-repair mechanisms following PARP-inhibitor treatment in HR-deficient cells leads to synthetic lethality due to the accumulation of DNA damage and will eventually result in cell death.[54, 56, 57]

VARIANTS OF UNCERTAIN SIGNIFICANCE (VUS) IN *BRCA1* AND *BRCA2*

It is more difficult to determine the cancer risk related to other sequence variants such as missense changes, small in-frame insertions and deletions, nucleotide substitutions that do not lead to amino acid changes and alterations in non-coding sequences. These changes are called variants of uncertain clinical significance (VUS).

In a population-based cohort of young women with contralateral (n=705) or unilateral breast cancer (n=1398), 470 unique sequence variants were identified in the *BRCA1/2* genes of which 113 were pathogenic variants. The remaining 357 VUS consisted of 185 missense changes, of which 60% were observed only once and 3% occurred with a frequency of >10%.[58]

In the Netherland, in general genetic screening of *BRCA1/2* is offered when the mutation detection chance is around 10% (www.oncoline.nl, www. www.nice.org.uk, accessed March 2017). Mutation carrier probability can be calculated based on the number and ages of affected individuals in the family. Different algorithms and web-based tools are available which can determine the probability of carriership such as BRCAPRO (http://bcb.dfci.harvard.edu/bayesmendel/brcapro.php, accessed April 2017), BOADICEA (http://ccge.medschl.cam.ac.uk/boadicea/, accessed April 2017) and the BRCA mutation risk calculator (BRCA Risk Calculator, http://www.myriadpro.com, accessed May 2017).

When the threshold of 10% is taken into account, around 10-15% of these tests result in identification of a VUS (personal communication with dr. J.T. Wijnen, molecular clinical geneticist). It has also previously been estimated that about 10% of *BRCA1/2* tests in Caucasians results in a VUS.[59] A higher percentage was reported in African Americans (44.2%)[60] and Hispanics (12%).[61] As more individuals are offered *BRCA1/2* screening, more data is becoming available and because of improvement in classification and communication guidelines the number of individuals receiving a VUS test results is becoming smaller. Myriad genetics claims that of all their *BRCA1/2* tests, only 2.1% is classified as a VUS using an algorithm which is most importantly based on family history.[62]

In the Netherlands there are around 293 unique variants identified in *BRCA1* and 492 in *BRCA2* and over 1,800 families are now known to carry a *BRCA1* and *BRCA2* VUS (personal communication with Frans Hogervorst, molecular clinical geneticist, National working group for Breast Cancer DNA Diagnostics (LOB)).

Almost 1,800 unique VUS are listed in the Breast Cancer Information Core database (http://research.nhgri.nih.gov/bic/, accessed March 2017), however this database is outdated and replaced by other databases such as LOVD and ClinVar (www.ncbi.nlm.nih.gov/clinvar, http://databases.lovd.nl/shared/genes, accessed March 2017).[63] ClinVar is a freely accessible, public archive of reports of the relationships among human variations and phenotypes, with supporting evidence. ClinVar search in April 2017 resulted in about 1,701 unique VUS in *BRCA1* and 2,871 unique VUS in *BRCA2* (https://www.ncbi.nlm.nih.gov/clinvar, accessed March 2017).

Classifying VUS is a great challenge for tailoring genetic counselling and disease prevention strategies. Patients in which a VUS is identified experience considerable psychological distress, not only due to the possibility that they may have a cancer risk as high as that for known pathogenic variants, but also due to the uncertainty of this cancer risk.[64, 65] Not only the person who is carrying the VUS can benefit from classification of the variant but also their relatives can benefit from classification. In case a variant is classified as pathogenic, then the family members will be offered cascade screening. They can be tested for the presence of the pathogenic variant. Carriers can enter screening programs for early cancer detection or consider prophylactic surgery (www.oncoline.nl, accessed April 2017). Moreover, affected carriers can benefit from personalized treatments with platinum agents or PARP-inhibitors.

In case a variant remains unclassified as a VUS, then according to the current guidelines none of the above measures can be offered to the patients and their relatives. For these group of patients and their families and for those in whom no variants are identified, the breast cancer risk is calculated using algorithms and web based tools such as 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), as mentioned previously. They are then stratified to high risk or moderate risk families and will be offered surveillance and/or prophylactic surgeries according to the local protocols as described previously (www.oncoline.nl, accessed April 2017).

CLASSIFICATION OF THE VARIANTS

Assessment of individual VUS-related characteristics

It is difficult to apply the usual genetic approach of linkage/segregation or association analysis for classification of the majority of the VUS because individual VUS are rare.[58] It

is therefore essential that methods would be developed that allow reliable assessment of the clinical significance of VUS and so provide VUS-carriers with the required information to make an informed decision. Reliable classification of VUS would considerably increase the clinical utility and cost-efficiency of DNA testing and alleviate the psychological burden on these families.[64, 65]

Different efforts have been undertaken to classify VUS using the different sources of data, which are listed below. Table 1 shows a summary of the advantages and disadvantages of these characteristics in using variant classification, as previously described by Goldgar et al.[66]

1. In silico analysis of variant characteristics
2. Co-occurrence with a deleterious variant
3. Prevalence of the variant in a control population
4. Cosegregation of the variant and disease within families
5. Clinical features and family history
6. Histopathology and genetic tumour characteristics
7. In vitro RNA analysis
8. Functional analysis

1. In silico analysis of variant characteristics

This analysis focuses on the predicted effect of the nucleotide and/or amino acid change. Amino acids, which are evolutionary strongly conserved across species, are probably residues essential for protein function. A change at that position is expected to seriously affect that function. Nucleotide changes might also be located in regions essential for accurate RNA splicing, and as a consequence might affect the protein function.[67-69] One of the tools which can be used for in silico analysis of the variants is Alamut® software (www.interactive-biosoftware.com/alamut-visual). It integrates several missense variant pathogenicity prediction tools and algorithms such as SIFT (http://sift.jcvi.org, accessed March 2017), PolyPhen (http://genetics.bwh.harvard.edu/pph2, accessed March 2017), AlignGVGD (http://agvgd.hci.utah.edu, accessed March 2017), MutationTaster (http://www.mutationtaster.org/, accessed March 2017) and Human Splicing Finder (HSF) (http://www.umd.be/HSF/, accessed April 2017).

2. Co-occurrence with a deleterious variant

Homozygosity or compound heterozygosity for deleterious variants in *BRCA1/2* are embryonic lethal (*BRCA1*) or associated with severe syndromes not related to breast cancer such as Fanconi Anemia.[59, 70-72] It should be noted that this approach is particularly powerful in identifying neutral variants, but less so in supporting potential pathogenicity of variants (i.e. the lack of co-occurrence does not have much discriminatory power in determining the pathogenicity of a VUS).

3. Prevalence of the variant in a control population

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% of a healthy population strongly argues against its pathogenicity.[73, 74] However, recently the expert panel in ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) consortium (https://enigmaconsortium.org/, accessed April 2017) has reviewed frequency of known pathogenic variants in ExAC and gnomAD population-specific datasets and has suggested adapting this threshold (personal communication with ENIGMA members). Both the Exome Aggregation Consortium (ExAC) and the Genome Aggregation Database (gnomAD) are resources developed by an international alliance of investigators, with the goal of "aggregating and harmonizing exome and/or genome sequencing data from various large-scale sequencing projects, and making summary data available for the wider scientific community" (http://exac.broadinstitute.org/, http://gnomad.broadinstitute.org/, accessed April 2017).

4. Cosegregation of the variant and disease within families

The presence of cosegregation (i.e. variant is present in all affected family members) provides strong evidence for pathogenicity within an autosomal dominant pattern that is normally associated with pathogenic *BRCA1/2* variants. This method can be particularly powerful when more than one family with the same VUS has been detected.

5. Clinical features and family history

Information on the number of first and second-degree relatives affected with breast and/or ovarian cancer, bilateral cancer and age of onset can predict the probability of having a *BRCA1/2* variant and on the basis of this information individual breast cancer risk can be estimated.[75, 76] Individuals carrying deleterious variants are expected to have more severe personal and family cancer histories than individuals carrying benign variants.[62] Based on this premise, and when extensive family data is available, algorithms can be built which can determine the probability of pathogenicity of a variant.

6. Histopathology and genetic tumour characteristics

Histopathology

It is well established that the histological phenotype of *BRCA1*-related breast tumours differ from non-*BRCA1* tumours. The breast tumour in carriers of *BRCA1* pathogenic variants are more likely to be high-grade and are shown to have increased mitotic count, pushing margins, lymphocytic infiltrate and necrosis.[77-79] The histological characteristics of tumours in *BRCA2* carriers are less distinctive compared to *BRCA1*-related breast tumours.[77, 80, 81] However, there are reports which show that *BRCA2* breast tumours are more likely ER (oestrogen receptor) positive, have high grades, less tubule formation and continuous pushing margins when compared to non-*BRCA2* tumours.[78, 80] Based on the commonly

measured histopathological features such as receptor status and grade ascertained from thousands of *BRCA* and non-*BRCA* tumours, likelihood ratio estimates are calculated which can be used for classification of the variants.[80, 82-85]

Loss of heterozygosity

Loss of heterozygosity (LOH) of the wild type allele is a common mechanism of inactivation in tumours of *BRCA1/2* carriers. This observation could potentially be used to classify the VUS.[66, 86] However, Beristain et al in a study saw that not all the pathogenic variants which should have shown LOH in the wild type allele did follow the expected pattern.[87]

They concluded that LOH analysis in a tumour is not suitable to distinguish between neutral and pathogenic variants.[87]

Comparative genomic hybridization (CGH)

BRCA1 protein is involved in the DNA damage response pathway and loss of BRCA1 function will result in the accumulation of DNA damage and chromosomal instability.[17] As a consequence the *BRCA1*-mutated tumours develop a distinct pattern of chromosomal aberration. The same method was used to develop a classifier by array-CGH for the *BRCA2* tumours.[88] Array-Comparative Genomic Hybridization (array-CGH) can be used as an effective method to distinguish *BRCA1* and *BRCA2*-mutated breast tumours from sporadic breast tumours. In 2002 the first CGH (chromosome) classifier for *BRCA1* was developed.[89]

In 2008 Joosse et al introduced a method for classification of breast tumours by array-CGH.[90]

7. *In vitro* RNA analysis

For the production of full-length mRNA, that will be translated into a functional protein, correct RNA splicing is necessary. Splice-site prediction programs have been developed to predict the effect of a variant on RNA splicing. These programs are very reliable in predicting whether variants in canonical sites affect splicing. However, predicting the functional effect variants such as changes in splice enhancers and silencers motifs is much more difficult. In vitro RNA analysis is therefore often essential to determine the effect of the variants on RNA splicing and to determine their clinical significance.[69, 91-94]

8. Functional analysis

As many VUS are rare, clinical and genetic data are usually insufficient to classify a variant. As an alternative approach, in vitro and in vivo assays can be used to study the effect of a VUS on the function of the protein to predict its pathogenicity.

BRCA1 and BRCA2 are multifunctional proteins that interact with tumour suppressors (other (breast) cancer genes) such as PALB2, RAD51, DNA repair proteins, and cell cycle regulators through their different domains.[95, 96]

Some of the functions of BRCA1 have been linked to specific domains of the protein.[97] Roy et al in a review article describe the different domains in BRCA1 and BRCA2 as follows

(Figure 3):[98] BRCA1 protein consists of an N-terminal RING domain that associates with BRCA1-associated RING domain protein 1 (BARD1) and a nuclear localization sequence (NLS). The central part of BRCA1 protein has a CHK2 (CHEK2) phosphorylation site on Serine988 (S988).[99] The C-terminal of BRCA1 protein consists of: a coiled-coil domain that associates with partner and localizer of BRCA2 (PALB2); a serine cluster domain (SCD) that contains approximately ten potential ataxia-telangiectasia mutated (ATM) phosphorylation sites and plays an important role in BRCA1-mediated G2/M and S-phase checkpoint activation and spans amino acid residues 1280–1524;[100, 101] and a BRCT domain that binds ATM-phosphorylated abraxas, CtBP-interacting protein (CtIP) and BRCA1-interacting protein C-terminal helicase 1 (BRIP1). The BRCA1-abraxas complex is associated with BRCA1 recruitment to sites of DNA damage.[102-105] The BRCA1-BRIP1 complex is linked to DNA repair during replication.[106] The BRCA1-CtIP complex causes ataxia-telangiectasia and Rad3-related (ATR) activation and homologous recombination (HR) by associating with the MRN complex and facilitating DNA double-strand break resection.[100] MRN complex itself consists of MRE11, RAD50 and Nijmegen breakage syndrome protein 1 (NBS1).

There are different roles describes for the function of the human BRCA2 protein such as DNA repair and genome stability,[107] control of micronuclei and centrosome amplification,[108] regulation of cell cycle progression,[109] regulatory role in the cytokinesis process[110] and it is shown to be a component and regulator in the midbody structure and function.[111] BRCA2 can be divided to three major regions: the N-terminal which binds PALB2 amino acids 21-39;[112] the eight BRC repeat region between amino acid residues 1009 and 2083 that bind RAD51. The BRCA2 DNA-binding domain consists of a helical domain (H), three oligonucleotide binding (OB) folds and a tower domain (T), which is thought to facilitate BRCA2 binding to both single-stranded DNA and double-stranded DNA;[113] and the C-terminal of BRCA2 contains an NLS and a cyclin-dependent kinase (CDK) phosphorylation site at Serine3291 that also binds RAD51.[114]

Based on the presence of different protein domains and the different functions of the BRCA1 and BRCA2 proteins, many in vitro and in vivo assays have been developed to evaluate the effect of VUS on protein function.[115] However, it is still not completely clear how the results from functional analysis, on their own or in combination with other sources of data, can be used for the classification of the variants.

CLASSIFICATION METHODS

In 2004, Goldgar et al introduced a multifactorial likelihood model (MLM) for the classification of the VUS in *BRCA1* and *BRCA2* in which the likelihoods of pathogenicity, obtained from various sources of data, could be combined. In general, when a VUS reached odds higher than 1,000:1 in favour of pathogenicity, it could be classified as pathogenic. In order to classify a variant as neutral, the threshold was set at 100:1 against pathogenicity. This threshold is set lower compared to the threshold in favour of pathogenicity. That is because declaring a variant as neutral has a less critical consequence for the patients and their families.[66]

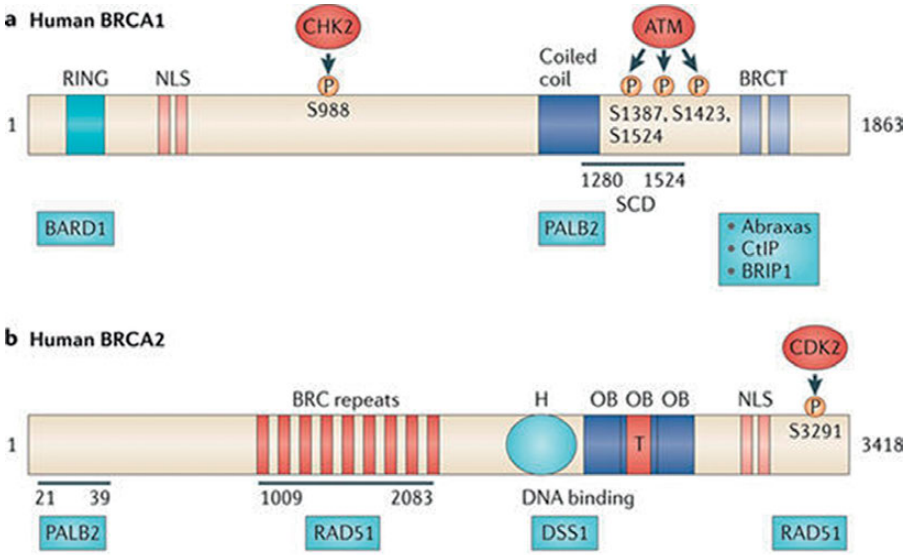

Figure 3. BRCA1 and BRCA2 functional domains. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Nat Rev Cancer. 2011 Dec 23;12(1):68-78.), copyright (2011).[98]

Table 1. Types of evidence potentially useful for variant classification. Reprinted and adapted from The American Journal of Human Genetics, 75, Goldgar DE, Easton DF, Deffenbaugh AM, Monteiro ANA, Tavtigian S, Couch FJ, Integrated Evaluation of DNA Sequence Variants of Unknown Clinical Significance: Application to BRCA1 and BRCA2, 535-544, Copyright (2004), with permission from Elsevier.[66]

Line of Evidence	Advantage(s)	Disadvantage(s)
In silico analysis of variant characteristics	• Can be applied to every possible missense change in the *BRCA1* and *BRCA2* genes • Does not require extensive family history • Complete conservation is predictive if enough evolutionary time sequence is available	• Indirectly related to disease risk • The magnitude of likelihood ratios is generally not sufficient to classify variants without additional information
Co-occurrence with a deleterious variant	• If homozygotes and compound heterozygotes are assumed to be embryonically lethal (or vanishingly rare), a variant can often be classified as neutral on the basis of a single observation	• Much less power to show causality • Quantification is dependent on the assumed fitness of the homozygous genotype, which is not known with precision

Table 1. (continued)

Line of Evidence	Advantage(s)	Disadvantage(s)
Prevalence of the variant in a control population	• Provides a direct estimate of associated cancer risk	• Variants are rare, so such studies would need to be very large
Cosegregation of the variant and disease within families	• Easily quantifiable and directly related to disease risk • Not susceptible to uncertainties in variant frequencies or population stratification	• Requires sampling of additional individuals in the pedigrees (particularly affected)
Clinical features and family history	• Usually available for most variants without additional data or sample collection • Potentially very powerful	• Depends on family ascertainment scheme • Power may be low for rare variants
Histopathology and genetic tumour characteristics	• Potentially powerful for *BRCA1* tumours in which the pathological characteristics are quite distinct	• Prediction is weak when routine pathology data are used, especially for *BRCA2* • Systematic evaluation requires tumour material
In vitro RNA analysis	• Powerful to show causality when there is no wild type transcript from the variant allele	• Specific blood samples are required • RNA splicing might be different in different tissues
Functional analysis	• Can evaluate the variant's effect on the protein functions of the *BRCA1* and *BRCA2*	• Not all the functions of the BRCA1/2 are known. A variant being neutral in a specific assay does not necessarily mean that it has no effect on cancer risk • Function tested might not be related to cancer causation • Still needs to be validated

In 2008, prior probability of pathogenicity of a variant based on its position and function was added to the MLM model[116, 117] (Figure 4). In this method, in essence, for each line of evidence, as mentioned previously, the Likelihood Ratio (LR) is calculated. 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.[118] To determine the "overall likelihood" for pathogenicity versus non-pathogenicity of a specific VUS, the LRs for the VUS from each independent component of the model are multiplied together. Using this approach, such likelihood ratios from different studies, if it is not originating from the same data and provided

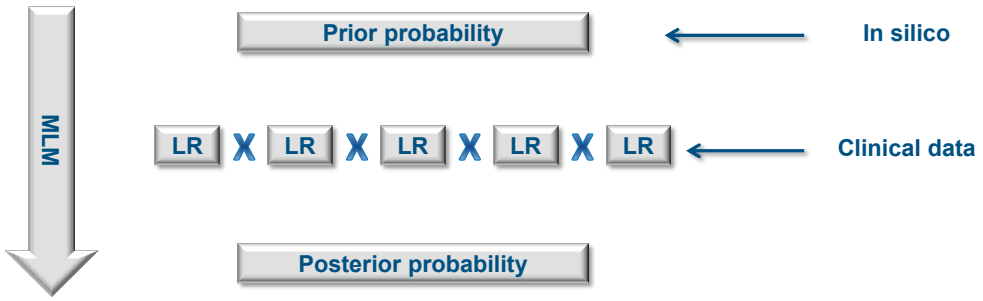

Figure 4. Multifactorial likelihood model (MLM). Posterior probability of pathogenicity = posterior odds/ (posterior odds + 1) and posterior odds= Overall LR × (prior probability/[1– prior probability]).

that the datasets are independent, can be multiplied to generate updated likelihood ratios. Then the probability of pathogenicity based on in silico data which is calculated previously based on position and conservation of the mutated nucleotide can be added to the calculation as the "prior probability" (Table 2).

