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

Moghadasi, S.

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

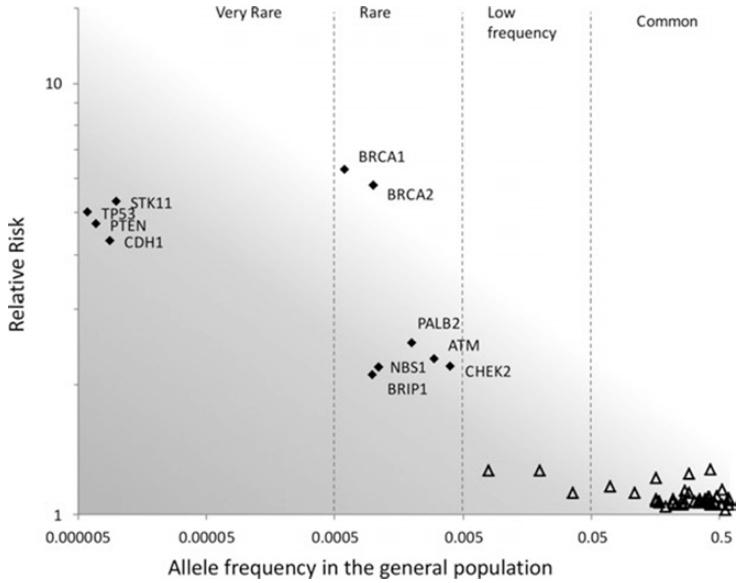

Figure 1. The genetic landscape of breast cancer. This figure shows the allele frequencies in the general population and relative risks for the known breast cancer risk genes and single nucleotide polymorphisms (SNPs). Reprinted from Clinical Genetics, 84, Hilbers FS, Vreeswijk MP, van Asperen C J, Devilee 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

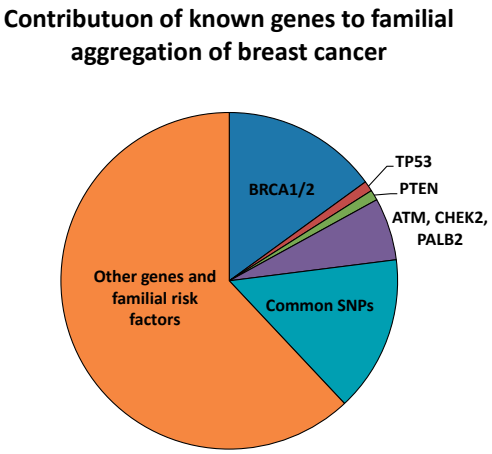

Figure 2. This figure shows the fraction of cases caused by genes known to contain pathogenic variants that predispose to breast cancer and by common genetic risk factors (common SNPs), adapted from Couch FJ. et al[52] and http://discoverysedge.mayo.edu/2015/10/07/breast-cancer-predicting-individual-risk,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

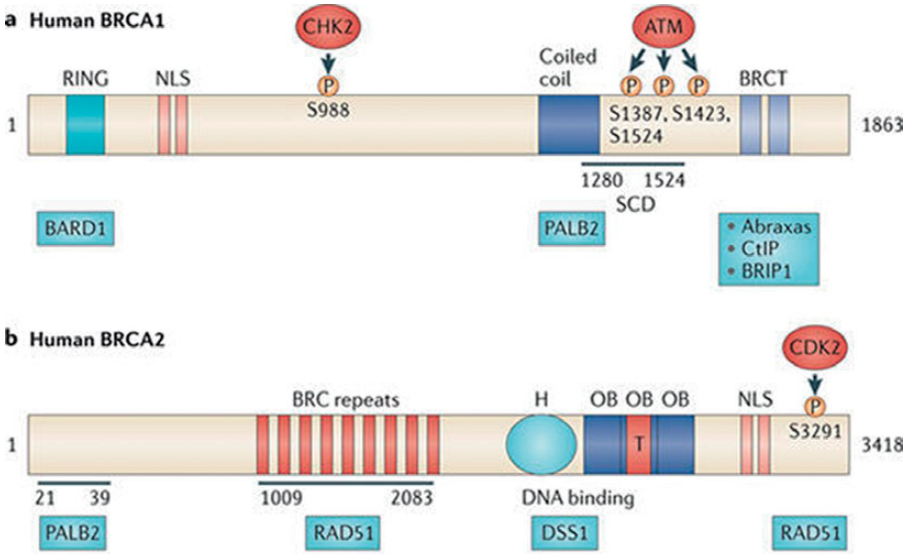

Figure 3. BRCA1 and BRCA2 functional domains. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Nat Rev Cancer. 2011 Dec 23;12(1):68-78.), copyright (2011).[98]