The in silico based "prior probability" and the "overall likelihood" estimates can be used to determine the "posterior probability" of a VUS being pathogenic, through first determining the "Posterior Odds of pathogenicity" by using this formula: **Posterior Odds = Likelihood ratio × [prior probability/(1-prior probability)].** Then the posterior probability of pathogenicity is calculated using Bayes theorem: **Posterior Probability = Posterior Odds /(Posterior Odds + 1).**[119] The scale of posterior probability is between 0 and 1.00 and is often expressed as a percentage.

Table 2. Prior probabilities associated with VUS graded by Align-GVGD or based on the position of the VUS. Reprinted and adapted from Human Mutation,33, Lindor NM, Guidugli L, Wang X, Vallee MP, Monteiro AN, 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), 8-21, Copyright (2011), with permission from John Wiley & Sons.[119]

Align-GVGD grade or the position of the variant	Prior Probability	95% Confidence Interval
C65	0.81	(0.61-0.95)
C35-C55	0.66	(0.34-0.93)
C15-C25	0.29	(0.09-0.56)
C0	0.03	(0.00-0.06)
Splicing consensus site alteration	0.96	(0.91-1.00)
Intronic variants outside the consensus dinucleotides	0.26	(0.15-0.39)

There was for a long time no consensus as how to handle the diagnosis of a VUS, in the clinical practice, in the *BRCA1/2* genes. In 2007, the UK Clinical Molecular Genetics Society together with the Dutch Society of Clinical Genetics Laboratory Specialists proposed a four class system for reporting the variants: (I) certainly not pathogenic, (II) unlikely to be pathogenic, (III) likely to be pathogenic, and (IV) certainly pathogenic "Good Practice Guidelines for the Interpretation and Reporting of Unclassified Variants in Clinical Molecular Genetics Laboratories" [Bell et al, 2007] (Table 3).

In 2008, the American College of Medical Genetics (ACMG) proposed a six-class system for interpretation and reporting of sequence variants: (1) sequence variation is previously reported and is a recognized cause of the disorder; (2) sequence variation is previously unreported and is of the type that is expected to cause the disorder; (3) sequence variation is previously unreported and is of the type which may or may not be causative of the disorder; (4) sequence variation is previously unreported and is probably not causative of disease; (5) sequence variation is previously reported and is a recognized neutral variant; and (6) sequence variation is previously not known or expected to be causative of disease, but is found to be associated with a clinical presentation.[120] The emphasis of this system is on appropriate reporting of sequence variations using standardized terminology and established databases. However, although both these two systems use basically the same *BRCA1* or *BRCA2* characteristics for variant classification, neither of them recommended using quantitative information for the classification and clinical management of variants. An expert working group, assembled at IARC (International Agency for research in Cancer, http://www.iarc.fr) in 2008, proposed a quantitative classification system applicable to variants in high risk cancer predisposition genes such as *BRCA1*, *BRCA2*, *MLH1* (MIM* 120436), and *MSH2* (MIM* 609309). This classification system interprets posterior probability from the MLM and translates these to recommendations for clinical practice (Table 4).[121]

Table 3 Bell's classification system.

Class	Description
I	Certainly not pathogenic
II	Unlikely to be pathogenic but cannot be formally proven
III	Likely to be pathogenic but cannot be formally proven
IV	Certainly pathogenic

Table 4 IARC classification system for sequence variants identified by genetic testing and recommendations associated with each class of variant. Reprinted and adapted from Human Mutation, 29, Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, Hogervorst FB, Hoogerbrugge N, Spurdle AB, Tavtigian SV, Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results, 1282-91, Copyright (2008), with permission from John Wiley & Sons.[121]

Class and description	Posterior probability of pathogenicity	Clinical testing	Surveillance recommendations if at-risk relative is positive	Research testing of family members
5 Definitely pathogenic	>0.99	Test at risk relatives for variant	Full high risk surveillance	Not indicated
4 Likely pathogenic	0.95-0.99	Test at risk relatives for variant	Full high risk surveillance	Maybe helpful to further classify variant
3 Uncertain	0.05-0.949	Do not use for predictive testing in at-risk individuals	Based on family history (and other risk factors)	Maybe helpful to further classify variant
2 Likely not pathogenic or of little clinical significance	0.001-0.049	Do not use for predictive testing in at-risk individuals	Treat as “no mutation detected” for this disorder	Maybe helpful to further classify variant
1 Not pathogenic or of no clinical significance	<0.001	Do not use for predictive testing in at-risk individuals	Treat as “no mutation detected” for this disorder	Not indicated

AIM OF THIS THESIS

This thesis is aimed at improving the classification of the variants of uncertain clinical significance in the *BRCA1/2* genes. Furthermore, it describes the optimization and standardisation of guidelines for communication of the VUS with the counselees in clinical practice.

OUTLINE OF THIS THESIS

In this thesis, an introduction to hereditary breast cancer, *BRCA1/2* genes, variants of uncertain significance and different classification methods and guidelines are described in **chapter 1**.

As mentioned above, previously a four-class system according to Bell (Table 3) was applied in most Dutch DNA diagnostic laboratories. The results of the classification of VUS based on only in silico characteristics was studied and compared to the results of classification when additional information was used (**chapter 2**). The results showed that VUS assigned to class III more frequently showed in silico indications of a pathogenic effect than class II VUS. Of the 46 VUS assigned to class II by in silico analysis alone, nearly half were eventually re-categorised as class I and 10% as class III when additional information was included. As in silico analysis alone is not always sufficient to unambiguously assign VUS to either class II or class III, the possibility of obtaining additional information from a family should be taken into account during the decision process preceding the communication of a VUS test result.[122]

The paper in **chapter 3** describes the cancer risks associated with the missense variant c.5096G>A, p.Arg1699Gln (R1699Q) in *BRCA1* in a large group of families ascertained internationally.[45, 46] The results showed that the risks associated with this variant, breast cancer: 20% and ovarian cancer: 6%, are lower than for the average truncating *BRCA1* variants and that this variant can be classified as an intermediate risk variant. Furthermore, cancer risks in families with this intermediate risk variants are likely to be influenced by additional genetic factors. Based on these risks recommendations for clinical management for female carriers were proposed.

In **chapter 4** mutation prediction performance of BOADICEA (http://ccge.medschl.cam.ac.uk/boadicea, accessed April 2017), BRCAPRO (http://bcb.dfci.harvard.edu/bayesmendel/brcapro.php, accessed March 2017) and Myriad *BRCA* risk calculator (http://www.myriadpro.com/brca-risk-calculator/calc.html, accessed March 2017) was tested in a large cohort of Dutch male breast cancer patients. The numbers of observed versus predicted mutation carriers were compared and the area under the receiver operating characteristic (ROC) curve (AUC) for each model was assessed. The results support the use of both BRCAPRO and BOADICEA for determining the probability of carrying a *BRCA1* or *BRCA2* pathogenic variants in MBC patients. Freely available, reliable prediction models such as BOADICEA and BRCAPRO play an important role in improving clinical care, especially in countries with limited health care resources.[123] Furthermore, the proven

prediction accuracy of both BOADICEA and BRCAPRO for *BRCA* carriership in males underlines the reliability of other function of these models which is the prediction of overall breast cancer risk.

Information on array-CGH in addition to other data based on different lines of evidence was used to (re)classify some of the most common *BRCA1* variants in the Netherlands (Figure 4). For the classification of the variants mainly in silico data, cosegregation of the variant and disease within families, histopathological tumour characteristics were used. Where available the results of classification were compared with functional analysis which is performed by our colleagues in the Netherlands Cancer Institute (NKI) in Amsterdam (**chapter 5**) (manuscript in preparation).

To improve the clinical utility of the current IARC classification system,[121] a pragmatic adaptation to clinical practice was suggested in **chapter 6**. The suggestion is that the laboratory specialists divide VUS class 3 into two subgroups: class 3A with a posterior probability of 0.05 to 0.499 and class 3B with a posterior probability of 0.5-0.949. The counsellors could then consider to communicate and test family members when the posterior probability of pathogenicity of a VUS is higher than 0.5 (i.e. category 3B) but not communicate variants in class 3A unless there is clinical benefit for counselee or for research. The purpose of the recommendations is to improve the clinical management of the counselees by a more precise classification of the variants without causing unnecessary stress for the counselees or additional costs for the health care system, while minimizing the risk of missing pathogenic variants in clinical practice.[124]

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

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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

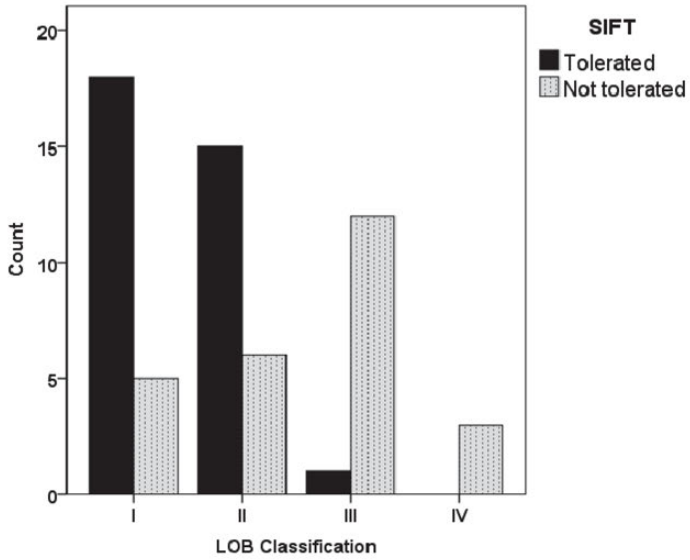

Figure 1. Sorting intolerant from tolerant (SIFT)-analysis of different Variants of Uncertain Significance classes classified by the National working group for Breast Cancer DNA Diagnostics (LOB). The bars represent the outcome of the SIFT-analysis depicted as tolerated or not tolerated.

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

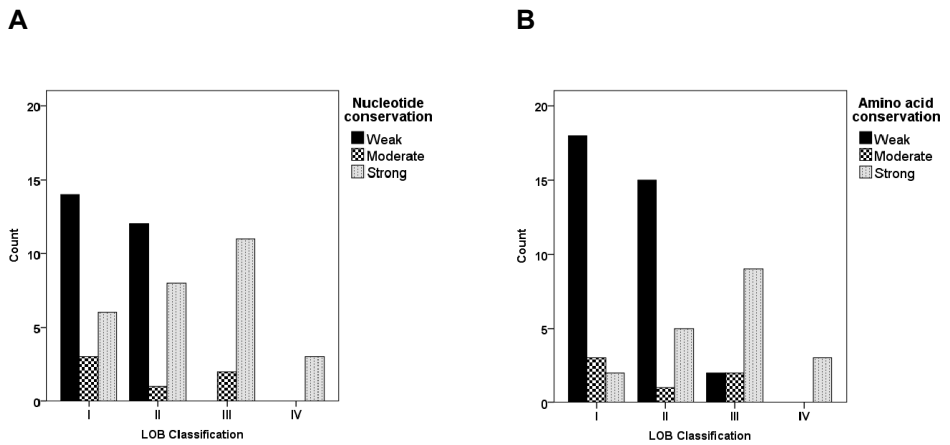

Figure 2. The nucleotide and amino acid conservation per Variants of Uncertain Significance class classified by the National working group for Breast Cancer DNA Diagnostics (LOB). (A) Nucleotide conservation based on Alamut output, which includes alignments of most published sequences and functional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

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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.

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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	

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The *BRCA1* c.5096G>A p.Arg1699Gln (R1699Q) intermediate risk variant: breast and ovarian cancer risk estimation and recommendations for clinical management from the ENIGMA consortium

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3

ABSTRACT

Background

We previously showed that the *BRCA1* variant c.5096G>A p.Arg1699Gln (R1699Q) was associated with an intermediate risk of breast cancer (BC) and ovarian cancer (OC). This study aimed to assess these cancer risks for R1699Q carriers in a larger cohort, including follow-up of previously studied families, to further define cancer risks and to propose adjusted clinical management of female *BRCA1**R1699Q carriers.

Methods

Data were collected from 129 *BRCA1**R1699Q families ascertained internationally by ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) consortium members. A modified segregation analysis was used to calculate BC and OC risks. Relative risks were calculated under both monogenic model and major gene plus polygenic model assumptions.

Results

In this cohort the cumulative risk of BC and OC by age 70 years was 20% and 6%, respectively. The relative risk for developing cancer was higher when using a model that included the effects of both the R1699Q variant and a residual polygenic component compared with monogenic model (for BC 3.67 vs 2.83, and for OC 6.41 vs 5.83).

Conclusion

Our results confirm that *BRCA1**R1699Q confers an intermediate risk for BC and OC. Breast surveillance for female carriers based on mammogram annually from age 40 is advised. Bilateral salpingo-oophorectomy should be considered based on family history.

INTRODUCTION

In 2008, the International Agency for Research on Cancer (IARC) proposed a standardised five-tier classification system applicable to sequence-based results in highly penetrant cancer predisposition genes and linked the likelihood of pathogenicity to clinical actions.[1] The multifactorial likelihood model (MLM) is commonly used to calculate the probability of pathogenicity[2] of individual *BRCA1* and *BRCA2* variants. It is used in the IARC five-tier classification system to categorise each variant into a specific class. The MLM combines complementary sources of data (ie, physicochemical proper- ties,[3] family history,[4] cosegregation of the variant with disease in a family[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) variants.

The *BRCA1* variant c.5096G>A p.Arg1699Gln (hereafter termed *BRCA1**R1699Q) was initially classified as class 3 (variant of uncertain significance) using the MLM method.[1] A subsequent study[7] included functional assays to assess pathogenicity, but did not yield conclusive results. Indeed this variant, located in the *BRCA1* carboxyl terminal region of the transcriptional transactivation domain, and at the interface of the phosphopeptide binding region, demonstrated ambiguous behaviour in a variety of functional assays, when compared with the pathogenic *BRCA1* variant c.5095C>T p.Arg1699Trp (*BRCA1**R1699W) at the same residue, wild-type *BRCA1* and other known pathogenic missense variants.[7] Other models based on family history analysis of *BRCA*-ness[8] or cosegregation within a family[5] also gave inconclusive results.

In 2012, members of the ENIGMA consortium (Evidence- based Network for the Interpretation of Germline Mutant Alleles)[9] reported on the family histories of 69 families carrying *BRCA1**R1699Q.[10] Comparison of *BRCA1* carrier prediction scores of probands using the BOADICEA risk prediction tool[11] showed that *BRCA1**R1699Q variant carriers had family histories that were less 'BRCA1-like' than *BRCA1**R1699W carriers but more 'BRCA1-like' than BRCA-X families (families with no detectable *BRCA1* or *BRCA2* pathogenic mutation). Second, modified segregation analysis was used in a subset of 30 families and showed lower risks of breast cancer (BC) or ovarian cancer (OC) (estimated cumulative risk to age 70: 24%) than *BRCA1**R1699W (58%) and the 'average' pathogenic *BRCA1* truncating variant (68%).[10] Due to the relatively small number of families with cosegregation data in that study, age-specific cancer risks could not be established with a high degree of precision.

The aim of the present study was to update the BC and OC risk estimates associated with *BRCA1**R1699Q in a larger series that included newly identified families, as well as some of the previously studied families, which had been updated with cosegregation data as a result of cascade screening. Based on these results, we propose recommendations for the clinical management of the carriers and their family members.

MATERIALS AND METHODS

Data collection

All families participating in this study included one or more individuals referred to a cancer family clinic because of a personal history of BC and/or OC, and/or a family history consistent with hereditary BC and/or OC.

Each index case had a confirmed *BRCA1**R1699Q variant. ENIGMA members, including those from centres that had contributed pedigrees to the previous study, were asked to provide updated pedigrees (if possible) and additional families segregating *BRCA1**R1699Q identified after the close of enrolment of the previous study. Pedigrees and patient-specific data such as ages at diagnoses and genotypes were collected from a total of 129 families from 11 different countries, of which 91 families had at least one additional person genotyped, and were thus informative for estimating BC and OC risks. From these 91 families, 30 had been included in the segregation analysis in our previous study[10] (see online supplementary table S1). When ages of diagnosis were missing, we conservatively assumed them to be age 65, and for unaffected women we imputed their age using other pedigree members using the PedPro suite of programs (www.bjfenglab.org, accessed 21 September 2016).

Statistical analysis

Data sets

In order to account for ascertainment bias, the likelihood of the pedigree phenotypes and *BRCA1**R1699Q genotypes was calculated conditional on the pedigree phenotypes and the *BRCA1**R1699Q genotype of the index case. Cancer risks were estimated using the following data sets:

The primary analysis (hereafter termed main analysis) included all 129 informative pedigrees from both the previous study and the present recruitment. The second analysis (subanalysis 1) was similar to the main analysis, except that for the genotypes and phenotypes from the previous study only information gathered since the previous study is included. In this analysis, the likelihood was conditioned on the genotype of the index case and pedigree phenotypes of the new families and all genotypes and pedigree phenotypes in the previous pedigrees as they were in the previous analysis in 2012. In fact the index patients carrier status and affected status are not used to estimate the hazard/ risk ratios on which the cumulative risks are based. The last analysis (subanalysis 2) included only the 60 pedigrees that were recruited for this study. Data from subanalyses 1 and 2 are shown in the online supplementary materials.

Cancer risk estimation methods

BC and OC risks were estimated using modified segregation analysis with the MENDEL package of programs.[12] For each data set, the analysis was performed under each of the following assumptions: (1) the relative risk (RR) across age groups was assumed to be constant; and (2) the RR was assumed to be a continuous, piecewise linear function of age,

which was constant before age 40 years and after age 60 years and linear between ages 40 and 60 years. For both models, baseline population incidence rates were assumed to be those for the UK 2003–2007 (Cancer Incidence in Five Continents Reports (IARC-WHO; update November 2010).[13]

For both these analyses we first used a model assuming a single major gene only (the *BRCA1**R1699Q variant) and second a model that included the major gene and a polygenic background effect. From the resulting estimates of BC and OC relative risk, age-specific cumulative risk estimates were calculated based on the cumulative incidence A(t): F(t)=1 − exp(A(t)), and the corresponding CIs were calculated using a parametric bootstrap with 5000 replications.[14]

RESULTS

Descriptive characteristics of the cohort

Our cohort included 129 separate families with a total of 4024 family members, from whom 309 women were proven *BRCA1**R1699Q carriers and 173 were proven non-carriers. For 91 families, in addition to genotyping data of the proband, at least one additional genotype was available (see online supplementary table S2). Descriptive characteristics of the cohort about BC and OC cancer history and age distribution are listed in the online supplementary table S2.

BC and OC risks

Online supplementary figure S1 and supplementary table S2 show the age distribution for BC and OC for the female carriers. The sharpest increase of BC occurred between ages 40 and 49. For OC this was between ages 50 and 59. The youngest case of BC was diagnosed at age 25, for OC this was age 35.

Cumulative risks for this variant by age 70 years are estimated to be 20% (95% CI 13% to 32%) for BC and 6% (95% CI 3% to 25%) for OC. The risks are lower than for high-risk *BRCA1* truncating variants and higher than for the general population in all the three data sets. Figure 1 shows the corresponding curves for the main analysis. Online supplementary figures S2 and S3 and supplementary tables S3 and S4 show comparable results for all the data sets under both assumptions.

Effect of other genetic factors on cancer risks

In order to study the effect of other (genetic) factors on risk, HRs were calculated based on the 'major gene only' model and the 'major gene and polygenic' model under both assumptions.

For the main analysis, HRs for BC are higher in the major gene plus polygenic model compared with the major gene only model, both when assuming constant RR across age groups, and when modelled as a continuous piecewise linear function of age.

HRs for OC are higher in the major gene plus polygenic model when assuming constant RR. When assuming RR as a continuous, piecewise linear function of age, the HR is higher

for the major gene plus polygenic model when the individual is older than 60 years old, suggesting that modifiers might be especially important for the late-onset disease (table 1). Online supplementary table S5 shows the HRs for the subanalyses.

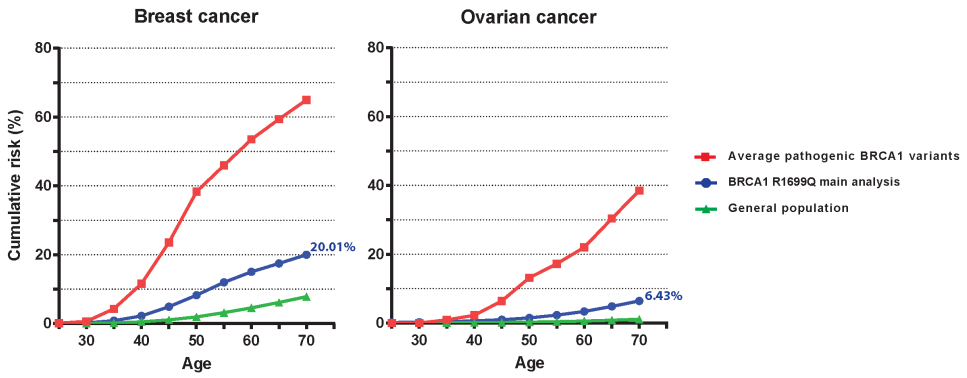

Figure 1. Cumulative risks (%) for breast cancer (left graph) and ovarian cancer (right graph) by age for carriers of *BRCA1**R1699Q based on the main analysis (blue line). The corresponding curves or the cumulative risk conferred by average pathogenic *BRCA1* variants (red line) and for the general population (green line) are also shown. Cumulative risks are calculated using segregation analysis, major gene model assuming relative risk as a continuous, piecewise linear function of age.

Table 1. Modified segregation analysis results from MENDEL in the main analysis a) assuming constant relative risk across age groups and b) assuming relative risk as a continuous, piecewise linear function of age.

	Model	HR (a)	Age	HR (b)
Breast	Major Gene Only	2.83 (1.76, 4.57)	< 40	4.72 (2.22, 10.02)
			> 60	1.75 (0.75, 4.05)
	Major and Polygenic	3.67 (1.97, 6.81)	< 40	5.05 (2.07, 12.34)
			> 60	2.71 (1.09, 6.75)
Ovarian	Major Gene Only	5.83 (2.19, 15.49)	< 40	5.91 (0.58, 60.20)
			> 60	5.81 (1.80, 18.76)
	Major and Polygenic	6.41(2.19, 18.75)	< 40	5.39 (0.48, 61.10)
			> 60	6.75 (1.96, 23.22)

DISCUSSION

After publication of the study by Spurdle *et al*[10] in 2012, many cancer clinics started offering cascade screening to relatives of carriers of the *BRCA1**R1699Q variant. However, in the absence of robust estimates of cancer risks, it was not clear whether available guidelines for *BRCA* carriers would also be suitable for female carriers of *BRCA1**R1699Q.

The cumulative risks estimated from the main analysis and the two subanalyses were lower than for the average *BRCA1* truncating pathogenic variant, yet still substantially higher than the rates in the general population. Cumulative risk by age 70 years was estimated to be 20% (95% CI 13% to 32%) for BC and 6% (95% CI 3% to 25%) for OC.

Our results strongly confirm our previous findings that this variant has reduced penetrance,[10] and can thus be termed an intermediate risk variant conferring risks lower than that for the average pathogenic variant in a high-risk cancer predisposition gene. These risk estimates are consistent with those reported for disease-associated variants in so-called 'moderate risk' genes, defined as genes in which pathogenic variants have an RR between 2 and 5. [15, 16]

Interestingly, our results show that the estimated HRs are in general slightly higher when the 'major gene plus polygenic' model is used compared with the 'major gene only' model, which is especially evident in the late-onset disease (>60 years) group. This means that in addition to *BRCA1**R1699Q, other genetic and/or environmental factors seem to contribute to the magnitude of the BC and OC risk in carriers. Indeed, recent literature[15-17] indicates that single nucleotide polymorphisms are important determinants of personal cancer risk in women carrying a deleterious disease-associated variant especially in moderate risk genes. As those factors are mostly unmeasured or unknown, an indirect estimation of clustering of risk factors can be deduced taking the family history into account. This is particularly relevant to consider when deciding surveillance for healthy relatives who are non-carriers of deleterious variants in the moderate risk genes, or non-carriers of intermediate risk variants in 'high-risk cancer predisposition genes' such as *BRCA1* or *BRCA2*.

The relevance of these findings for clinical management of *BRCA1**R1699Q carriers and their relatives was considered during the Clinical Working Group meeting at the April 2016 ENIGMA conference, held in Prague, which was attended by 38 members with expertise in laboratory research, statistics and clinical genetics. Recommendations for *CHEK2* c.1100delC carriers[17, 18] and country-specific guidelines including Oncoline (The Netherlands: http://www.oncoline.nl, accessed 21 September 2016), National Institute for Health and Care Excellence (UK: https:// www.nice.org.uk, accessed 21 September 2016) and National Comprehensive Cancer Network (USA: https://www.nccn.org, accessed 21 September 2016) were used as a framework to guide discussion. A consensus and majority-based discussion led to the following opinions and recommendations:

Female non-carriers of *BRCA1**R1699Q from BRCA1*R1699Q families

Surveillance should depend on (family) history of cancer, for example, on the risk calculated using programs like BOADICEA.[11]

Female carriers of *BRCA1**R1699Q

A cumulative risk of BC (20% (95% CI 13% to 32%)) does not by itself justify preventive mastectomy or breast MRI. Breast surveillance for female carriers based on annual mammogram from age 40 up to 50 years and inclusion in population screening afterwards is advised.

Combining with family history, the BC risk might be estimated to be higher than the risk conferred by the variant alone. If this is the case, the surveillance advice for *BRCA1**R1699Q carriers can be 'overruled' by the higher family history risk and additional genetic testing can be considered.

The specific genes included will vary across countries dependent on testing practices, which incorporate availability and extent of panel-based testing, eligibility for health insurance or state-based testing, clinical guidelines for ascertainment including number and types of cancer reported in families, etc (ENIGMA, unpublished findings). Genetic testing for variants in other genes using a panel approach for a range of BC/OC susceptibility genes may offer some additional genotype-based information about risk in those cases; however, penetrance estimates for the majority of other genes beyond *BRCA1*, *BRCA2* and *PALB2* are imprecise.[16] Furthermore, it is still unclear how genetic risks are best combined to produce more accurate, individualised, risk estimates.

The *BRCA1**R1699Q variant carriers have lower OC risk (6% (95% CI 3% to 25%)), compared with that for *BRCA1* carriers (39% (95% CI 22% to 51%)) and *BRCA2* carriers (11% (95% CI 4.1% to 18%)).[19] Bilateral salpingo-oophorectomy (BSO) is the standard preventive treatment in the Netherlands for high- risk pathogenic variant carriers, performed at age 35–40 for *BRCA1* and 40–45 for *BRCA2* (http://www.oncoline.nl). Routine surveillance for OC is not effective and is no longer offered to carriers.[20] The magnitude of OC risk for R1699Q carriers suggests that BSO, if performed, may be postponed until age 50. We advise BSO surgery should be offered at age 50, based on the age-related cumulative risks for OC obtained from the study. The cumulative lifetime risk of OC for someone in the general population is approximately 1.5%, but the vast majority of risk occurs after 50 years of age. From our study the cumulative OC risk for *BRCA1**R1699Q carriers by age 50 is lower than the cumulative population risk for OC and rises significantly after age 55. Although BSO surgery could be offered at any age after the genetic risk is identified, we base our guidance on a pragmatic balance between cancer prevention and minimum adverse effects from early oestrogen deprivation, achieved if the surgery is timed around the current average age for the menopause in the Western society (52 years).

However, as for BC risk management, and considering the wide CI for the estimated risk of OC, information about cancer history in the family should be taken into account for decision making.

CONCLUSION

Our analysis of a large cohort of 129 families, using several analytical approaches, confirms that the *BRCA1**R1699Q variant is associated with intermediate cancer risks (compared

with the average *BRCA1* truncating variant). It also provides evidence that cancer risk in carriers is likely to be influenced by other genetic factors. Based on our findings, we propose recommendations for the clinical management of *BRCA1**R1699Q carriers and non-carriers. We recommend that follow-up and screening in these families are performed in a research setting in order to enable future assessment of the utility of the proposed surveillance.

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SUPPLEMENTARY DATA

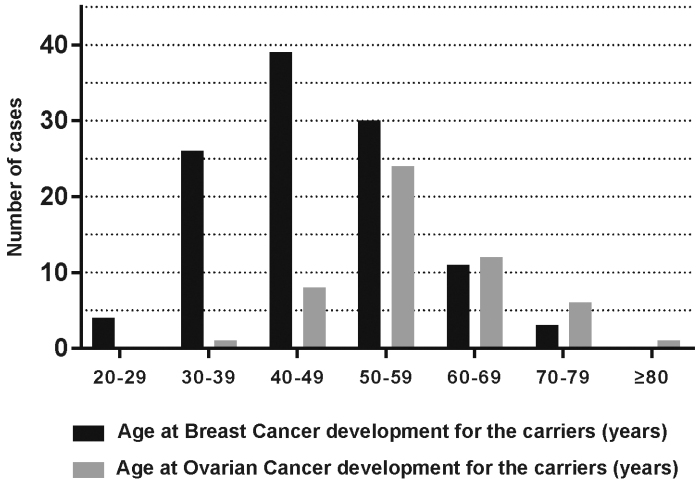

Figure S1. Age at breast cancer development (black bars) and ovarian cancer development (grey bars) for the carriers of *BRCA1**R1699Q.

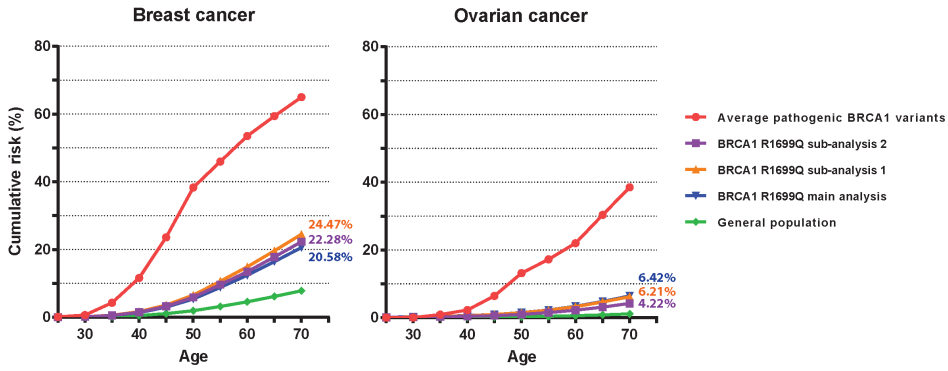

Figure S2. Cumulative risks (%) for breast cancer (left graph) and ovarian cancer (right graph) by age for carriers of *BRCA1**R1699Q based on the main analysis (blue line), sub-analysis 1 (orange line) and sub-analysis 2 (purple line). The corresponding curves or the cumulative risk conferred by average pathogenic *BRCA1* variants (red line) and for the general population (green line) are also shown. Cumulative risks are calculated using segregation analysis, major gene model assuming constant relative risk.

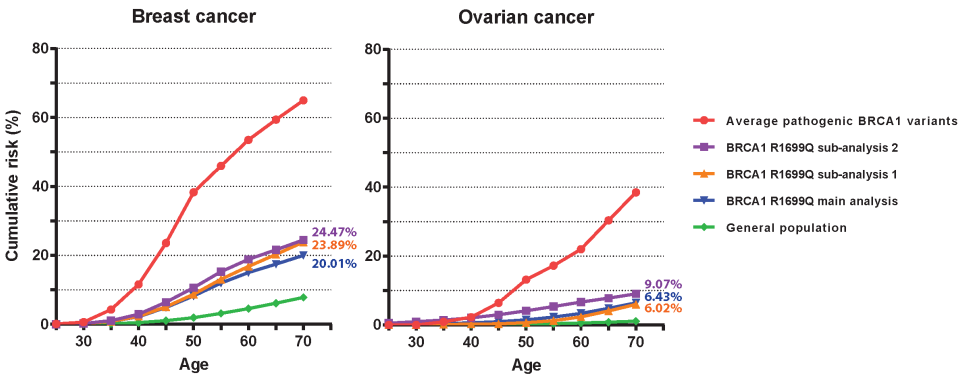

Figure S3. Cumulative risks (%) for breast cancer (left graph) and ovarian cancer (right graph) by age for carriers of *BRCA1**R1699Q based on the main analysis (blue line), sub-analysis 1 (orange line) and sub-analysis 2 (purple line). The corresponding curves or the cumulative risk conferred by average pathogenic *BRCA1* variants (red line) and for the general population (green line) are also shown. Cumulative risks are calculated using segregation analysis, major gene model assuming relative risk as a continuous, piecewise linear function of age.

Table S1. Number and origin of families in the previous study and current study (previous plus newly included families) .

	Previous study[10]		Current study	
Country	# Families	# Families with additional genotyping (*)	# Families	# Families with additional genotyping (*)
Australia	6	2	6	2
The Netherlands	12	3	20	15
Belgium	3	2	8	6
Denmark	10	4	22	19
France	5	3	14	7
Germany	5	1	19	10
South Africa	1	1	1	1
Sweden	14	5	20	17
Switzerland	0	0	1	1
United Kingdom	4	2	4	2
U.S.A.	9	7	14	11
Total	69	30	129	91

(*): additional genotyping means at least one other relative tested in addition to the index.

Table S2. Descriptive characteristics of the 129 families.

	Unknown Carriership			Non-carriers			Carriers		
Age	Total	BC[#]	OC[&]	Total	BC	OC	Total	BC	OC
<30	2935	1	0	100	0	0	105	4	0
30-39	89	18	2	8	1	0	37	26	1
40-49	94	26	5	27	5	0	60	39	8
50-59	124	40	26	24	4	1	53	30	24
60-69	122	36	15	6	2	0	39	11	12
70-79	96	16	7	4	2	1	11	3	6
>=80	82	10	2	4	1	0	4	0	1
Total	3542	147	57	173	15	2	309	113	52

#BC: Breast cancer
&OC: Ovarian cancer

Table S3. Cumulative risk (95% Confidence Interval) using segregation analysis, major gene models assuming constant relative risk.

	Main Analysis Cumulative risk (95% Confidence Interval)		Sub-Analysis 1 Cumulative risk (95% Confidence Interval)		Sub-Analysis 2 Cumulative risk (95% Confidence Interval)	
Age	Breast cancer	Ovarian cancer	Breast cancer	Ovarian cancer	Breast cancer	Ovarian cancer
25	0.017 (0.010, 0.024)	0.15 (0.06, 0.24)	0.02 (0.01, 0.03)	0.14 (0.04, 0.24)	0.02 (0.01, 0.03)	0.10 (0.03, 0.17)
30	0.13 (0.07, 0.18)	0.26 (0.12, 0.41)	0.16 (0.07, 0.24)	0.26 (0.10, 0.41)	0.14 (0.05, 0.23)	0.17 (0.06, 0.28)
35	0.49 (0.31, 0.68)	0.42 (0.21, 0.62)	0.60 (0.32, 0.88)	0.40 (0.17, 0.63)	0.54 (0.23, 0.85)	0.27 (0.11, 0.43)
40	1.34 (0.90, 1.78)	0.63 (0.34, 0.92)	1.63 (0.95, 2.31)	0.61 (0.29, 0.93)	1.47 (0.72, 2.21)	0.41 (0.18, 0.63)
45	2.97 (2.08, 3.85)	0.95 (0.52, 1.38)	3.60 (2.23, 4.96)	0.92 (0.45, 1.38)	3.24 (1.74, 4.72)	0.62 (0.29, 0.95)
50	5.40 (3.96, 6.82)	1.49 (0.81, 2.17)	6.54 (4.31, 8.71)	1.44 (0.69, 2.18)	5.89 (3.45, 8.28)	0.97 (0.45, 1.49)
55	8.82 (6.68, 10.90)	2.28 (1.25, 3.29)	10.63 (7.36, 13.79)	2.20 (1.08, 3.31)	9.61 (5.99, 13.09)	1.49 (0.70, 2.27)
60	12.38 (9.72, 14.97)	3.36 (1.89, 4.81)	14.87 (10.81, 18.74)	3.25 (1.64, 4.83)	13.47 (8.96, 17.75)	2.20 (1.07, 3.32)
65	16.42 (13.23, 19.49)	4.81 (2.78, 6.79)	19.62 (14.80, 24.16)	4.65 (2.42, 6.82)	17.82 (12.43, 22.87)	3.15 (1.58, 4.70)
70	20.58 (16.95, 24.05)	6.42 (3.87, 8.90)	24.47 (19.04, 29.53)	6.21 (3.41, 8.92)	22.28 (16.17, 27.95)	4.22 (2.24, 6.17)

Table S4. Cumulative risk (95% Confidence Interval) using segregation analysis, major gene models assuming relative risk as a continuous, piecewise linear function of age.

	Main Analysis Cumulative risk (95% Confidence Interval)		Sub-Analysis 1 Cumulative risk (95% Confidence Interval)		Sub-Analysis 2 Cumulative risk (95% Confidence Interval)	
Age	Breast cancer	Ovarian cancer	Breast cancer	Ovarian cancer	Breast cancer	Ovarian cancer
25	0.03 (0.01, 0.06)	0.15 (0.02, 1.57)	0.03 (0.01, 0.08)	0.06 ($3.94x10^{-4}$, 7.50)	0.04 (0.01, 0.10)	0.56 (0.003, 3.73)
30	0.21 (0.10, 0.45)	0.27 (0.03, 2.79)	0.22 (0.08, 0.57)	0.10 ($7.01x10^{-4}$, 12.98)	0.28 (0.08, 0.73)	0.96 (0.005, 6.56)
35	0.82 (0.38, 1.75)	0.42 (0.04, 4.34)	0.86 (0.32, 2.21)	0.16 ($1.10x10^{-3}$, 19.63)	1.09 (0.31, 2.81)	1.44 (0.008, 10.11)
40	2.22 (1.04, 4.70)	0.64 (0.06, 6.49)	2.32 (0.87, 5.92)	0.24 ($1.66x10^{-3}$, 28.11)	2.95 (0.85, 7.49)	2.08 (0.01, 14.87)
45	4.89 (2.30, 10.19)	0.96 (0.10, 9.66)	5.10 (1.94, 12.72)	0.36 ($2.52x10^{-3}$, 39.32)	6.41 (1.89, 15.95)	2.98 (0.02, 21.63)
50	8.22 (4.22, 16.40)	1.51 (0.30, 13.68)	8.72 (3.86, 20.34)	0.67 (0.12, 51.16)	10.62 (3.69, 24.88)	4.13 (0.13, 29.53)
55	11.99 (6.82, 22.50)	2.30 (0.66, 17.66)	13.05 (6.73, 28.18)	1.29 (0.36, 60.48)	15.25 (6.06, 32.87)	5.39 (0.34, 36.61)
60	15.02 (9.35, 26.46)	3.39 (1.21, 20.74)	16.82 (9.53, 33.35)	2.37 (0.80, 66.01)	18.87 (8.15, 37.55)	6.65 (0.66, 41.30)
65	17.46 (11.37, 28.80)	4.83 (1.99, 21.94)	20.28 (12.18, 36.99)	4.10 (1.51, 66.40)	21.64 (9.93, 40.72)	7.80 (1.14, 41.82)
70	20.01 (13.26, 32.01)	6.43 (2.78, 24.53)	23.89 (14.51, 42.42)	6.02 (2.25, 66.73)	24.47 (11.29, 46.03)	9.07 (1.58, 42.50)

Table S5. Modified segregation analysis results from MENDEL in the sub-analysis 1 and sub-analysis 2, a) assuming constant relative risk across age groups and b) assuming relative risk as a continuous, piecewise linear function of age.