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

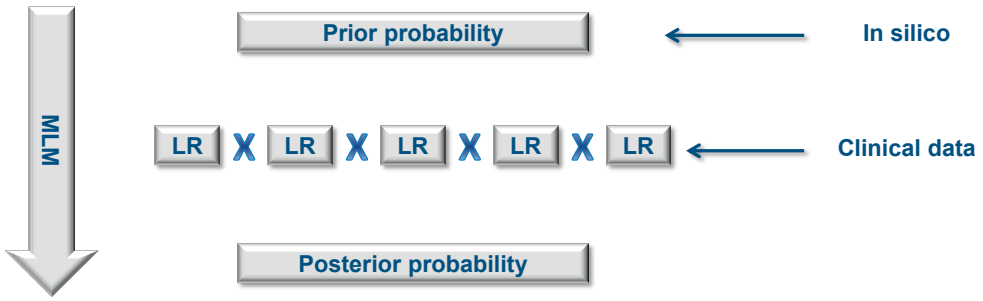

Figure 4. Multifactorial likelihood model (MLM). Posterior probability of pathogenicity = posterior odds/ (posterior odds + 1) and posterior odds= Overall LR × (prior probability/[1– prior probability]).

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]

REFERENCES

1. Cancer CGoHFiB. Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies. *Lancet Oncol*. 2012;13(11):1141-1151.
2. Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer. *Lancet*. 1997;350(9084):1047-1059.
3. Rossouw JE, Anderson GL, Prentice RL, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *Jama*. 2002;288(3):321-333.
4. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. *Cancer Epidemiol Biomarkers Prev*. 2006;15(6):1159-1169.
5. John EM, Kelsey JL. Radiation and other environmental exposures and breast cancer. *Epidemiol Rev*. 1993;15(1):157-162.
6. Morimoto LM, White E, Chen Z, et al. Obesity, body size, and risk of postmenopausal breast cancer: the Women's Health Initiative (United States). *Cancer Causes Control*. 2002;13(8):741-751.
7. Hamajima N, Hirose K, Tajima K, et al. Alcohol, tobacco and breast cancer--collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. *Br J Cancer*. 2002;87(11):1234-1245.
8. Lambe M, Hsieh C, Trichopoulos D, et al. Transient increase in the risk of breast cancer after giving birth. *N Engl J Med*. 1994;331(1):5-9.
9. Cancer. CGoHFiB. Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease. *Lancet*. 2002;360(9328):187-195.
10. Furberg H, Newman B, Moorman P, Millikan R. Lactation and breast cancer risk. *Int J Epidemiol*. 1999;28(3):396-402.
11. Bernstein L, Henderson BE, Hanisch R, Sullivan-Halley J, Ross RK. Physical exercise and reduced risk of breast cancer in young women. *J Natl Cancer Inst*. 1994;86(18):1403-1408.
12. Thune I, Brenn T, Lund E, Gaard M. Physical activity and the risk of breast cancer. *N Engl J Med*. 1997;336(18):1269-1275.
13. Colditz GA, Kaphingst KA, Hankinson SE, Rosner B. Family history and risk of breast cancer: nurses' health study. *Breast Cancer Res Treat*. 2012;133(3):1097-1104.
14. Lichtenstein P, Holm NV, Verkasalo PK, et al. Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *N Engl J Med*. 2000;343(2):78-85.
15. Antoniou AC, Easton DF. Models of genetic susceptibility to breast cancer. *Oncogene*. 2006;25(43):5898-5905.
16. Easton DF, Pharoah PD, Antoniou AC, et al. Gene-panel sequencing and the prediction of breast-cancer risk. *N Engl J Med*. 2015;372(23):2243-2257.
17. Nielsen FC, van Overeem Hansen T, Sorensen CS. Hereditary breast and ovarian cancer: new genes in confined pathways. *Nat Rev Cancer*. 2016;16(9):599-612.
18. Vos JR, Hsu L, Brohet RM, et al. Bias Correction Methods Explain Much of the Variation Seen in Breast Cancer Risks

of BRCA1/2 Mutation Carriers. *J Clin Oncol*. 2015;33(23):2553-2562.