	Analysis	Model	HR (a)	Age	HR (b)
Breast	Sub-Analysis 1	Major Gene Only	3.45 (1.88, 6.34)	< 40	4.93 (1.87, 12.99)
				> 60	2.56 (0.96, 6.82)
		Major and Polygenic	4.14 (1.93, 8.91)	< 40	4.66 (1.53, 14.23)
				> 60	3.75 (1.32, 10.72)
	Sub-Analysis 2	Major Gene Only	3.10 (1.48, 6.49)	< 40	5.50 (1.80, 16.81)
				> 60	1.59 (0.36, 7.09)
		Major and Polygenic	3.93 (1.56, 9.90)	< 40	5.07 (1.26, 20.43)
				> 60	2.91 (0.47, 14.92)
Ovarian	Sub-Analysis 1	Major Gene Only	5.63 (1.86, 17.03)	< 40	2.18 (0.01, 337.05)
				> 60	6.92 (1.80, 26.58)
		Major and Polygenic	5.96 (1.83, 19.44)	< 40	1.50 (0.01, 406.07)
				> 60	7.86 (1.99, 31.09)
	Sub-Analysis 2	Major Gene Only	3.79 (1.20, 11.96)	< 40	4.32 (0.12, 159.24)
				> 60	3.68 (0.89, 15.13)
		Major and Polygenic	4.06 (1.99, 8.26)	< 40	3.48 (0.05, 259.56)
				> 60	4.19 (0.95, 18.47)

HR: hazard ratio

Performance of *BRCA1/2* mutation prediction models in male breast cancer patients

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ABSTRACT

To establish whether existing mutation prediction models can identify which male breast cancer (MBC) patients should be offered *BRCA1* and *BRCA2* diagnostic DNA screening, we compared the performance of BOADICEA (Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm), BRCAPRO (BRCA probability) and the Myriad prevalence table ("Myriad"). These models were evaluated using the family data of 307 Dutch MBC probands tested for *BRCA1/2*, 58 (19%) of whom were carriers. We compared the numbers of observed vs predicted carriers and assessed the Area Under the Receiver Operating Characteristic (ROC) Curve (AUC) for each model. BOADICEA predicted the total number of *BRCA1/2* mutation carriers quite accurately (observed/predicted ratio: 0.94). When a cut-off of 10% and 20% prior probability was used, BRCAPRO showed a non-significant better performance (observed/predicted ratio BOADICEA: 0.81, 95% confidence interval [CI]: [0.60-1.09] and 0.79, 95% CI: [0.57-1.09], vs. BRCAPRO: 1.02, 95% CI: [0.75-1.38] and 0.94, 95% CI: [0.68-1.31], respectively). Myriad underestimated the number of carriers in up to 69% of the cases. BRCAPRO showed a non-significant, higher AUC than BOADICEA (0.798 vs 0.776). Myriad showed a significantly lower AUC (0.671). BRCAPRO and BOADICEA can efficiently identify MBC patients as *BRCA1/2* mutation carriers. Besides their general applicability, these tools will be of particular value in countries with limited healthcare resources.

KEYWORDS

BOADICEA, BRCA1, BRCA2, BRCAPRO, male breast cancer, Myriad prevalence table

INTRODUCTION

Female carriers of a mutation in *BRCA1* (OMIM* 113705) or *BRCA2* (OMIM* 600185) are at increased risk of developing breast and ovarian cancer and require specific clinical management such as extra surveillance and/or preventive surgery and strategies such as platinum-based therapy[1] or PARP inhibitors.[2]

The cumulative risk of breast cancer at age 70 for male carriers of a pathogenic *BRCA1* or *BRCA2* mutation is estimated to be 1.2% and 6.8%, respectively.[3] Male carriers may also be at increased risk for other types of cancer such as prostate, colon and pancreatic cancer.[4,5] Although some expert groups recommend that male carriers of a pathogenic mutation should undergo regular mammography in addition to surveillance for prostate cancer, the value of these surveillance strategies is still unproven.[6] For these reasons, male mutation carriers generally do not receive extra surveillance and rarely undergo prophylactic mastectomy of the breasts. Nonetheless, it is of vital importance to determine whether a male breast cancer (MBC) patient is a carrier of a pathogenic *BRCA1/2* mutation. Not only is this important as a determinant of chemotherapy choices such as treatment with platinum[1] or PARP inhibitors,[2] but also it provides the opportunity to identify other mutation carriers in the family through cascade screening, thus enabling prevention.

The NICE (National Institute for Health and Care Excellence) guideline proposes that genetic testing should be offered to female probands when the combined probability of being a *BRCA1* and *BRCA2* mutation carrier is 10% or higher.[7] However, this guideline is more ambiguous when it comes to genetic testing for MBC patients. In the Netherlands, every male affected with breast cancer is offered *BRCA1/2* testing regardless of age or family history. Previous studies have shown that 4%-40% of MBC patients carry mutations in one of the *BRCA* genes, with *BRCA2* mutations being the most common.[8] This obviously means that *BRCA1/2* account for only a minority of MBC patients, and thus many individuals are tested unnecessarily. As well as being cost-inefficient against a background of limited healthcare resources, testing may also lead to adverse psychological effects, as shown for female patients offered *BRCA1/2* diagnostic testing.[9]

Over the last 2 decades, various algorithms, tables and more sophisticated web-based tools have been developed to calculate the prior probability of *BRCA1* or *BRCA2* mutation carriership.[10–13]

The performance of these models has generally been evaluated in mostly female probands with various ethnic backgrounds.[14–26] We now wish to establish whether these models can also accurately select MBC probands for DNA testing. To date, this question has only been addressed in 2 small studies. In 2010, Zanna et al[27] evaluated the discriminatory capacity of the Myriad prevalence table ("Myriad"), the Ontario Family History Assessment Tool (FHAT), BRCAPRO (BRCA probability) 4.0 and 5.0 and the Italian Consortium (IC) model in a cohort of 102 MBC cases from Tuscany, Italy. They found that BRCAPRO 5.0 showed the best combination of sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) for combined *BRCA1/2* probability. BRCAPRO 5.0 was

also superior in the discrimination of *BRCA2* mutations and it was especially useful in dealing with non-familial MBC patients. More recently, Mitri et al[28] studied the accuracy of BRCAPRO 6.0 in 146 MBC cases. They concluded that BRCAPRO is a useful aid in selecting MBC cases for mutation analysis. Both studies only evaluated the discriminatory ability of the models.

In this study, Myriad,[29] BRCAPRO 6.0 (CaGene6) and BOADICEA 3.0 (Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm) were chosen for evaluation due to their ability to calculate the mutation prediction probability for an affected male proband, the frequent (international) use of these tools in both clinical and research settings, and their free availability. The internationally known International Breast Cancer Intervention Study (IBIS) model[12] was not used in this study because in IBIS the index case can only be female.

Including 307 Dutch MBC patients under the age of 80 years, to the best of our knowledge, the present study is the largest and the only nationwide study to evaluate the predictive accuracy of several different mutation carrier probability models. In addition, BOADICEA has not yet been validated in a population of MBC patients.

The aim of this study was to evaluate the diagnostic accuracy of these models by investigating and comparing their discriminatory ability and calibration within a population of MBC patients. We were interested to know whether these models can accurately predict mutations in MBC individuals and thus increase diagnostic yield, opening the way to their use in the selection of MBC cases for DNA testing in a clinical setting.

MATERIALS AND METHODS

Families

All MBC patients who were diagnosed in the Netherlands between 1989 and 2009 (n = 1487) were identified via the Dutch National Cancer Registry. Affected males who had been referred for genetic testing of *BRCA1* and *BRCA2* to 1 of the 9 genetic cancer centres in the Netherlands were then used for this study (N = 364). The pedigrees and results of genetic testing were collected from the Amsterdam Medical Centre (AMC, n = 14), Erasmus Medical Centre (EMC, n = 37), Leiden University Medical Centre (LUMC, n = 40), Maastricht University Medical Centre (MUMC, n = 30), Dutch Cancer Institute (NKI, n = 28), Radboud University Medical Centre (RadboudUMC, n = 77), University Medical Centre Groningen (UMCG, n = 61), University Medical Centre Utrecht (UMCU, n = 44) and VU University Medical Centre (VUMC, n = 33). From these families, 57 patients were excluded from the study for the following reasons: disease or mutation status or pedigree unavailable (n = 23), the proband was diagnosed with Ductal carcinoma in situ (n = 1), probands were carriers of a class 2 or 3 variant of uncertain significance (VUS). According to the International Agency for Research on Cancer (IARC) classification they had a posterior probability of pathogenicity between 0.1% and 94.9%[30] (n = 6). The age at diagnosis of breast cancer in the proband was above 80 years (cancer diagnoses that occur

after 80 years of age are not included in BOADICEA because of a lack of data to constrain the model) (n = 18). Nine pedigrees were known in 2 different cancer genetic centres, so each was included only once.

A final total of 307 cases were included. The proband was always a male and affected with at least breast cancer. In total 364 of 1487 families (24%) had undergone a DNA test. Table S1, in the Supporting Information, shows how many probands were tested every year. Data quality control and imputation rules for missing data are described in Supporting Information. The collection of data was approved by local ethics committees.

Mutation testing

BRCA1 and *BRCA2* mutation analysis was performed at the various cancer genetics centres in the Netherlands. Diverse mutation screening methods such as denaturing gradient gel electrophoresis, high resolution melting curve analysis, Sanger sequencing and/or multiplex ligation-dependent probe amplification were used, followed by confirmation of aberrant samples by Sanger sequencing. Variant classification was performed by the molecular clinical geneticists at the time of the genetic testing, according to internationally recognized criteria (https://enigmaconsortium.org/wp-content/uploads/2016/06/ENIGMA_Rules_2015-03-26.pdf, accessed April 2017 and the Breast cancer core database https://research.nhgri.nih.gov/bic/, accessed April 2017). VUS were re-evaluated for the present study and the 6 probands who were carriers of a VUS were excluded from the study (Clinvar database: [https://www.ncbi.nlm.nih.gov/clinvar/],accessed April 2017 and LOVD database: [http://databases.lovd.nl/shared/variants], accessed April 2017).[30,31]

Risk prediction models

The BOADICEA model assumes that genetic susceptibility to breast cancer is due to *BRCA1* and *BRCA2* mutations but also takes a polygenic component into account.[5,10,32] This algorithm allows predicted mutation probabilities and cancer risks in individuals to be estimated. Apart from first and second breast and ovarian cancer, it also includes prostate and pancreatic cancer in the calculations.[33] BRCAPRO is a comparable model which, taking into account family history, calculates the likelihood of carrying a *BRCA1* or *BRCA2* gene mutation.[34] In this study, we used BOADICEA version 3.0 and BRCAPRO 6.0 (CaGene6). The Myriad tables provide the combined probability of detecting a *BRCA1* and *BRCA2* mutation in counselees.[29] In contrast to BOADICEA and BRCAPRO which both provide a continuous number for the probability of finding a mutation, probabilities in Myriad for MBC are stratified into specific groups, namely 6.9%, 15.9%, 17.4%, 28.3%, 33.3% and 36.6%.[35] The probabilities in these tables are based on the observation of deleterious mutations in the counselees tested by Myriad Genetics Laboratories. We used the latest version of the tables, which was updated in February 2010 and is based on 162 914 tests.[35] The probability that a mutation remained undetected due to limitations of the sequencing technology was taken into account in the analysis. During the first

years of *BRCA1/2* screening and up to 2007, a very restricted mutation screening took place. The average mutation screening sensitivity increased when modern sequencing technology became available. The mutation screening sensitivity was assumed to be 95% for all those screened at and after 2007. For the tests performed before 2007, we used mutation search sensitivities of 0.7 for *BRCA1* and 0.8 for *BRCA2*.[20]

Statistical evaluation

We evaluated the calibration and discrimination of the risk prediction models. Calibration tests whether BOADICEA, BRCAPRO and Myriad can accurately predict the total number of *BRCA1* and *BRCA2* mutation carriers in the sample set. The calibration of these models was tested in the whole cohort for different categories of predicted mutation carrier probabilities. To compute the number of mutations predicted under these models, we averaged the probabilities of detecting a *BRCA1/2* mutation across all families in each category and then calculated the number of predicted mutation carriers (the predicted or expected number). Categories with carrier probability >20% were grouped together because the groups were small. These were compared with the actual number of mutations detected (the observed number) by calculating the observed/expected (predicted) ratio (O/E ratio). The exact 95% confidence intervals (CI) for the O/E were calculated under a Poisson assumption for the number of observed mutations.[36,37] Discrimination is the ability of the model to distinguish between a mutation carrier and a non-carrier at the individual level. This was assessed using the Area Under the Receiver Operating Characteristic (ROC) Curve (AUC). Confidence intervals and tests for comparing AUCs were based on the DeLong et al[38] method. Furthermore, we compared the sensitivity, specificity, NPV and PPV of the models at 10% and 20% carrier probability thresholds.

RESULTS

Table 1 shows the characteristics of the 307 probands and families. Almost 19% of the patients were carrier of either a *BRCA1* (2.9%) or a *BRCA2* (16%) mutation. The average age of the onset of breast cancer among male carriers was 59.83 years.

Calibration

The observed and predicted total number of mutations in each gene is shown in Table 2. The calibration of BOADICEA in terms of total number of mutations was better than the other models. Overall, 58 probands were carriers of a pathogenic mutation, whereas BOADICEA predicted 62 mutations (O/E: 0.94, 95% CI: [0.73-1.22]). BOADICEA predicted 5 *BRCA1* and 57 *BRCA2* mutation carriers compared with 9 and 49 observed, respectively (O/E ratio for *BRCA1*: 1.91, 95% CI: [0.99-3.66] and O/E ratio for *BRCA2*: 0.86, 95% CI: [0.65-1.14]). For BRCAPRO, the total number of predicted mutations was lower than observed (58 observed vs 48 predicted, O/E: 1.20, 95% CI: [0.93-1.56]). BRCAPRO predicted 8 *BRCA1* and 40 *BRCA2* mutation carriers among probands compared with 9

Table 1. Characteristics of the 307 probands and families

	Characteristics	Carriers Number (% or mean per family)	Non-carriers Number (% or mean per family)
Probands	Carrier of a *BRCA1* or *BRCA2* mutation	58/307 (18.9%) *BRCA1*: 9 (2.9%) *BRCA2*: 49 (16%)	249/307 (81%)
	Unilateral breast cancer	58 (100%)	249 (100%)
	Bilateral breast cancer	5 (8.6%)	8 (3.2%)
	Breast cancer and prostate cancer	2 (3.4%)	14 (5.6%)
	Average age of onset of breast cancer	59.83 yrs	60.09 yrs
Families	Unilateral breast cancer in family including proband	202 (3.48)	567 (2.28)
	Bilateral breast cancer in family including proband	24 (0.41)	30 (0.12)
	Breast cancer and prostate cancer in family including proband	3 (0.05)	41 (0.16)
	Only prostate cancer	11 (0.19)	27 (0.11)
	Breast cancer and ovarian cancer in family	0	2 (0.008)
	Only ovarian cancer	11 (0.19)	13 (0.05)

and 49 observed, respectively (O/E ratio for *BRCA1*:1.16, 95% CI: [0.61-2.24] and O/E ratio for *BRCA2*: 1.21, 95% CI: [0.92-1.60]). In none of the cases the difference between O/E ratios was significant. The Myriad tables provide a combined probability of detecting a *BRCA1* or *BRCA2* mutation and underestimated the total number of mutations (58 observed vs 34 predicted, O/E: 1.69, CI: [1.30-2.18]).

Discrimination

ROCs are presented in Figure 1 for (A) BOADICEA *BRCA1/2*, BRCAPRO *BRCA1/2* and Myriad *BRCA1/2*, (B) BOADICEA *BRCA1* and BRCAPRO *BRCA1*, and (C) BOADICEA *BRCA2* and BRCAPRO *BRCA2*. Corresponding AUCs, or the likelihood that a mutation carrier will score higher than a non-carrier, are reported in Table 3. A value of 0.5 suggests that the test is no better than tossing a coin and a value of 1 indicates perfect discriminatory power. The AUC for BOADICEA was 0.776 (95% CI: [0.708-0.845]), for BRCAPRO it was 0.798 (95% CI: [0.726-0.871]), and for Myriad it was 0.671 (95% CI: [0.599- 0.743]), the latter being significantly lower than the AUCs for BOADICEA and BRCAPRO (*P*-value = 0.0072 for comparison for AUCs of Myriad and BOADICEA, *P*-value = 0.00029 for comparison for AUCs of Myriad and BRCAPRO). When predicting *BRCA1* or *BRCA2* mutations separately, BOADICEA and BRCAPRO both showed better discrimination for *BRCA1* than for *BRCA2* (Table 3). Table 4 shows the performance of the different models at a carrier probability

Table 2. Observed and expected number of mutations by predicted carrier probability

		Observed, n				Expected, n					
Model	Carrier probability (%)[a]	No mutation	*BRCA1*	*BRCA2*	Either	No mutation	*BRCA1*	*BRCA2*	Either	O/E [b]	95% Confidence interval
BOADICEA	<5	97	0	6	6	100.31	0.14	2.56	2.69	2.23	1.001-4.96 [c]
	5-10	56	2	6	8	59.25	0.23	4.53	4.75	1.68	0.84-3.36
	10-15	35	0	2	2	32.43	0.15	4.42	4.57	0.44	0.11-1.75
	15-20	12	0	5	5	14.12	0.14	2.74	2.88	1.74	0.72-4.17
	>20	49	7	30	37	39.25	4.07	42.68	46.75	0.79	0.57-1.09
	Total	**249**	**9**	**49**	**58**	**245.36**	**4.72**	**56.91**	**61.64**	**0.94**	**0.73-1.22**
BRCAPRO	<5	148	2	9	11	155.98	0.30	2.72	3.02	3.65	2.02-6.58 [c]
	5-10	51	0	5	5	52.02	0.37	3.61	3.98	1.26	0.52-3.02
	10-15	15	0	5	5	17.52	0.21	2.27	2.48	2.02	0.84-4.85
	15-20	7	0	2	2	7.45	0.15	1.40	1.55	1.29	0.32-5.17
	>20	28	7	28	35	25.86	6.69	30.45	37.14	0.94	0.68-1.31
	Total	**249**	**9**	**49**	**58**	**258.83**	**7.72**	**40.44**	**48.17**	**1.20**	**0.93-1.56**
Myriad	<5	0	0	0	0	0	NA	NA	0	NA	NA
	5-10	193	3	23	26	203.89	NA	NA	15.11	1.72	1.17-2.53 [c]
	10-15	0	0	0	0	0	NA	NA	0	NA	NA
	15-20	44	1	18	19	52.16	NA	NA	10.84	1.75	1.12-2.75 [c]
	>20	12	5	8	13	16.60	0	0	8.40	1.55	0.90-2.67
	Total	**249**	**9**	**49**	**58**	**272.64**	**NA**	**NA**	**34.36**	**1.69**	**1.30-2.18 [c]**

Abbreviations:
NA: Not available.
[a] Classes of carrier probability calculated with the respective model.
[b] Observed/Expected ratio, observed number of mutation carriers divided by number of mutation carriers expected according to the respective model.
[c] The 95% Confidence Interval (CI) for O/E does not include 1.

of 10% and 20% for BOADICEA and BRCAPRO and the equivalent threshold score of 6.9 and 17.4 for Myriad. At a 10% threshold, BOADICEA showed the highest sensitivity (77.2%) and the lowest specificity (61.4%) for *BRCA1* and *BRCA2* combined. At a 20% threshold, BOADICEA again had the highest sensitivity (64.9%) and the lowest specificity (80.3%). At 10% threshold for *BRCA1*, BOADICEA had a lower sensitivity compared to BRCAPRO (33.3% vs 55.5%, respectively), however, specificities were comparable (98.7 vs 97.0). At 10% threshold for *BRCA2*, sensitivity of BOADICEA was higher than sensitivity of

Table 3. Area under the ROC curve for each model

	ROC area (95% Confidence Interval)		
Model	Either *BRCA1* or *BRCA2*	*BRCA1*	*BRCA2*
BOADICEA	0.776 (0.708-0.845)	0.848 (0.700-0.996)	0.743 (0.667-0.819)
BRCAPRO	0.798 (0.726-0.871)	0.857 (0.708-0.999)	0.768 (0.687-0.849)
Myriad	0.671 (0.599-0.743)	NA	NA

Abbreviations:
NA: Not available.
ROC: Receiver Operating Characteristic Curve

Table 4. Diagnostic performance of BOADICEA, BRCAPRO and Myriad at different threshold levels

Outcome	Cut-off	Model	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
BRCA1	10%	BOADICEA	33.3	98.7	42.9	98.0
		BRCAPRO	55.5	97.0	35.7	98.6
		Myriad	NA	NA	NA	NA
BRCA2	10%	BOADICEA	75.0	61.2	26.4	92.9
		BRCAPRO	72.9	79. 4	39.7	94.0
		Myriad	NA	NA	NA	NA
Either *BRCA1* or *BRCA2*	10%	BOADICEA	77.2	61.4	31.4	92.1
		BRCAPRO	73.7	79.9	45.7	93.0
		Myriad (6.9)	54.4	77.5	35.6	88.1
Either *BRCA1* or *BRCA2*	20%	BOADICEA	64.9	80.3	43.0	90.9
		BRCAPRO	61.4	88.8	55.6	90.9
		Myriad (17.4)	22.8	95.2	52.0	84.3

Abbreviations:
NA: Not available.
Outcome calculated for total and 10% and 20% threshold and equivalent threshold score of 6.9 and 17.4 for Myriad, for *BRCA1* or *BRCA2* separately if available, or for both genes.