19. Antoniou A, Pharoah PD, Narod S, et al. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. *Am J Hum Genet*. 2003;72(5):1117-1130.
20. Cancer risks in BRCA2 mutation carriers. *J Natl Cancer Inst*. 1999;91(15):1310-1316.
21. Kirchhoff T, Kauff ND, Mitra N, et al. BRCA mutations and risk of prostate cancer in Ashkenazi Jews. *Clin Cancer Res*. 2004;10(9):2918-2921.
22. van Asperen CJ, Brohet RM, Meijers-Heijboer EJ, et al. Cancer risks in BRCA2 families: estimates for sites other than breast and ovary. *J Med Genet*. 2005;42(9):711-719.
23. Risch HA, McLaughlin JR, Cole DE, et al. Population BRCA1 and BRCA2 mutation frequencies and cancer penetrances: a kin-cohort study in Ontario, Canada. *J Natl Cancer Inst*. 2006;98(23):1694-1706.
24. Tryggvadottir L, Vidarsdottir L, Thorgeirsson T, et al. Prostate cancer progression and survival in BRCA2 mutation carriers. *J Natl Cancer Inst*. 2007;99(12):929-935.
25. Edwards E, Yearwood C, Sillibourne J, Baralle D, Eccles D. Identification of a de novo BRCA1 mutation in a woman with early onset bilateral breast cancer. *Fam Cancer*. 2009;8(4):479-482.
26. Ford D, Easton DF, Bishop DT, Narod SA, Goldgar DE. Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. *Lancet*. 1994;343(8899):692-695.
27. Brose MS, Rebbeck TR, Calzone KA, et al. Cancer risk estimates for BRCA1 mutation carriers identified in a risk evaluation program. *J Natl Cancer Inst*. 2002;94(18):1365-1372.
28. Thompson D, Easton DF. Cancer Incidence in BRCA1 mutation carriers. *J Natl Cancer Inst*. 2002;94(18):1358-1365.
29. Thompson D, Easton D. Variation in cancer risks, by mutation position, in BRCA2 mutation carriers. *Am J Hum Genet*. 2001;68(2):410-419.
30. Tai YC, Domchek S, Parmigiani G, Chen S. Breast cancer risk among male BRCA1 and BRCA2 mutation carriers. *J Natl Cancer Inst*. 2007;99(23):1811-1814.
31. Easton DF. How many more breast cancer predisposition genes are there? *Breast Cancer Res*. 1999;1(1):14-17.
32. Campeau PM, Foulkes WD, Tischkowitz MD. Hereditary breast cancer: new genetic developments, new therapeutic avenues. *Hum Genet*. 2008;124(1):31-42.
33. Garber JE, Goldstein AM, Kantor AF, et al. Follow-up study of twenty-four families with Li-Fraumeni syndrome. *Cancer Res*. 1991;51(22):6094-6097.
34. Birch JM, Alston RD, McNally RJ, et al. Relative frequency and morphology of cancers in carriers of germline TP53 mutations. *Oncogene*. 2001;20(34):4621-4628.
35. FitzGerald MG, Marsh DJ, Wahrer D, et al. Germline mutations in PTEN are an infrequent cause of genetic predisposition to breast cancer. *Oncogene*. 1998;17(6):727-731.
36. Tan MH, Mester JL, Ngeow J, et al. Lifetime cancer risks in individuals with germline PTEN mutations. *Clin Cancer Res*. 2012;18(2):400-407.
37. Boardman LA, Thibodeau SN, Schaid DJ, et al. Increased risk for cancer in patients with the Peutz-Jeghers syndrome. *Ann Intern Med*. 1998;128(11):896-899.
38. Lim W, Olschwang S, Keller JJ, et al. Relative frequency and morphology of cancers in STK11 mutation carriers. *Gastroenterology*. 2004;126(7):1788-1794.
39. Pharoah PD, Guilford P, Caldas C. Incidence of gastric cancer and breast cancer in CDH1 (E-cadherin) mutation carriers from hereditary diffuse gastric cancer families. *Gastroenterology*. 2001;121(6):1348-1353.

40. Walsh T, Casadei S, Coats KH, et al. Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer. *Jama*. 2006;295(12):1379-1388.

41. Antoniou AC, Casadei S, Heikkinen T, et al. Breast-cancer risk in families with mutations in PALB2. *N Engl J Med*. 2014;371(6):497-506.