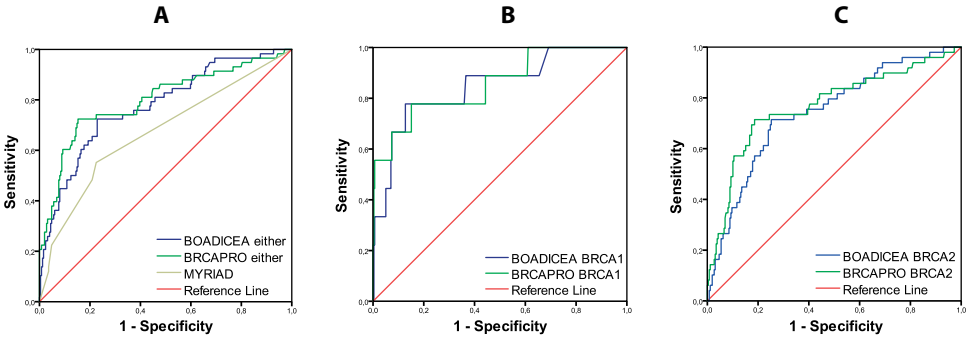

Figure 1. Receiver operating characteristic curves (ROC) curves. Receiver operating characteristic curves for (A) BOADICEA *BRCA1/2*, BRCAPRO *BRCA1/2* and Myriad *BRCA1/2*, (B) BOADICEA *BRCA1* and BRCAPRO *BRCA1* (C), BOADICEA *BRCA2* and BRCAPRO *BRCA2*, all at 10% cut-off.

BRCAPRO (75.0% vs 72.9%) while its specificity was lower (61.2% vs 79.4%). Both models had a lower sensitivity and higher specificity for *BRCA1* compared to *BRCA2*.

DISCUSSION

Using a cohort consisting of 307 MBC cases assembled from 9 genetic counselling centres, this is the largest study to date to evaluate the performance of the 3 most commonly used mutation prediction models, BOADICEA, BRCAPRO and Myriad, in the estimation of *BRCA1* and *BRCA2* mutation-carrier probabilities in MBC patients. We also provide the first validation of the use of BOADICEA in MBC patients. In contrast to previous studies, we not only studied discrimination but also examined calibration of the prediction models.

The reported prevalence of *BRCA1/2* mutations in MBC patients varies considerably between different populations and cancer genetic centres, ranging from 4% to 40% for *BRCA2* and up to 4% for *BRCA1* genes.[8] Our study found that about 19% (58/307) of all MBC patients actually carry a *BRCA* mutation. In the Netherlands all affected male individuals are currently offered *BRCA1/2* screening. As testing all patients might cause unnecessary additional distress in patients and relatives, a tool that can accurately determine the prior probability of MBC mutation carriers would therefore be of great clinical value. Moreover, testing all patients at the moment is cost-inefficient, given limited healthcare resources, especially in nonwestern countries. However, we acknowledge that, regarding the price and availability of population-wide gene panel testing, we might soon be at the stage where it is actually cost-effective to screen all patients.

Every MBC patient in our study who was referred to a cancer genetics centre was offered a DNA test, regardless of family history or the prior probability of being a carrier. However, many of the originally identified MBC patients (n = 1487, diagnosed between 1989 and 2009) were not referred to cancer genetics centres, primarily because *BRCA1/2* testing was only implemented in clinical practice in the late 1990's. At that time some

clinicians were either unaware of the possibility of *BRCA1/2* testing of male patients or had a different pattern of referral criteria. It is also possible that in the early years, clinicians only referred patients with a strong family history or younger age at diagnosis. The average age for the 307 patients who were referred is significantly lower than those who were not referred (60.04 vs 68.06, *P*-value 0.0009). Table S1 shows that the number of *BRCA1/2* screenings has increased in recent years. It also shows that genetic tests were performed in some men several years after their diagnosis. Studies of the pathological features of *BRCA1/2* MBC tumours showed that these tumours display distinct characteristics compared with *BRCA1/2* female breast cancer tumours (eg, high histologic grade in *BRCA2* MBC patients), which suggested greater biological aggressiveness.[39,40] Although it is not directly proven for MBC caused by *BRCA1/2* mutations, it might be the case that some patients in this specific group were not tested because they did not survive the disease. These factors partly explain why only 364 probands among the 1487 MBC patients actually received a DNA test, and the relatively high percentage of mutation carriers reported in the study (19%). Although this study is the largest study to date performed for prediction of mutation carrier probability in MBC patients, it is still a small cohort. The number of patients has limited the power of this study and as a result, in many cases, the differences are not significant.

Calibration

In our cohort, BOADICEA showed the best calibration for the overall number of *BRCA1* and *BRCA2* mutations. When a cut-off of 10% and 20% prior probability was used, BRCAPRO showed a non-significant better performance (observed/predicted ratio BOADICEA: 0.81, 95% CI: [0.60-1.09] and 0.79, 95% CI: [0.57-1.09], vs BRCAPRO: 1.02, 95% CI: [0.75-1.38] and 0.94, 95% CI: [0.68-1.31], respectively).

Discrimination

BOADICEA and BRCAPRO both showed good discrimination of mutation carriers vs non-carriers, whereas Myriad had a significantly lower AUC. Both BOADICEA and BRCAPRO showed better AUCs for *BRCA1* than for *BRCA2*, these differences did not, however, reach statistical significance (*P*-value = 0.2187 for comparison of AUCs of BOADICEA, *P*-value = 0.3075 for comparison of AUCs of BRCAPRO). As BOADICEA and BRCAPRO were developed for female patients it seems likely that several factors included in these models result in better prediction of *BRCA1* mutations. For example, *BRCA1* mutations are associated with a higher ovarian cancer risk compared to *BRCA2* mutations, and with an earlier age at diagnosis of breast cancer.[41] As expected, the number of *BRCA1* mutations observed in our cohort was much lower than the number of *BRCA2* mutations (9 vs 49, respectively). This resulted in wide CIs for *BRCA1* in both BOADICEA and BRCAPRO (Table 3). Nonetheless, both models showed good discrimination of *BRCA1* and *BRCA2* carriers and non-carriers, although discrimination of carriers of either mutation and of

non-carriers is of limited utility in clinical practice because the overall carrier probability determines the decision to screen for mutations. Nevertheless, while probands are always tested simultaneously for *BRCA1* and *BRCA2* mutations in the Netherlands, the accurate discrimination of *BRCA1* and *BRCA2* carriers may be of considerable importance in countries with fewer financial resources.

In contrast to the Myriad prevalence data, BOADICEA and BRCAPRO both appear to be well calibrated and show a high discriminatory power to identify male *BRCA1/2* mutation carriers. However, both models could still be improved. At the time of this study, estimates of *BRCA1* and *BRCA2* mutation frequencies based on a large Dutch series were unavailable and there were no specific penetrance estimates for cancers affecting sites other than the breast, so none of the models included incidence rates for Dutch population. We presume that incorporating data on Dutch incidences into the models would improve their accuracy in the present cohort.

Furthermore, the inclusion of other genetic and non-genetic risk factors known to be important in MBC such as radiation exposure, alcohol use, obesity, hormonal imbalances, disease and medical treatments leading to hyperestrogenism might also improve the accuracy of these models.[8]

CONCLUSION

In the largest cohort of MBC cases studied to date, we found that BOADICEA and BRCAPRO both showed good discriminatory ability for male *BRCA1/2* carriers. In terms of total number of carriers, BOADICEA showed the best calibration, and BRCAPRO displayed a non-significant better fit when a mutation probability threshold of 10% or 20% was used. Myriad tables showed a significantly lower calibration and discrimination compared to the two other models.

Both BOADICEA and BRCAPRO are valuable tools when deciding whether to offer *BRCA1* and *BRCA2* DNA mutation screening to MBC patients and will be of considerable value in countries with limited healthcare resources that cannot offer testing to all MBC patients. However, both models could potentially be improved through the incorporation of population-specific parameters and risk factors for MBC.

BOADICEA is currently the first choice for calculation of mutation carrier probability in many countries[42] and the developers are planning to include other breast cancer-related genes such as PALB2 (OMIM* 610355) and CHEK2 (OMIM+ 604373),[43] breast cancer-associated Single Nucleotide Polymorphism (SNPs), and environmental factors and risks in the algorithm. A model that incorporates additional MBC-related factors in a user-friendly tool will eventually be the preferred choice for the calculation of the mutation carrier probability in MBC patients.

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SUPPLEMENTARY DATA

- A study by R. Sijmons et al.[1] shows that for breast cancer the accuracy of the family history of all degrees of kinship together is 93%. Although very time consuming, in the Netherlands family medical histories of all the probands, and if available the family medical history of the affected family members, are routinely verified. However, in this study cancers were only included when there was no uncertainty in the recorded data. For example: an individual with a record of "B?" or "B(?):68" was not included as a breast cancer case in the analysis.

- The completeness of the data on the probands was checked and corrected at the centre where the proband was known. If there was any ambiguity in the proband data, the pedigree was excluded. Therefore, all the probands in this study had complete information about cancer and age at diagnosis of cancer.

- If age at diagnosis was not known for a family member, age at death or age at the moment of last interview was used.

- Eligible families were the families whose family mutation status was not known when genetic testing was started.[2] When the male proband was not affected but referred for DNA screening, we concluded that a BRCA1/2 mutation was already identified in the family, so we excluded the family in this study (n=21).

- For data standardizing purposes, only first, second, and when information was available, also third-degree relatives were included in the input pedigrees.

- For BOADICEA, the year of birth and age at diagnosis of the index and family members are required for calculation of mutation carrier probability. Therefore, we imputed the missing values for YOB of individuals with breast cancer and for age at diagnosis according to the following rules:

Imputation rules Year of Birth (YOB):

1. If the year of birth (YOB) of one of parents or children was unknown, YOB was imputed based on Dutch average ages at pregnancy. (Figure 3, average age of mother at birth of first child, 1950-2012, Central Agency for Statistics, CBS: Population survey).

2. If YOB of family members in the same generation was unknown, we removed 2 years for each following sibling if there were 5 siblings in total, and we removed 1 year if there were more than 5 siblings in the family.

Previous large studies had estimated carrier probabilities in BOADICEA, with and without the imputed data, and compared the results. There were improvements in model performance after imputing missing year of birth and age at diagnosis for affected family members.[3] In this study the results are based on pedigrees with imputation.

Table S1. Number of BRCA1/2 tests per year

		Tests are performed in																					
Year of diagnosis	Number of tests per year	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	Unknown
1989	1																		1				
1990	2									1						1							
1991	5				1					1						2			1				
1992	2										1					1							
1993	4																	1	1		1		1
1994	9	2			1				1	1		1				1			1	1			
1995	11		2	1						1	2	3				1	1						
1996	8			1	1	1				1		2		1		1							
1997	8				1			1		2	1	1	1						1				
1998	10					2						3	1			2		2					
1999	11						1	2			1	4	1	1		1							
2000	10								2	1	1	3	1						2				
2001	15								2	2	2	3	4				1	1					
2002	18									7	3	2	1		1	2	1			1			
2003	20										2	10	3		2	1	1		1				
2004	28											12	8			3	1	1	1	1			1
2005	30												4	13	2	3	3	3	1	1			
2006	18													7	7	1	1		1	1			
2007	25														8	7	7	2		1			
2008	34															15	11	5	1	1		1	
2009	35																17	14	2		1		1
unknown	3													1			1	1					
Total	307																						

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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

Reference

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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

Classification and Clinical Management of Variants of Uncertain Significance in High Penetrance Cancer Predisposition Genes

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ABSTRACT

In 2008, the International Agency for Research on Cancer (IARC) proposed a system for classifying sequence variants in highly penetrant breast and colon cancer susceptibility genes, linked to clinical actions. This system uses a multifactorial likelihood model to calculate the posterior probability that an altered DNA sequence is pathogenic. Variants between 5%–94.9% (class 3) are categorized as variants of uncertain significance (VUS). This interval is wide and might include variants with a substantial difference in pathogenicity at either end of the spectrum. We think that carriers of class 3 variants would benefit from a fine-tuning of this classification. Classification of VUS to a category with a defined clinical significance is very important because for carriers of a pathogenic mutation full surveillance and risk-reducing surgery can reduce cancer incidence. Counselees who are not carriers of a pathogenic mutation can be discharged from intensive follow-up and avoid unnecessary risk-reducing surgery. By means of examples, we show how, in selected cases, additional data can lead to reclassification of some variants to a different class with different recommendations for surveillance and therapy. To improve the clinical utility of this classification system, we suggest a pragmatic adaptation to clinical practice.

BACKGROUND

Evaluation of the Pathogenicity of Variants of Uncertain Significance

Besides classical pathogenic mutations that truncate or inactivate the protein, the continuous development of various sequence-based technologies in DNA diagnostic laboratories is resulting in the detection of an increasing number of variants for which the clinical significance is unknown. These variants, also referred to as variants of uncertain significance (VUS), include missense variants, small in-frame deletions or insertions, synonymous nucleotide substitutions, certain truncating mutations (such as mutations in the last exons of genes), as well as alterations in noncoding sequences or in untranslated regions.

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[1, 2] or the predicted effect on messenger RNA splicing.[3] Some other methods measure the direct association of the variant with disease, and include cosegregation of the variant with disease in a family,[4, 5] family history,[6-8] co-occurrence of the variant with pathogenic mutations on the second allele,[9, 10] tumor pathology,[2, 11, 12] and analysis of the tumor DNA (e.g., array comparative genomic hybridization and genomic methylation).[13-16] There are also functional studies that assess the impact of genetic variants on the activity of the protein in vitro.[17-20]

In 2004, Goldgar et al introduced a multifactorial likelihood model (MLM) for the classification of the VUS in *BRCA1* (MIM #113705) and *BRCA2* (MIM #600185) in which the odds of causality, obtained from different methods under the assumption of independence, could be combined. In general, when a VUS reached odds higher than 1,000:1 in favor of pathogenicity, it could be classified as pathogenic, and when it was lower than 1:100 against pathogenicity, the variant could be classified as neutral.[21] This model was improved in 2008 by the addition of the prior probability of pathogenicity of a variant based on its position and function.[1, 22]

In 2007, the UK Clinical Molecular Genetics Society and the Dutch Society of Clinical Genetics Laboratory Specialists proposed "Good Practice Guidelines for the Interpretation and Reporting of Unclassified Variants in Clinical Molecular Genetics Laboratories".[23] It proposed reporting variants in four classes: (I) certainly not pathogenic, (II) unlikely to be pathogenic, (III) likely to be pathogenic, and (IV) certainly pathogenic. In 2008, the American College of Medical Genetics (ACMG) proposed a six-class system for interpretation and reporting of sequence variants, with an emphasis on the importance of appropriate reporting of sequence variations using standardized terminology and established databases: (1) sequence variation is previously reported and is a recognized cause of the disorder; (2) sequence variation is previously unreported and is of the type that is expected to cause the disorder; (3) sequence variation is previously unreported and is of the type which may or may not be causative of the disorder; (4) sequence variation is previously unreported and is probably not causative of disease; (5) sequence variation is previously reported and is a recognized neutral variant; and (6) sequence variation is

previously not known or expected to be causative of disease, but is found to be associated with a clinical presentation.[24] However, neither of these two systems recommended using quantitative information for the classification and clinical management of variants. An expert working group, convened at IARC (http://www.iarc.fr) in 2008, proposed a standardized classification system applicable to sequence-based results in highly penetrant cancer predisposition genes such as *BRCA1*, *BRCA2*, *MLH1* (MIM #120436), and *MSH2* (MIM #609309). This classification system interprets results from the MLM and translates these to recommendations for clinical practice.[25]

Current Clinical Management of the VUSs

According to the IARC classification (Table 1), the counselees who carry a variant in class 1 should be counselled as if no mutation was detected for this disorder. The carriers in class 5 should be counselled as those who are carriers of the conventional pathogenic mutations. Variants in class 2 and 4 should be clinically managed as variants in class 1 and 5, respectively[25]. The DNA alterations that are in class 3 are classified as VUS, which means that the laboratory interpreted the DNA alteration based on standard evidence at the time of the test (mostly *in silico* and literature review) and found that there was insufficient evidence to classify the alteration as either pathogenic (deleterious) or neutral. Within this classification, a VUS should not be used for predictive testing in at-risk individuals and the surveillance should be based on family history. The authors suggested that the research testing of the family members might be helpful to further classify variants (Table 1).[25]

Options for Communication of the VUSs

Based on this classification, we think there are currently broadly two approaches in clinical practice for communication of an identified VUS to the tested individual and their family members: communicating all the VUSs or communicating none of the VUSs.

1. Communicating all the VUSs

From a research point of view, collecting as much evidence from all sources as possible for all the VUS will allow the reclassification of the maximum number of variants. As summarized by Spurdle et al the majority of *BRCA1* and *BRCA2* variants submitted to ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles, http://www.enigmaconsortium.org) as of September 2010 were missense variants (61% and 64%, respectively).[26] In the study of Easton et al, 1,177 out of 1,433 (82%) variants were either missense variants or in-frame deletions or insertions. Of all the missense variants and in-frame deletions or insertions in *BRCA1/2*, about 12% are estimated to be pathogenic (based on combined likelihood ratios [LR])[6]. Furthermore, several studies have shown that counselees and the family members of those who know themselves to be a carrier of a VUS experience considerable distress due to the possibility that they face a high cancer risk and due to the uncertainty surrounding this risk[27, 28]. When *BRCA* VUS reports

Table 1. IARC classification system for sequence variants identified by genetic testing and recommendations associated with each class of variant. This table is adapted with permission from authors[1]

Class and description	Probability of pathogenicity	Clinical testing	Surveillance recommendations if at-risk relative is positive	Research testing of family members
1 Not pathogenic or of no clinical significance	<0·001	Do not use for predictive testing in at-risk individuals	Treat as "no mutation detected" for this disorder	Not indicated
2 Likely not pathogenic or of little clinical significance	0·001-0·049	Do not use for predictive testing in at-risk individuals	Treat as "no mutation detected" for this disorder	Maybe helpful to further classify variant
3 Uncertain	0·05-0·949	Do not use for predictive testing in at-risk individuals	Based on family history (and other risk factors)	Maybe helpful to further classify variant
4 Likely pathogenic	0·95-0·99	Test at risk relatives for variant	Full high risk surveillance	Maybe helpful to further classify variant
5 Definitely pathogenic	>0·99	Test at risk relatives for variant	Full high risk surveillance	Not indicated

are interpreted by clinicians with minimal training in genetics, misunderstandings are compounded[29]. Moreover, for a variant in class 3 with a prior probability of for example, 0.05 (lower end of probability of pathogenicity in class 3), a likelihood ratio of 361.2 (19/0.0526) toward pathogenicity is needed to ascend to a posterior probability of 0.95 (lower end of probability of pathogenicity in class 4) (Figure 1A), with a clinical consequence for the patients. To achieve this LR, a lot of additional data such as histopathological information and extensive segregation data are necessary. The same variant in class 3 with a prior probability of 0.05 can easily descend to class 2, but this does not have any clinical consequence for the carriers (Table 1).