42. Thompson D, Duedal S, Kirner J, et al. Cancer risks and mortality in heterozygous ATM mutation carriers. *J Natl Cancer Inst*. 2005;97(11):813-822.

43. CHEK2*1100delC and susceptibility to breast cancer: a collaborative analysis involving 10,860 breast cancer cases and 9,065 controls from 10 studies. *Am J Hum Genet*. 2004;74(6):1175-1182.

44. Shimelis H, Mesman RL, Von Nicolai C, et al. BRCA2 hypomorphic missense variants confer moderate risks of breast cancer. *Cancer Res*. 2017.

45. Spurdle AB, Whiley PJ, Thompson B, et al. BRCA1 R1699Q variant displaying ambiguous functional abrogation confers intermediate breast and ovarian cancer risk. *J Med Genet* 2012;49(8):525-532.

46. Moghadasi S, Meeks HD, Vreeswijk MP, et al. 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. *J Med Genet*. 2017.

47. Hilbers FS, Vreeswijk MP, van Asperen CJ, Devilee P. The impact of next generation sequencing on the analysis of breast cancer susceptibility: a role for extremely rare genetic variation? *Clin Genet*. 2013;84(5):407-414.

48. Michailidou K, Hall P, Gonzalez-Neira A, et al. Large-scale genotyping identifies 41 new loci associated with breast cancer risk. *Nat Genet*. 2013;45(4):353-361, 361e351-352.

49. Antoniou AC, Spurdle AB, Sinilnikova OM, et al. Common breast cancer-predisposition alleles are associated with breast cancer risk in BRCA1 and BRCA2 mutation carriers. *Am J Hum Genet*. 2008;82(4):937-948.

50. Tung N, Domchek SM, Stadler Z, et al. Counselling framework for moderate-penetrance cancer-susceptibility mutations. *Nat Rev Clin Oncol*. 2016.

51. Adank MA, Verhoef S, Oldenburg RA, et al. Excess breast cancer risk in first degree relatives of CHEK2 *1100delC positive familial breast cancer cases. *Eur J Cancer*. 2013;49(8):1993-1999.

52. Couch FJ, Nathanson KL, Offit K. Two decades after BRCA: setting paradigms in personalized cancer care and prevention. *Science*. 2014;343(6178):1466-1470.

53. Pennington KP, Walsh T, Harrell MI, et al. Germline and somatic mutations in homologous recombination genes predict platinum response and survival in ovarian, fallopian tube, and peritoneal carcinomas. *Clin Cancer Res*. 2014;20(3):764-775.

54. Tan DS, Kaye SB. Chemotherapy for Patients with BRCA1 and BRCA2-Mutated Ovarian Cancer: Same or Different? *Am Soc Clin Oncol Educ Book*. 2015:114-121.

55. Lord CJ, Ashworth A. PARP inhibitors: Synthetic lethality in the clinic. *Science*. 2017;355(6330):1152-1158.

56. Fong PC, Boss DS, Yap TA, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med*. 2009;361(2):123-134.

57. Li S. Inhibition of poly(ADP-ribose) polymerase in BRCA mutation carriers. *N Engl J Med*. 2009;361(17):1707; author reply 1707-1708.

58. Borg A, Haile RW, Malone KE, et al. Characterization of BRCA1 and BRCA2 deleterious mutations and variants of unknown clinical significance in unilateral and bilateral breast cancer: the WECARE study. *Hum Mutat*. 2010;31(3):E1200-1240.

59. Frank TS, Deffenbaugh AM, Reid JE, et al. Clinical characteristics of individuals with germline mutations in BRCA1 and

BRCA2: analysis of 10,000 individuals. *J Clin Oncol*. 2002;20(6):1480-1490.

60. Nanda R, Schumm LP, Cummings S, et al. Genetic testing in an ethnically diverse cohort of high-risk women: a comparative analysis of BRCA1 and BRCA2 mutations in American families of European and African ancestry. *JAMA*. 2005;294(15):1925-1933.
61. Weitzel JN, Lagos V, Blazer KR, et al. Prevalence of BRCA mutations and founder effect in high-risk Hispanic families. *Cancer Epidemiol Biomarkers Prev*. 2005;14(7):1666-1671.
62. Eggington JM, Bowles KR, Moyes K, et al. A comprehensive laboratory-based program for classification of variants of uncertain significance in hereditary cancer genes. *Clin Genet*. 2014;86(3):229-237.
63. Spurdle AB, Healey S, Devereau A, et al. ENIGMA—Evidence-based network for the interpretation of germline mutant alleles: An international initiative to evaluate risk and clinical significance associated with sequence variation in BRCA1 and BRCA2 genes. *Hum Mutat*. 2011;33(1):2-7.
64. Vos J, Gómez-García E, Oosterwijk JC, et al. Opening the psychological black box in genetic counseling. The psychological impact of DNA testing is predicted by the counselees' perception, the medical impact by the pathogenic or uninformative BRCA1/2-result. *Psycho-Oncology*. 2010;21(1):29-42.
65. Vos J, Otten W, van Asperen C, et al. The counsellees' view of an unclassified variant in BRCA1/2: recall, interpretation, and impact on life. *Psychooncology*. 2008;17(8):822-830.
66. Goldgar DE, Easton DF, Deffenbaugh AM, et al. Integrated Evaluation of DNA Sequence Variants of Unknown Clinical Significance: Application to BRCA1 and BRCA2. *Am J H Genet*. 2004;75(4):535-544.
67. Grantham R. Amino acid difference formula to help explain protein evolution. *Science*. 1974;185(4154):862-864.
68. Tavtigian SV, Deffenbaugh AM, Yin L, et al. Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. *J Med Genet*. 2006;43(4):295-305.
69. Vreeswijk MPG, Kraan JN, van der Klift HM, et al. Intronic variants in BRCA1 and BRCA2 that affect RNA splicing can be reliably selected by splice-site prediction programs. *Hum Mutat*. 2009;30(1):107-114.
70. Howlett NG, Taniguchi T, Olson S, et al. Biallelic inactivation of BRCA2 in Fanconi anemia. *Science*. 2002;297(5581):606-609.
71. Wagner JE, Tolar J, Levran O, et al. Germline mutations in BRCA2: shared genetic susceptibility to breast cancer, early onset leukemia, and Fanconi anemia. 2004. p. 3226-3229.
72. Sawyer SL, Tian L, Kahkonen M, et al. Biallelic mutations in BRCA1 cause a new Fanconi anemia subtype. *Cancer Discov*. 2015;5(2):135-142.
73. Deffenbaugh AM, Frank TS, Hoffman M, Cannon-Albright L, Neuhausen SL. Characterization of Common BRCA1 and BRCA2 Variants *Genetic Testing*. 2002;6(2):119-121.
74. Claes K, Poppe B, Machackova E, et al. Differentiating pathogenic mutations from polymorphic alterations in the splice sites of BRCA1 and BRCA2. *Genes Chromosomes Cancer*. 2003;37(3):314-320.
75. Amir E, Freedman OC, Seruga B, Evans DG. Assessing women at high risk of breast cancer: a review of risk assessment models. *J Natl Cancer Inst*. 2010;102(10):680-691.
76. Easton DF, Deffenbaugh AM, Pruss D, et al. A Systematic Genetic Assessment of 1,433 Sequence Variants of Unknown Clinical Significance in the BRCA1 and BRCA2 Breast Cancer-Predisposition Genes. *Am J Hum Genet*. 2007;81(5):873-883.
77. Armes JE, Egan AJ, Southey MC, et al. The histologic phenotypes of breast carcinoma occurring before

age 40 years in women with and without BRCA1 or BRCA2 germline mutations: a population-based study. *Cancer*. 1998;83(11):2335-2345.