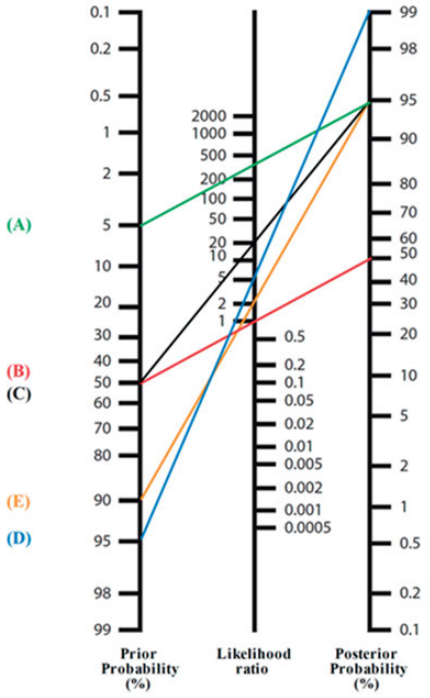

Figure 1. Nomogram for Bayes theorem.[30] Copyright© (1975) Massachusetts Medical Society. Reproduced from Fagan[30] with permission from Massachusetts Medical Society. A line drawn from prior probability on the left of Figure 1 through the likelihood ratio in the center of the figure gives the posterior probability on the right side of the figure (explanation of 1A-1E in the text). Likelihood ratio (LR) is a measure of accuracy of a diagnostic test. The LR of any 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.[31] Posterior probability of pathogenicity can be calculated as: posterior odds/ (posterior odds + 1) and the posterior odds are calculated as: LR × (prior probability/[1−prior probability]).[32] For example, for a variant in class 3 with a prior probability of 0·05, to ascend to a posterior probability of 0·95 (class 4), a LR of 361.2 is needed. [Posterior probability = 0·95 = [posterior odds/(posterior odds +1)]➔ posterior odds = 19; and posterior odds = LR x [prior probability/(1-prior probability)]➔ 19 = LR x[0·05/(1-0·05)]➔ 19 = LR x 0·0526 ➔ LR = 19/ 0·0526 = 361.2].

2. Communicating none of the VUSs

Previous studies have shown that VUS may be recategorized when additional information becomes available, and although basic in silico categorization is fairly robust, it has also been shown that additional information is central to an accurate appraisal[33]. Communication of a VUS test result provides the opportunity to discuss collection of additional information and material with the counselee. A consequence of not communicating the variants is that potentially pathogenic variants will go unrecognized and remain categorized as class 3 VUS, patients and their family members are then advised based on the family history. Some may choose risk-reducing surgery that could be avoided if a genetic test can be offered and they are shown not to have inherited a clearly pathogenic variant.

Recommendations for the Communication of a Variant and Examples

Since, (1) the odds are low that a random VUS in class 3 is pathogenic,[6] (2) most of the variants after inclusion of additional data will be classified as likely not pathogenic, (3) communication of any VUS can lead to psychological distress,[27, 28] (4) misinterpretation of a VUS may have significant adverse sequelae in terms of inappropriate decisions,[34] and as a consequence (5) an increase in overall costs to the health care system and the individual,[35] we believe that communicating all class 3 variants in a health care setting is unhelpful and may be harmful. However, communication within a research setting is clearly a different and potentially useful option.

When a VUS is identified in a high-risk cancer gene, a molecular geneticist in the DNA diagnostic laboratory, in collaboration with national and international colleagues, provides the classification (Figure 2). For a better clinical management of the VUS, our suggestion is that the laboratory specialists divide VUS class 3 into two subgroups: class 3A with a posterior probability of 0.05–0.499 and class 3B with a posterior probability of 0.5–0.949. We put forward these recommendations for the classification of high penetrance cancer predisposition genes because these genes are most commonly and completely analyzed and a lot of clinical data about these genes are available that can be used in the statistical classification of their variants. In principle, any high penetrance cancer susceptibility gene can be classified by this model. However, the model needs to be adapted to quantify the posterior probability based on different lines of evidence that are used to classify the variant.[25] Since its introduction for *BRCA1/2* in 2008, convening expert panels such as ENIGMA have continuously updated and fine-tuned the MLM. Members of the InSiGHT committee (International Society for Gastrointestinal Hereditary Tumours, http://insight-group.org) reviewed the types of data available for each mismatch repair (MMR) gene and developed quantitative scores for these different types of data. As a result, MLM was used for the classification of VUS in MMR genes in 2013. [2] It is expected that, in the future, other international groups adapt the model for use in the classification of other cancer predisposition genes.

We suggest communication and testing of family members when the posterior probability of pathogenicity of a VUS is higher than 0.5 (i.e., category 3B) but no

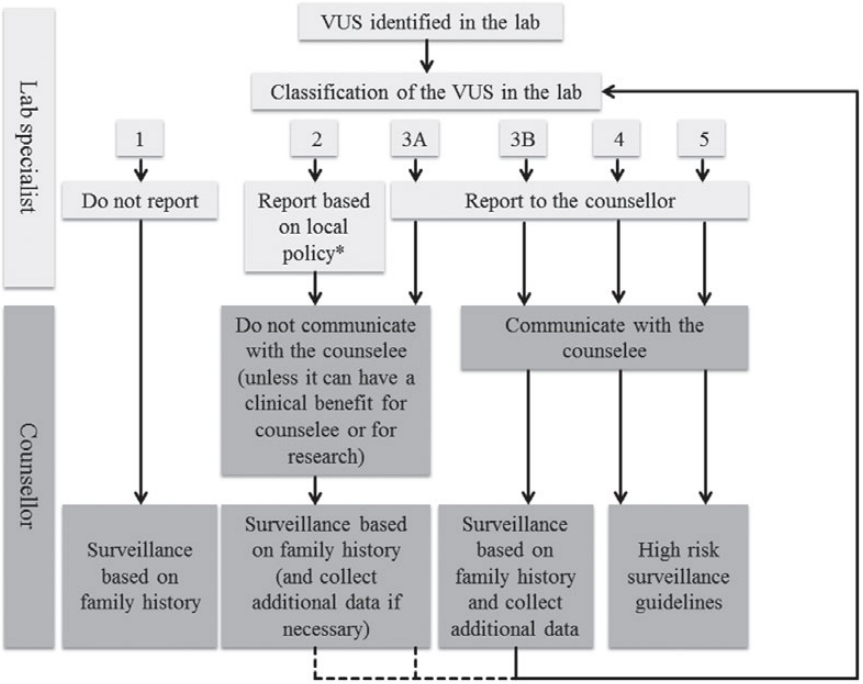

Figure 2. Schematic view for the laboratory and clinical management of the variants. *According to the "Practice Guidelines for the Evaluation of Pathogenicity and the Reporting of Sequence Variants in Clinical Molecular Genetics" by the UK Association for Clinical Genetic and the Dutch Society of Clinical Genetic Laboratory Specialists Science, local policy will determine whether class 2 variants are reported to the counsellors.

communication of variants in class 3A, unless the counsellor has a reason to expect a clinical benefit for the counselee or, for example, when there is an opportunity for research among many affected family members. Furthermore, the counsellor should inform recipients of any inconclusive genetic test result to seek contact with the cancer genetics center within a few years so that the pedigree can be reassessed and (additional) DNA testing can be offered, should there be new insights into cancer genetics or new DNA sequencing technologies available.

Based on estimations in previous studies, only about 20% of all the variants in *BRCA1* and *BRCA2* are pathogenic.[6] Therefore we believe that the number of variants in classes 3A and 3B is not equal and rather few variants have a posterior probability above 50% (3B). We chose 50% as threshold because for this probability there is an equal chance that a variant is pathogenic or neutral (odds 1:1) (Figure 1B). For a variant with a 50% risk of pathogenicity, a LR of 19:1 is sufficient to reach a posterior probability of 95% (class 4) (Figure 1C). This can be obtained by addition of some pathological data from a few tumors and evidence of cosegregation of the variant with cancer (assuming that most of the additional data are in favor of pathogenicity). For example, it is estimated that

the LR increases 4.41-fold for every carrier of a *BRCA1* VUS who is diagnosed with breast cancer at the age of 50 years or older with a triple negative tumor (negative estrogen and progesterone receptor status and no amplification of *HER2*).[12] LR of cosegregation is highly dependent on the exact family information such as number of affected and unaffected individuals in the family, age of diagnosis, and the degree of kinship. For example, if the index is a female who has breast cancer at the age of 29 years, and carries a specific variant in *BRCA1*, and her sister also carries the same variant and is affected with ovarian cancer at the age of 41 years, and there are two healthy untested siblings at about the age of 50 years with healthy parents, then the LR of cosegregation for this family will be about 2.[5]In general, genotypes of distantly related individuals with very early onset of cancer or old healthy individuals give the strongest LRs in favor of or against pathogenicity[5]. Also, each MSI high tumor with a VUS in one of the MMR genes increases the LR 6.96-fold toward pathogenicity.[2] An example is c.1852_1854delAAG, p.Lys618del in the *MLH1* gene for which the prior probability of being pathogenic was 0.5. After addition of LR for cosegregation and tumor characteristics, the variant was classified as pathogenic with posterior probability of 1.0.[2] Another example is c.5066T>G, p.Met1689Arg in *BRCA1* that had a prior probability of pathogenicity of 0.66. After addition of other information such as family history and co-occurrence data, the probability of pathogenicity reached 0.989 that led to reclassification of this variant to class 4 (likely pathogenic) and allowed family members to be offered meaningful predictive genetic testing. [6, 32]

Caveats

The examples given above are only to illustrate how additional information can change the classification. The thresholds for classification are carefully set by IARC.[25] Because reclassification of a variant from class 3 to class 4 or class 5 can have serious clinical consequences for the carriers of the variant, the upper range of class 3 in the IARC classification is set very high (0.95). However, if prior is 0.5, to ascend from posterior probability of 0.95–0.99, a 5.3-fold (99/18.6) increase in LR is needed (Figure 1D), whereas from 0.90 to 0.95 only a 2.1-fold (18.6/9) increase in the LR is sufficient (Figure 1E). So, for the same increase of about 5% in the posterior probability, much less information is needed and the classification can in some cases easily change from one class to another.

It is important to emphasize that collection of information by the counsellors should not be selective, which means that the counsellor needs to collect all available evidence, not just evidence that supports the pathogenic status of the variant or just to the point at which a high posterior probability is reached. Failure to do this may lead to an overestimation of the LR through selection bias. Furthermore, all the collected information, when not strictly confidential, should be shared with the molecular geneticists who are responsible for classification. Also, it is important to appreciate that confidence in a posterior probability increases as multiple additional data sources from diverse resources increase. Moreover, probabilities might be based on misinterpretation due to

incorrect underlying assumptions in the model, exceptions to certain rules, incomplete knowledge of some underlying biophysical property of the gene or protein, or to many other factors.[36] For example, *BRCA1* c.594-2A>C also known as *BRCA1* IVS9-2A>C that was presumed to be pathogenic based on predicted impact of base change on splicing and biochemical evidence but eventually is proven to be benign based on other biological evidences.[37] Lindor et al also suggested keeping these possibilities in mind and integrating them into discussions with the counselees who are actually involved in making personal medical decisions.[36]

DISCUSSION AND CONCLUSION

As previously mentioned, there is still no universally accepted international guideline for genetic counsellors regarding the communication and research testing of the family members of the carriers of VUS.[34]

Communication of a VUS test result provides the opportunity to discuss collection of additional information and material with the counselee that can eventually lead to a better assessment of the variants. However, there is a fine balance between on the one hand causing additional stress for the counselees and extra costs for the health care system and on the other hand a reduction in morbidity and mortality through better screening, possibility for prophylactic surgery, and personalized chemotherapy (such as, if proven effective, treatment with platinum and PARP inhibitors in *BRCA1/2*mutation carriers)[38, 39] when a variant can be classified as pathogenic.

If, after collection of additional information, a variant is downgraded from class 3 to 2, this will not change the clinical management of the carriers. For these reasons, we propose that in a primarily clinical setting, counsellors are not obliged to communicate all VUS. Since reclassification of a VUS with posterior probability >0·5 has a realistic chance of leading to a change in clinical management, we consider that communication of information to counselees who are carriers of class 3B variants would encourage the collection of additional information in the family and would thus represent a worthwhile investment of resources given the potential gains in clinical utility.

It is important to mention that the assumption that is valid in the MLM for the classification of the variants is that the variant under study is either neutral with respect to cancer risk or has the same risk as known highly penetrant pathogenic mutations. The IARC system was developed for highly penetrant risk genes and therefore it is probably not suited for classifying low or intermediate penetrance variants either in known genes such as *BRCA1* (e.g., c.5096G>A, p.Arg1699Gln)[40] or moderate risk genes such as *CHEK2* (MIM #604373). For such cases, there is still no clear guideline for communication or clinical management of the counselees and their family members. More insight into the exact cancer risk associated with such variants is needed to determine a suitable approach to classification of lower risk variants.

In this paper, we propose an extension to the existing classification system,[25] currently used for VUS in the high-risk cancer predisposing genes, and we suggest a new

communication protocol. The purpose of these recommendations is to improve the clinical management of the counselees by a more precise classification of the variants without causing unnecessary stress for the counselees or additional costs for the health care system, while minimizing the risk of missing pathogenic mutations in clinical practice.

National and international collaborative research consortia such as the HEBON (HEreditary Breast and Ovarian cancer research in the Netherlands, http://www.hebon.nl), InSiGHT, and ENIGMA play an extremely valuable role in improving cancer risk estimates by assisting definitive classification through collection of all available information on variants and associated phenotypes, and by working closely with clinical groups in many countries to further enhance the value of genetic testing for patients.

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Discussion

7

Genetic risk assessment in families with breast cancer is mainly based on genetic screening of the *BRCA1* (MIM* 113705) and *BRCA2* (MIM* 600185) genes. If a pathogenic variant is found, an advice is given for surveillance and risk reducing surgeries following national guidelines (www.oncoline.nl, accessed May 2017). Up to 10% of all the *BRCA1/2* tests lead to identification of a variant of uncertain clinical significance (VUS). VUS are sequence changes such as missense variants, small in-frame insertions and deletions, nucleotide substitutions that do not lead to amino acid changes and alterations in non-coding sequences for which the clinical significance is uncertain. Classifying VUS and determining cancer risk associated with these variants is a great task for personalized genetic counselling and preventive strategies.[1] Patients in whom a VUS has been identified experience considerable psychological distress, caused by the uncertainty that they may face a cancer risk as high as that for known pathogenic variants.[2, 3] If a VUS is classified as pathogenic or likely pathogenic,[4] the counselee will have a screening/surgery advice according to the guidelines whereas if the variant is classified as benign or likely benign, the counselee will be treated as if not having any pathogenic variants. The risk of (second) breast cancer or ovarian cancer, for her and her female family members, will then be calculated based on the age and number of affected individuals in her family, using different breast or ovarian cancer models such as the models by Stratton et al[5] and van Asperen et al,[6] 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). The risks are then stratified and for each class a specific surveillance and/or advice for prophylactic surgery is given (www.oncoline.nl, http://www.stoet.nl/artsen-informatie/ accessed May 2017).

Although *BRCA1/2* are discovered since mid-1990s and in spite of intensive national and international collaborations to classify these variants, there are still thousands of variants waiting to be classified (Breast Cancer Information Core database: http://research.nhgri.nih.gov/bic/, ClinVar: www.ncbi.nlm.nih.gov/clinvar, LOVD: http://databases.lovd.nl/shared/genes, accessed March 2017 accessed May 2017).

This thesis is aimed at improving the classification of the variants of uncertain clinical significance in the *BRCA1/2* genes. Furthermore, it describes the optimization and standardisation of guidelines for communication of the VUS with the counselees in clinical practice. Progress in the classification of the variants would improve accuracy of advice involving surveillance and risk-reducing strategies, reduce counselee's and their families' psychological stress, reduce unnecessary health care costs and ultimately improve patient care.

To this end, the results of the classification of VUS based on only in silico characteristics was studied and compared to the results of classification when additional information was used (**Chapter 2**). Breast and ovarian cancer risks for the *BRCA1* c.5096G>A, p.Arg1699Gln (R1699Q) carriers were assessed in a large cohort and adjusted clinical management

recommendations for female carriers were proposed (**Chapter 3**). To study the sensitivity and specificity of *BRCA1/2*-carrier prediction- of the existing mutation- models for male breast cancer, the performance of three commonly used *BRCA1/2* models, i.e., BOADICEA, BRCAPRO and the Myriad Pro Calculator were compared for a large cohort of male breast cancer patients (**Chapter 4**). A subset of the most common Dutch *BRCA1* variants were analysed using a multifactorial likelihood model (MLM) (**Chapter 5**). This 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. In **chapter 6** the current IARC (International Agency for Research on Cancer) classification system was discussed and adaptations to this system were proposed regarding clinical management of carriers of VUS in high penetrance cancer predisposition genes.

PITFALLS OF THE CURRENT CLASSIFICATION

Classification models

The multifactorial likelihood approach, as described in the introduction of this thesis, can be applied to VUS, not only in *BRCA1* and *BRCA2* but also in other high risk cancer-predisposition genes.

In the MLM, the assumption is that the variants under study are either neutral in regard to cancer risks, or that they have the same age- and site-specific breast/ovarian cancer risks as the average *BRCA1*-pathogenic variants.[7] Antoniou et al estimated the average cumulative risks in *BRCA1*-athogenic variant carriers by age 70 years were estimated to be 65% (95% confidence interval (CI): 44%-78%) for breast cancer and 39% (95% CI: 18%-54%) for ovarian cancer. The corresponding estimates for *BRCA2* were 45% (95% CI: 31%-56%) and 11% (95% CI: 2.4%-19%).[8] In its current state therefore, the MLM can only predict the probability of pathogenicity of a variant in a high risk cancer gene.

The MLM is particularly powerful if different types of data (cosegregation, tumour pathology, co-occurrence, etc.) are available from many families carrying the same variant. However, if a particular variant is associated with a lower risk compared to the average truncating pathogenic variants, in spite of the availability of a large amount of data, the model might provide inconclusive evidence, and/or there would be conflicts between the results from different sources of evidence.[7] An example of such a variant is the *BRCA1* c.5093G>A, P.Arg1699Gln (R1699Q) which was initially classified as VUS using the MLM method.[9] For this variant functional tests to assess pathogenicity did not lead to conclusive results.[10] Other models, based on family history analysis of *BRCA*-ness[11] or cosegregation within a family,[12] also gave inconclusive results. In such cases adaptations to the model are required to determine the probability of pathogenicity.[9] The results from a large cohort of carriers of this variant showed that the risks associated with this variant- 20% lifetime risk for breast cancer and 6% lifetime risk for ovarian cancer - are lower than for the average *BRCA1* variant. Hence R1699Q can be classified as an intermediate risk variant[13] (relative risk (RR) 2-5).[14, 15]

Lack of sufficient clinical data for most of the *BRCA1* and *BRCA2* VUS and the inability to reliably assess intermediate risk alleles, has led researchers to focus on the results of functional tests. During the ENIGMA Consortium Meeting on 15-17 January 2017 in Limassol, Cyprus (https://enigmaconsortium.org, accessed April 2017) participants agreed that functional data on *BRCA1/2* VUS can be used, provided that it is not the sole data on which a classification is based. The main argument for the latter provision is that as functional assays do not measure cancer risk directly, they still should be calibrated for sensitivity and specificity against variants of known clinical significance in *BRCA1/2* genes which are located in the relevant functional domains.[16, 17] In case of some specific types of variants (e.g. missense variants) this can be particularly challenging, usually because the number of variants reliably classified as pathogenic or non-pathogenic in the validation set is limited. Once properly calibrated, the use of functional test results in the MLM will allow the translation of functional effects to cancer risk. Different research teams developed a model for *BRCA2* VUS using results from a Homology-directed repair (HDR) assay.[18, 19] Likelihood ratios (LRs) could then be calculated for inclusion in the multifactorial likelihood model, next to data from other sources, such as family history and cosegregation, which eventually give a posterior probability of pathogenicity.[20] Furthermore, because these genes have different cellular functions, not all of which are known, a negative result for a particular functional assay (i.e., no functional defect detected) does not indicate low or absence of cancer predisposition. In order to deal with this problem, a panel of different assays representing different functions of the gene should be used.[21] Moreover, highly quantitative assays are needed to discriminate between variants that totally inactivate or only partially inactivate protein function, such as seen for the intermediate risk variant *BRCA1* R1699Q.[9] Nonetheless, in time, functional assay data on its own or combined with clinical/genetics data will be used for the evaluation of pathogenicity of VUS. In this way, functional assays will become a crucial tool for the assessment of the clinical significance of VUS.[21]

Classification systems

Different classification systems have been proposed in the last years based on the probability or possibility of the association of the variant with cancer.

In 2007, the UK Clinical Molecular Genetics Society and the Dutch Society of Clinical Genetics Laboratory Specialists proposed reporting variants in four classes depending on their pathogenicity [Bell et al, 2007]. In 2008, the American College of Medical Genetics (ACMG) proposed a six class system for interpretation and reporting of sequence variants, with an emphasis on the importance of appropriate reporting of sequence variations using standardized terminology and established databases.[22] However, neither of these systems recommended using quantitative information for the classification of variants, nor did they recommend clinical management of the carriers based on the variant's pathogenicity class. An expert working group, convened at IARC (International Agency for Research on Cancer, Lyon, France, http://www.iarc.fr, accessed May 2017) in 2008, proposed a standardized

five-tier classification system applicable to sequence-based results in highly penetrant cancer predisposition genes. This classification system interprets posterior probabilities from the MLM and translate these to recommendations for clinical practice.[4] This system has served the community very well the past decade, but the continuing increase in our knowledge on the *BRCA1* and *BRCA2* genes, their protein functions and the increasingly more refined variant-classification methods, have recently revealed one of its major shortcomings, i.e., how to handle variants of intermediate risk in the high penetrance cancer predisposition genes. For example, although *BRCA1* R1699Q variant is pathogenic, it confers a lower risk compared with the average pathogenic variants in the *BRCA1* and therefore it might not be appropriate to clinically manage these carriers in the same way as the carriers of the average pathogenic variants in *BRCA1*. Using the term "pathogenic" for such variants can be very confusing, especially for the not-genetically trained clinicians and might cause misinterpretation of the data, and as a result, potential mismanagement of the carriers. It is therefore highly important to define an internationally-acknowledged terminology and a clinically-relevant classification for reporting and discussing genetic test results. Currently, international investigators are developing a classification system, designed to not only give information about the probability of pathogenicity[4] but also its associated cancer risk.

FUTURE PERSPECTIVE

Gene panel screening and consequences

Nowadays, new genomics technologies have defined the genetic architecture of cancer beyond the classic high risk cancer syndromes. These technologies have resulted in identification of more moderate risk (RR 2-5) and low risk (RR <2) genes.[14, 15] Internationally many breast cancer-associated genes are being tested such as *ATM* (MIM* 607585), *BARD1* (MIM* 601593), *BRCA1, BRCA2, BRIP1*(MIM* 605882), *CDH1* (MIM* 192090), *CHEK2*(MIM+ 604373), *MLH1* (MIM* 120436), *MRE11* (MIM* 600814), *MSH2* (MIM* 609309), *NBN* (MIM* 602667), *NF1* (MIM# 162200), *PALB2* (MIM* 610355), *PTEN* (MIM+ 601728), *RAD50* (MIM* 604040), *RAD51C* (MIM* 602774), *RAD51D* (MIM* 602954), *STK11* (MIM* 602216), *TP53* (MIM* 191170), *XRCC2* (MIM* 600375) (www.fulgentgenetics.com, www.ambrygen.com, http://www.ambrygen.com, accessed May 2017); in the near future whole exome or genome sequencing (WES, WGS) will be applied in the cancer clinics on an unprecedented scale. The diagnostic laboratories in the Netherlands are nonetheless reluctant to offer these services. Centres for disease control and prevention established a model for evaluating genetic tests; the ACCE. "ACCE, takes its name from the four main criteria for evaluating a genetic test — analytic validity, clinical validity, clinical utility and associated ethical, legal and social implications. It is a model process that includes collecting, evaluating, interpreting, and reporting data about DNA-testing for disorders with a genetic component in a format that allows policy makers to have access to up-to-date and reliable information for decision making" (https://www.cdc.gov/genomics/

gtesting/acce, accessed June 2017). In the Netherlands, although the technology is available, the clinical and molecular geneticists are reserved regarding sequencing all the known cancer genes in all the patients. Since September 2014, in addition to *BRCA1* and *BRCA2* testing, genetic testing of the risk allele 1100delC in *CHEK2* is offered in all the genetic diagnostic laboratories in the Netherlands. In some laboratories several breast cancer genes (e.g. *ATM, BRCA1, BRCA2, CHEK2, NBN, PALB2* (http://www.dnadiagnostiek.nl, accessed May 2017) are offered as a gene panel. For most of these genes the risk of breast cancer is still not reliably established, nor are the cellular gene functions.[14] That makes it very difficult to determine the clinical actionability of the test result and the clinical management of the carriers. Moreover, gene panel testing and WES will certainly increase the numbers of uncovered VUS in these cancer-related genes. Hilbers et al[23] (Figure 1) calculated the number of variants of uncertain significance for the gene panel sequencing under the assumption that the rate of VUS/base pair for the additional genes would be equal to that of *BRCA1/2* which were previously calculated by Frank et al.[24] The authors noticed a small increase in the amount of pathogenic variants compared to the strong increase in the number of VUS.

Tung et al[25] assessed the frequency of pathogenic variants in 25 cancer predisposition genes in a cohort of patients with stage I to III breast cancer. The genes tested were *BRCA1*, *BRCA2*, *ATM*, *BARD1*, *BRIP1, CDH1*, *CHEK2*, *NBN*, *PALB2, PTEN*, *STK11*, *TP53*, *APC* (MIM* 611731), *BMPR1A* (MIM* 601299), *CDK4* (MIM* 123829), *CDKN2A* (MIM* 600160), *EPCAM* (MIM*185535), *MLH1*, *MSH2, MSH6* (MIM* 600678), *MUTYH* (MIM* 604933), *PMS2* (MIM* 600259), *RAD51C*, *RAD51D*, *SMAD4* (MIM* 600993). In their study pathogenic variants were identified in 10.7% of the patients. 6.1 % were in *BRCA1/2*, of which 5.1% in non-Ashkenazi Jewish patients, and 4.6% in other breast/ovarian cancer predisposition genes.[25]

MLM, as explained above, based on its current assumptions and without adaptations, is not applicable for moderate risk and low risk genes. The functional approach for classification of the VUS also should still be developed for the newly discovered moderate and low risk genes. As the functions of the proteins encoded by these genes are not yet fully known, designing the various assays for testing the function of the wildtype and VUS becomes a major problem. The classification of these variants will therefore be one of the most important challenges of clinical genetics in the coming decade.

User-friendly web-based tools and personalized risk prediction models

In order to classify VUS with cosegregation, Petersen et al[26] developed a simple Bayesian method to assess pathogenicity of VUS in 1998. Later, Thompson et al[12] provided a more general method based on the full pedigree likelihood. All available genotype information from the family is used. The first method used a defined penetrance in carriers *versus* non-carriers and ignored the age of onset whereas the latter specified liability classes which defined the age-range of family members in intervals for which the breast/ovarian cancer

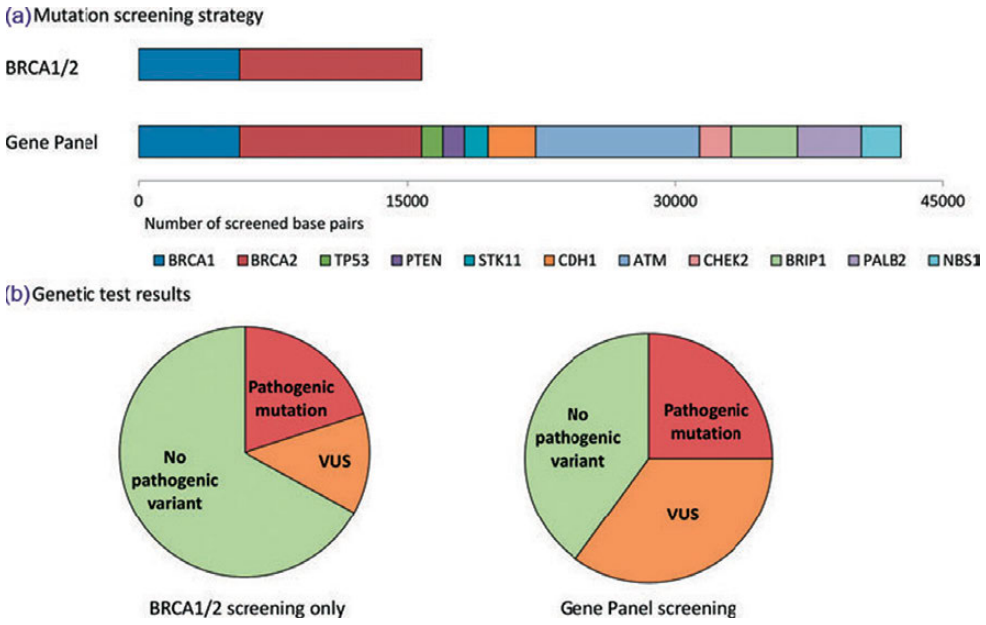

Figure 1. Test results from different genetic screening strategies in the clinic.(a) The screened genes for the different genetic screening strategies and the corresponding number of screened coding base pairs. (b) The distribution of test results for *BRCA1/2* screening based on Frank et al[24] The number of variants of uncertain clinical significance (VUS) for the gene panel screening was calculated under the assumption that the rate of VUS/base pair for the additional genes would be similar to that of *BRCA1* and *BRCA2*. Reprinted from Clinical Genetics, 84, Hilbers FS, Vreeswijk MP, van Asperen C J, Devilee P, The impact of next generation sequencing on the analysis of breast cancer susceptibility: a role for extremely rare genetic variation?, 407-14, Copyright (2013), with permission from John Wiley & Sons.[23]

risk is supposed to be constant.[27] The department of Clinical Genetics at the Leiden University Medical Centre (LUMC) in collaboration with the department of medical statistics previously developed an algorithm which calculates the likelihood ratio of a VUS being pathogenic based on all the available genotype data. Penetrance was used as a function of age of onset.[27] Thereby, they also developed a user-friendly web-based tool which makes calculation of LR for the cosegregation in *BRCA1* and *BRCA2* in small families available also to non-statisticians (https://www.msbi.nl/cosegregation/, accessed May 2017). There is however, no possibility to adapt the penetrance in this tool. The best model for cosegregation analysis will be a flexible tool which has a possibility to adjust the penetrance of the gene and takes frequency of the pathogenic variant in the population and year of birth-dependent incidence of breast cancer into account (breast cancer incidence is not constant and seemed to be increasing until 2010 in the Netherlands (http://www.cijfersoverkanker.nl, accessed May 2017). If the data on these parameters are known, such a model can also be used for calculation of cosegregation for other high risk autosomal dominant cancer genes.

As explained above, different models have been developed to calculate the cancer risk for family members, based either on the presence of a pathogenic variant in *BRCA1* or *BRCA2,* or on the number of affected family members if a pathogenic variant is absent. Examples of these models are 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). BOADICEA has been validated for predicting *BRCA1/2* carrier status in large cohorts of families from different international genetics clinics.[28-33] It is recommended as a risk assessment tool for the management of women with a family history of breast cancer in several important guidelines, including the Dutch Oncoline (www.oncoline.nl, accessed May 2017), NICE guidelines (www.nice.org.uk/guidance, National Institute for Health and Care Excellence clinical guideline in the UK, accessed May 2017) and guidelines of the American Cancer Society (www.cancer.org, accessed May 2017). BOADICEA is also chosen as the standard for analyses in ENIGMA consortium facilitating the exchange of data. This model currently incorporates the effects of *BRCA1* and *BRCA2*, family history, and the effect of common genetic variants (SNPs) on breast cancer risk. When available, data about *BRCA1*- and *BRCA2*-associated breast tumour pathology can be used in the calculations. The risk estimates for some of the moderate/high risk breast cancer genes such as *PALB2*, *CHEK2* and *ATM* are now incorporated in the BOADICEA[34] (https://pluto.srl.cam.ac.uk/cgi-bin/bd4/v4beta14/bd.cgi, accessed May 2017). BOADICEA is also being extended to include the effects of other known breast cancer risk factors, including breast density, reproductive history, BMI and hormone replacement therapy as part of the Dutch UK BRIDGES (Breast Cancer Risk after Diagnostic Gene Sequencing, https://bridges-research.eu, accessed May 2017) project.

As BOADICEA is currently the standard tool for risk assessment and is continuously being refined and updated, it forms a great platform for incorporation of MLM. Theoretically, the model can also use the pedigree information to calculate the likelihood ratio of cosegregation and family history of breast cancer for different high and moderate risk cancer predisposition genes. Based on the probability of pathogenicity and the pedigree data, in combination with life style factors and polygenic risk (based on the SNP data), it can calculate personalized breast cancer risk estimates. These estimates can then guide specific surveillance strategies for the family members.

Characteristics of *BRCA*-deficient tumours

Both *BRCA1* and *BRCA2* are required for DNA double-strand break repair by homologous recombination (HR-based DNA repair).[17, 21] Pathogenic variants in *BRCA1* and *BRCA2* inactivate protein function. Furthermore, in cancer the wild-type BRCA allele is almost always lost. These will result in a defect in HR-based DNA repair in the cancer. Due to this deficiency in homologous recombination, *BRCA1* and *BRCA2* related -tumours exhibit

genomic instability, which can be measured using different methods such as methods based on copy number variations (array-Comparative Genomic Hybridization, array-CGH)[35-37] and methods studying DNA mutational signatures also called genetic scars.[38, 39]

BRCA1 and *BRCA2*-related tumours show very specific gains and losses of large regions of DNA. These copy number alterations can be identified by Array-CGH and this method has been shown as an effective way to distinguish breast tumours caused by *BRCA1 or BRCA2* mutations from sporadic breast tumours. In chapter 5 of this thesis we have used this approach as a new component of the MLM in classification of the *BRCA1* VUS (manuscript in preparation).

Davies et al[38] recently published a method in which they use whole genome sequencing technology to identify a mutational signature predictive of *BRCA1/2* deficiency.

They developed a weighted model called HRDetect to identify *BRCA1/2* deficient tumours based on base substitution signature, large deletions with microhomology at the junctions and specific rearrangements. This model, if used routinely, could in the future be used directly or incorporated in the multifactorial likelihood model to determine the pathogenicity of the VUS. It could also help to select those patients most likely to respond to PARP-inhibitor or Platinum treatments in the absence of a *BRCA* germline mutation (personalized therapy).

Personalized therapy

The absence of homologous recombination in *BRCA*-related tumours make them vulnerable for treatment with specific drugs. *BRCA1* and *BRCA2*-deficient tumours are highly sensitive to platinum based chemotherapy both in vitro and in vivo.[40-42] Platinum chemotherapy generates inter-strand cross-links which can only be properly repaired by HR-based DNA repair. In a cell in which HR-based DNA repair is deficient, this will lead to cell death.

Recently, a new class of drugs, so-called Poly (ADP-ribose) polymerase (PARP)-inhibitors, have proven to be very successful to treat *BRCA*-related tumours. PARP inhibitors induce synthetic lethality in HR deficient cells (Figure 2). Patients with *BRCA1* and *BRCA2*-related breast and ovarian tumours respond very well to treatment with PARP-inhibitors. Since the tumour cells are HR deficient whereas the normal cells of the patient are HR proficient, this therapy is highly targeted to the tumour cells.[43] Recently, several PARP-inhibitors have been registered for the treatment of patients with *BRCA*-related high grade serous ovarian cancer.[44]

Patients carrying a *BRCA1/2* VUS will benefit from classification of the variants, as these might predict responsiveness of their tumours to targeted therapy such as PARP-inhibitors. Extensive research is required to study whether treatment with *BRCA1/2*-specific treatments for the carriers of intermediate risk variants such as *BRCA1* R1699Q[9, 46] has the same effect on the patients as on the carriers of the average pathogenic variants. Many other proteins involved in homologous recombination repair such as *ATM*, *CHEK2*, *BARD1*, *BRIP1*,

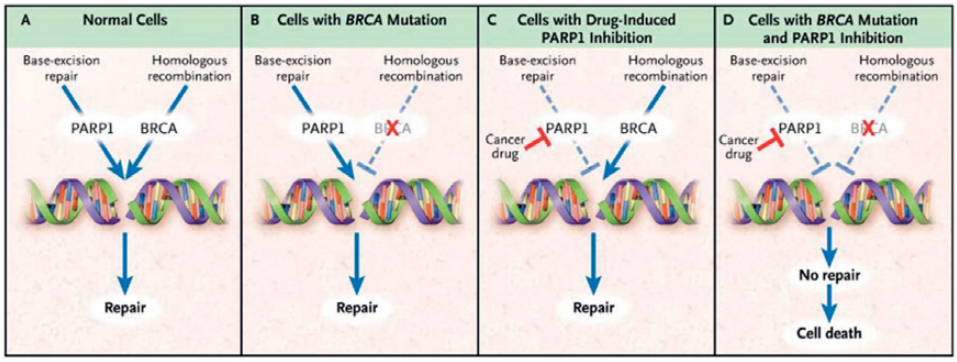

Figure 2. Mechanism of Cell Death from Synthetic Lethality, as Induced by Inhibition of Poly Adenosine Diphosphate [ADP]–Ribose) Polymerase 1 (PARP1). Reused with permission from Iglehart et al. N Engl J Med 2009; 361:189-191,[45] Copyright Massachusetts Medical Society.

RAD50, *RAD51C*, *RAD51D* and *PALB2* are now known to contribute to hereditary cancer risk.[47] In the same way, carriers of pathogenic variants in these genes could theoretically benefit from treatments with PARP-inhibitors and Platinum chemotherapy.

Conclusion

In the near future, through large-scale research initiatives using NGS (Next-Generation Sequencing), new disease predisposition genes will be identified. Screening of these genes will inevitably result in identification of an enormous number of VUS.

This thesis outlines the challenges regarding classification of the VUS in general and in particular in *BRCA1* and *BRCA2* breast cancer genes and the clinical management of patients carrying the VUS. It describes different methods which, when integrated, can be used for classification of the VUS in *BRCA1* and *BRCA2*. Furthermore, it describes different classification systems and proposes adaptations to the currently commonly-used IARC classification system.

As more variants will be identified in the future, the establishment of their associated disease risk will be important. Most rare variants will be unique to a population and there will not be sufficient genetic data for classification purposes. Research initiatives and international collaborations coordinated by consortia such as ENIGMA are essential to facilitate collection of extensive datasets and in this way reliably determine the pathogenicity of the variants. Long term follow-up and screening of carriers of VUS in a research setting are necessary to enable future assessment of the reliability of the classifications and utility of the proposed surveillance, especially for the intermediate risk variants in the high risk cancer predisposition genes and the newly identified moderate risk genes.

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Appendix

SUMMARY

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 or when the mutation detection chance is around 10%. (www.oncoline.nl)

In case a pathogenic variant in either of these genes is found, intensive surveillance and risk reducing surgeries can be offered to the carriers. Furthermore in some cases proven carriers can benefit from personalized treatments with platinum salts (carboplatin and cisplatin) or poly ADP-ribose polymerase (PARP)-inhibitors.

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 changes are called variants of uncertain clinical significance (VUS) and include missense changes, in-frame deletions or insertions, synonymous nucleotide substitutions, as well as alterations in non-coding sequences or in untranslated regions. In the Netherlands there were in 2012 around 293 unique variants identified in *BRCA1* and 492 in *BRCA2*. ClinVar is a freely accessible, public archive of reports of the relationships among human variations and phenotypes, with supporting evidence. ClinVar search in 2017 resulted in about 1700 unique VUS in *BRCA1* and 2800 unique VUS in *BRCA2*.

Classifying VUS is a great challenge for tailoring genetic counselling and disease prevention strategies. Patients in which a VUS is identified experience considerable psychological distress, not only due to the possibility that they may have a cancer risk as high as that for known pathogenic variants, but also due to the uncertainty of this cancer risk. Not only the persons who are carrying the VUS, but also their relatives can benefit from classification of the VUS. In case a variant is classified as pathogenic, then the family members will be offered cascade screening. They can be tested for the presence of the pathogenic variant. Carriers can enter screening programs for early cancer detection or consider prophylactic surgery. (www.oncoline.nl)

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

AIM OF THIS THESIS

This thesis is aimed at improving the classification of the variants of uncertain clinical significance in the BRCA1/2 genes. Furthermore, it describes the optimization and standardisation of guidelines for communication of the VUS with the counselees in clinical practice.

In this thesis, an introduction to hereditary breast cancer, *BRCA1* and *BRCA2* genes, variants of uncertain significance and different classification methods and guidelines is given in **chapter 1**.

In **chapter 2** the results of the classification of VUS based on only in silico characteristics was studied and compared to the results of classification when additional information was used. Of the 46 VUS assigned to class II by in silico analysis alone, nearly half were eventually re-categorised as class I and 10% as class III when additional information was included. As in silico analysis alone is not always sufficient to unambiguously assign VUS to either class II or class III, the possibility of obtaining additional information from a family should be taken into account during the decision process preceding the communication of a VUS test result.

The paper in **chapter 3** describes the cancer risks associated with the missense variant c.5096G>A, p.Arg1699Gln (R1699Q) in *BRCA1* in a large group of families ascertained internationally. The results showed that the risks associated with this variant, breast cancer: 20% and ovarian cancer: 6%, are lower than for the average truncating *BRCA1* variants and that this variant can be classified as an intermediate risk variant. Furthermore, cancer risks in families with this intermediate risk variant are likely to be influenced by additional genetic factors. Based on these risks recommendations for clinical management for female carriers were proposed.

In **chapter 4** mutation prediction performance of BOADICEA, BRCAPRO and Myriad *BRCA* risk calculator was tested in a large cohort of Dutch male breast cancer patients. The numbers of observed versus predicted mutation carriers were compared and the area under the receiver operating characteristic (ROC) curve (AUC) for each model was assessed. The results support the use of both BRCAPRO and BOADICEA for determining the probability of carrying a *BRCA1* or *BRCA2* pathogenic variants in MBC patients. Freely available, reliable prediction models such as BOADICEA and BRCAPRO play an important role in improving clinical care, especially in countries with limited health care resources. Furthermore, the proven prediction accuracy of both BOADICEA and BRCAPRO for *BRCA* carriership in males underlines the reliability of other function of these models which is the prediction of overall breast cancer risk.

Information on array-CGH in addition to other data based on different lines of evidence was used to (re)classify some of the most common *BRCA1* variants in the Netherlands. For the classification of the variants mainly in silico data, cosegregation of the variant and disease within families and histopathological characteristics of the tumour were used. Where available the results of classification based on the MLM were compared

with functional analysis which is performed by our colleagues in the Netherlands Cancer Institute in Amsterdam (NKI). Comparing functional analysis with our study, 7 out of 8 variants for the results matched. Results from this study have direct implications for genetic counselling and medical management of the carriers. Furthermore, this 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. (**Chapter 5**)

To improve the clinical utility of the current five-tier IARC classification system, a pragmatic adaptation to clinical practice was suggested in **chapter 6**. The suggestion is that the laboratory specialists divide VUS class 3 into two subgroups: class 3A with a posterior probability of 0.05 to 0.499 and class 3B with a posterior probability of 0.5-0.949. The counsellors could then consider to communicate and test family members when the posterior probability of pathogenicity of a VUS is higher than 0.5 (i.e. category 3B) but not communicate variants in class 3A unless there is clinical benefit for counselee or for research. The purpose of the recommendations is to improve the clinical management of the counselees by a more precise classification of the variants without causing unnecessary stress for the counselees or additional costs for the health care system, while minimizing the risk of missing pathogenic variants in clinical practice.

NEDERLANDSE SAMENVATTING

DNA-onderzoek van de 'hoog risico' genen *BRCA1* (MIM* 113705) en *BRCA2* (MIM* 600185) wordt steeds vaker aangeboden indien er sprake is van een verdenking op een erfelijke vorm van borst- en/of eierstokkanker. Het gaat dan om situaties met meerdere gevallen van deze aandoening(en) binnen een familie of bij één patiënte. Dit wordt aangeboden wanneer de mutatiedetectiekans ongeveer 10% is. (www.oncoline.nl)

De uitslag van het DNA-onderzoek naar mutaties in de *BRCA1*-en *BRCA2*-genen kan worden onderverdeeld in drie categorieën:

- "Positief", d.w.z. er is een verandering in het DNA aangetroffen waarvan wordt aangenomen dat het een pathogene variant betreft (d.w.z. het BRCA1- of BRCA2- eiwit wordt erdoor geïnactiveerd).
- "Negatief", d.w.z. er is geen verandering in het DNA gevonden, dan wel er is een verandering gevonden waarvan wordt aangenomen dat het géén effect heeft op de functie van het BRCA1- of BRCA2-eiwit (zgn. DNA-polymorfismen).
- "Variant of Uncertain Significance" oftewel "VUS"; deze verandering in het DNA is nog niet in te classificeren als "positief" of "negatief". Het pathogene effect van deze mutatie is (nog) niet bekend.

Pathogene varianten in de *BRCA1*- en *BRCA2*-genen veroorzaken hoge risico's op het krijgen van borst- en/of eierstokkanker. In het geval dat er een pathogene variant in één van deze genen wordt gevonden, komen de draagsters in aanmerking voor deelname aan intensieve screeningprogramma's van de borsten vanaf een jonge leeftijd of kunnen ze overwegen om de borst en/of eierstokken preventief te laten verwijderen. Bovendien kunnen draagsters die een behandeling nodig hebben voor borst- of eierstokkanker in een aantal situaties profiteren van gepersonaliseerde therapieën met platinum (carboplatine en cisplatine) of poly-ADP-ribose polymerase (PARP)-remmers.

De voortdurende ontwikkeling van sequencing technologie in DNA-diagnostische laboratoria resulteert echter ook in het detecteren van een toenemend aantal varianten in de *BRCA1*- en *BRCA2*-genen waarvoor het klinische significantie onbekend is. Deze varianten worden Variants of Uncertain Significance (VUS) genoemd. Het zijn missense varianten, in-frame deleties of inserties, synonieme nucleotide substituties, evenals veranderingen in niet-coderende sequenties of in niet-getransleerde regio's. Deze uitslag kan hoge risico's voor het ontwikkelen van borst- en of eierstokkanker inhouden of kan helemaal geen klinische significantie hebben. Zowel BRCA1 als BRCA2 zijn grote multifunctionele eiwitten. Het is daarom te verwachten dat sommige van deze varianten de normale cellulaire eiwitfunctie beïnvloeden of inactiveren. Andere veranderingen zullen echter neutraal zijn.

Bij de start van dit onderzoek evenaarde het percentage VUS-uitslagen het aantal pathogene mutaties. In 10-15% van de families werd een VUS gevonden. In Nederland waren er in 2012 ongeveer 293 unieke varianten geïdentificeerd in *BRCA1* en 492 in *BRCA2*. Op basis van ClinVar, een vrij toegankelijke site voor informatie over humane

genoom varianten en fenotypen, werden in 2017 ruim 1700 unieke VUS in *BRCA1* en ruim 2800 unieke VUS in *BRCA2* geregistreerd. (https://www.ncbi.nlm.nih.gov/clinvar)

Het classificeren van de VUS is een grote uitdaging voor de genetische counseling. Patiënten bij wie een VUS is geïdentificeerd, ervaren aanzienlijke psychologische stress, niet alleen door de mogelijkheid op een hoge kans op kanker, maar ook door de onzekerheid over dit kankerrisico. Naast de personen die een VUS dragen, kunnen ook hun familieleden profiteren als de varianten een duidelijke classificatie hebben. Als een variant als pathogeen wordt geclassificeerd, dan wordt genetisch onderzoek ook aan de familieleden aangeboden. Ze kunnen getest worden op de aanwezigheid van de pathogene variant. Dragers komen ook in aanmerking voor screeningsprogramma's voor vroegtijdige detectie van kanker of kunnen overwegen om profylactische operaties te ondergaan. Tevens kunnen ze profiteren van de specifieke chemotherapieën zoals hierboven genoemd.

Multifactorial likelihood model

Het ontwikkelen van het multifactorial likelihood model (MLM) voor *BRCA1*- en *BRCA2*-varianten was een belangrijke doorbraak in de studie van de VUS. De bouwstenen in het MLM zijn de Likelihood Ratio's (LR). LR is een maat voor het berekenen van de nauwkeurigheid van een diagnostische test. De LR van een klinische bevinding is de kans op die bevinding wanneer een conditie aanwezig is, gedeeld door de kans op dezelfde bevinding wanneer de conditie afwezig is. LR wordt berekend voor verschillende soorten van data (zoals in silico, familiegeschiedenis, co-segregatie van de variant met de ziekte in een familie, histopathologische kenmerken van de tumor en segregatie van de variant met een pathogene *BRCA1*- of BRCA2-variant in trans). Het MLM combineert deze LRs om de "probability" van de pathogeniciteit van een variant te bepalen.

DOEL VAN HET ONDERZOEK

Het doel van dit proefschrift was het verbeteren en optimaliseren van de bestaande methoden om de klinische relevantie van verschillende VUS te analyseren. In dit proefschrift werd in **hoofdstuk 1** erfelijke borstkanker, *BRCA1*- en *BRCA2*-genen, VUS en verschillende classificatiemethoden en richtlijnen beschreven.

In **hoofdstuk 2** werden een aantal VUS op basis van alleen in silico-eigenschappen geclassificeerd en deze classificatie werd vergeleken met de resultaten van classificatie wanneer aanvullende informatie werd gebruikt. Van de 46 VUS die aanvankelijk in klasse II waren geclassificeerd op basis van alleen in silico-analyse, werden uiteindelijk bijna de helft geclassificeerd als klasse I en 10% als klasse III wanneer er extra informatie werd gebruikt. Op basis van dit hoofdstuk werd geconcludeerd dat een variant niet altijd met zekerheid geclassificeerd kan worden naar klasse II of III alleen op basis van de in silico data.

Hoofdstuk 3 beschrijft de borst- en eierstokkankerrisico's van de missense variant c.5096G>A, p.Arg1699Gln (R1699Q) in *BRCA1*. Voor dit onderzoek werden meerdere

families internationaal geïncludeerd die drager zijn van deze variant. De resultaten toonden aan dat de risico's verbonden aan deze variant, borst kanker: 20% en eierstokkanker: 6%, lager zijn dan voor de gemiddelde truncerende *BRCA1*-varianten en dat deze variant een "intermediate risico" geeft op borst- en/of eierstokkanker voor een vrouwelijke draagster van deze variant. Daarnaast werd in deze studie aangetoond dat de kankerrisico's in deze families waarschijnlijk mede beïnvloed worden door aanvullende genetische factoren. Op grond van deze risico's werden klinische aanbevelingen voor vrouwelijke dragers voorgesteld.

BOADICEA, BRCAPRO en de Myriad Pro-calculator zijn drie modellen die veel gebruikt worden om de kans op het vinden van een *BRCA* mutatie te kunnen vaststellen. Het aantal met een DNA-test aangetoonde *BRCA1/BRCA2* mutatiedragers werd vergeleken met het aantal voorspelde mutatiedragers op basis van de modellen, binnen een groot cohort mannelijke borstkankerpatiënten (MBC) (**hoofdstuk 4**). Sensitiviteit en specificiteit van deze modellen werden met elkaar vergeleken. Hiertoe werd gebruik gemaakt van de "area under the receiver operating characteristic (ROC) curve (AUC)". De resultaten laten zien dat zowel BRCAPRO als BOADICEA betrouwbare modellen zijn voor het voorspellen van de kans op het dragerschap van een *BRCA1*- of *BRCA2*-pathogene variant in MBC-patiënten. Vrij beschikbare en betrouwbare algoritmes voor het vaststellen van de kans om een mutatie aan te tonen zoals BOADICEA en BRCAPRO spelen een belangrijke rol bij het verbeteren van de klinische zorg. Dit geldt met name voor landen met een beperkt gezondheidszorgsysteem en beperkte financiële middelen. Voorts benadrukt de bewezen voorspellingsnauwkeurigheid van zowel BOADICEA als BRCAPRO voor *BRCA* dragerschap in mannen de betrouwbaarheid van andere functie van deze modellen; het voorspellen van het risico op borstkanker.

Een aantal van de meest voorkomende Nederlandse *BRCA1*-VUS werden geanalyseerd met behulp van het multifactorial likelihood model (MLM) (**hoofdstuk 5**). Voor de classificatie van deze varianten werd voornamelijk data op basis van in silico analyse, co-segregatie van de variant met de ziekte binnen de families en histopathologische kenmerken van de tumor gebruikt. Deze data zijn in het MLM gecombineerd om de posterior probability van pathogeniciteit te berekenen. Voor het eerst zijn de resultaten van de array-Comparative Genomic Hybridization (array-CGH) geïncorporeerd in het MLM en werd het in combinatie met andere data gebruikt voor de classificatie van de varianten. Onze collega's in het Nederlands Kanker Instituut (NKI) te Amsterdam hebben in 2013 een aantal veelvoorkomende *BRCA1*-varianten met behulp van functionele analyses geclassificeerd. Wanneer deze functionele data beschikbaar waren voor een te onderzoeken variant, werd de resultaten van de classificatie op basis van MLM vergeleken met de resultaten van de functionele analyse. Voor 7 van de 8 varianten waarvoor functionele data beschikbaar was, is de classificatie op basis van MLM consistent met de resultaten van functionele analyse. Deze analyse zal de zorg verbeteren voor de dragers van deze varianten. Er zijn momenteel een beperkt aantal missense varianten in *BRCA1* die als pathogeen zijn geclassificeerd.

Deze studie voegt meer varianten toe aan deze set pathogene varianten. Deze varianten kunnen worden gebruikt als kalibratie set in de toekomstige studies waarin de resultaten van de functionele analyses van de varianten in het MLM model worden geïncorporeerd.

In **hoofdstuk 6** werd een pragmatische aanpassing van het huidige IARC classificatiesysteem voorgesteld dat bestaat uit vijf categorieën voor classificatie. Het voorstel is dat de laboratoriumspecialisten VUS klasse 3 verdelen in twee subgroepen: klasse 3A met de kans van 0,05 tot 0,499 en klasse 3B met de kans van 0,5-0,949. Binnen de Klinische Genetica valt te overwegen om de identificatie van de VUS aan de adviesvrager te communiceren wanneer de kans op pathogeniciteit van een VUS hoger is dan 0,5 (dat wil zeggen categorie 3B), maar de varianten in klasse 3A niet te communiceren, tenzij er een klinisch voordeel is voor de adviesvrager of voor onderzoek. Het doel van deze aanbevelingen is om de zorg voor de adviesvragers te verbeteren door een nauwkeurige indeling van de varianten zonder onnodige stress voor de dragers of extra kosten voor het gezondheidszorgsysteem, terwijl het risico op het missen van de pathogene varianten in de klinische praktijk wordt geminimaliseerd.

CURRICULUM VITAE

Setareh Moghadasi is born on 6th September, 1979 in Tehran, Iran where she completed her secondary school education. Since 2000 she lives in the Netherlands. She started studying Biology and Medical Laboratory Research at the University of Applied Sciences in Leiden in 2001. After gaining her propaedeutic diploma, she started studying Biomedical Sciences at the Leiden University Medical Centre (LUMC) in 2002. For her Bachelor of Science graduation assignment (2005) she took part in a research project to study the role of Transferrin receptor mutation in the MMTV- (Mouse mammary tumour virus) linked human breast cancer (dr. A. Pasternak and prof. dr. P. Devilee) at the department of Human Genetics at the LUMC. Setareh continued her Master of Science in Biomedical Sciences and performed two more research projects namely "Introduction of apoptosis via different signalling pathways in colorectal cancer cell lines" at the department of surgical oncology (dr. P.J.K. Kuppen) and "Down regulation of vertebrate Tel (ETV6) and Drosophila Yan is facilitated by an evolutionarily conserved mechanism of F-box-mediated ubiquitination" (dr. D.A. Baker), both at the LUMC. During her master she additionally followed a four-months course in Science and Research Based Business (SBB) in the faculty of science in Leiden University. She started studying Medicine in 2006 (alongside master in Biomedical Sciences). In 2011 she graduated in both Master of Science in Biomedical Sciences and Medicine. She started working as a resident (ANIOS) in the department of Clinical Genetics at the LUMC in 2011 and at the same year she received the Mosaic research fund from the Netherlands Organisation for Scientific Research (NWO) for her PhD project. She started her PhD in October 2011 at the department of Clinical Genetics at the LUMC. During her PhD-period, from 2012 to 2014, Setareh also obtained her Master of Science degree in Genetic Epidemiology at the Netherlands Institute for Health Sciences in Rotterdam, the Netherlands. In October 2015 she started as Clinical Geneticist in training (AIOS) at the Leiden University Medical Centre. She is married to Farshid Alemdehy, post-doctoral fellow in the Dutch Cancer Institute (NKI) and is mother of Ryan. Together they live happily in Nootdorp.

LIST OF PUBLICATIONS

S. Moghadasi, L.A.M. Janssen, P. Bouwman, P.M. Nederlof , M. Rodríguez-Girondo, C.M. Kets, A.R. Mensenkamp, M.G. Ausems, R.B. van der Luijt, C.M. Aalfs, L.E. van de Kolk, F.B.L. Hogervorst, M.A. Adank, J.J.P. Gille, E.B. Gomez Garcia, M.J. Blok, J.C. Oosterwijk, A.H. van der Hout, R. van Eijk, G.R. Vink, J.T. Wijnen, P. Devilee, M.P.G. Vreeswijk, C.J. van Asperen
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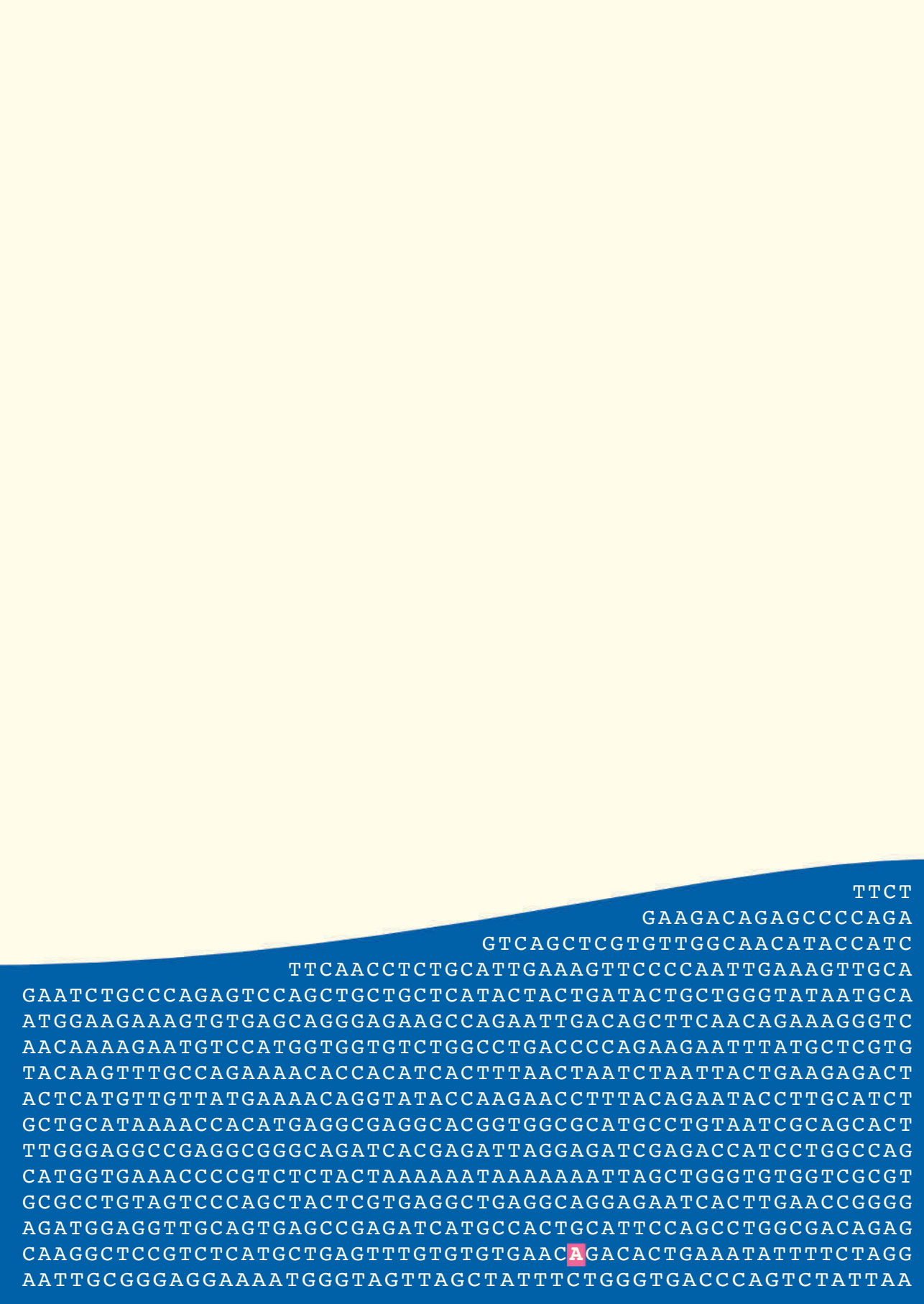
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