78. Lakhani SR, Jacquemier J, Sloane JP, et al. Multifactorial analysis of differences between sporadic breast cancers and cancers involving BRCA1 and BRCA2 mutations. *J Natl Cancer Inst*. 1998;90(15):1138-1145.
79. Southey MC, Ramus SJ, Dowty JG, et al. Morphological predictors of BRCA1 germline mutations in young women with breast cancer. *Br J Cancer*. 2011;104(6):903-909.
80. Bane AL, Beck JC, Bleiweiss I, et al. BRCA2 mutation-associated breast cancers exhibit a distinguishing phenotype based on morphology and molecular profiles from tissue microarrays. *Am J Surg Pathol*. 2007;31(1):121-128.
81. Lakhani SR, Gusterson BA, Jacquemier J, et al. The pathology of familial breast cancer: histological features of cancers in families not attributable to mutations in BRCA1 or BRCA2. *Clin Cancer Res*. 2000;6(3):782-789.
82. Honrado E, Benitez J, Palacios J. Histopathology of BRCA1- and BRCA2-associated breast cancer. *Crit Rev Oncol Hematol*. 2006;59(1):27-39.
83. Hofstra RM, Spurdle AB, Eccles D, et al. Tumor characteristics as an analytic tool for classifying genetic variants of uncertain clinical significance. *Hum Mutat*. 2008;29(11):1292-1303.
84. Spurdle AB, Couch FJ, Parsons MT, et al. Refined histopathological predictors of BRCA1 and BRCA2 mutation status: a large-scale analysis of breast cancer characteristics from the BCAC, CIMBA, and ENIGMA consortia. *Breast Cancer Res*. 2014;16(6):3419.
85. Spearman AD, Sweet K, Zhou X-P, et al. Clinically Applicable Models to Characterize BRCA1 and BRCA2 Variants of Uncertain Significance. *J Clin Oncol*. 2008;26(33):5393-5400.
86. Chenevix-Trench G, Healey S, Lakhani S, et al. Genetic and Histopathologic Evaluation of BRCA1 and BRCA2 DNA Sequence Variants of Unknown Clinical Significance. *Cancer Res*. 2006;66(4):2019-2027.
87. Beristain E, Guerra I, Vidaurrazaga N, Burgos-Bretones J, Tejada MI. LOH analysis should not be used as a tool to assess whether UVs of BRCA1/2 are pathogenic or not. *Fam Cancer*. 2010;9(3):289-290.
88. Joosse SA, Brandwijk KI, Devilee P, et al. Prediction of BRCA2-association in hereditary breast carcinomas using array-CGH. *Breast Cancer Res Treat*. 2012;132(2):379-389.
89. Wessels LF, van Welsem T, Hart AA, et al. Molecular classification of breast carcinomas by comparative genomic hybridization: a specific somatic genetic profile for BRCA1 tumors. *Cancer Res*. 2002;62(23):7110-7117.
90. Joosse SA, van Beers EH, Tielen IH, et al. Prediction of BRCA1-association in hereditary non-BRCA1/2 breast carcinomas with array-CGH. *Breast Cancer Res Treat*. 2009;116(3):479-489.
91. Spurdle AB, Couch FJ, Hogervorst FB, Radice P, Sinilnikova OM. Prediction and assessment of splicing alterations: implications for clinical testing. *Hum Mutat*. 2008;29(11):1304-1313.
92. Tosi M, Stamm S, Baralle D. RNA splicing meets genetic testing: detection and interpretation of splicing defects in genetic diseases. *Eur J Hum Genet*. 2010;18(6):737-738.
93. Colombo M, Blok MJ, Whiley P, et al. Comprehensive annotation of splice junctions supports pervasive alternative splicing at the BRCA1 locus: a report from the ENIGMA consortium. *Hum Mol Genet*. 2014;23(14):3666-3680.
94. Fackenthal JD, Yoshimatsu T, Zhang B, et al. Naturally occurring BRCA2

alternative mRNA splicing events in clinically relevant samples. *J Med Genet*. 2016;53(8):548-558.

95. Deng CX, Brodie SG. Roles of BRCA1 and its interacting proteins. *Bioessays*. 2000;22(8):728-737.
96. Huen MS, Sy SM, Chen J. BRCA1 and its toolbox for the maintenance of genome integrity. *Nat Rev Mol Cell Biol*. 2010;11(2):138-148.
97. Millot GA, Carvalho MA, Caputo SM, et al. A guide for functional analysis of BRCA1 variants of uncertain significance. *Hum Mutat*. 2012;33(11):1526-1537.
98. Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat Rev Cancer*. 2011;12(1):68-78.
99. Zhang J, Willers H, Feng Z, et al. Chk2 phosphorylation of BRCA1 regulates DNA double-strand break repair. *Mol Cell Biol*. 2004;24(2):708-718.
100. Yu X, Wu LC, Bowcock AM, Aronheim A, Baer R. The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in the CtBP pathway of transcriptional repression. *J Biol Chem*. 1998;273(39):25388-25392.
101. Xu B, O'Donnell AH, Kim ST, Kastan MB. Phosphorylation of serine 1387 in Brca1 is specifically required for the Atm-mediated S-phase checkpoint after ionizing irradiation. *Cancer Res*. 2002;62(16):4588-4591.
102. Wang B, Matsuoka S, Ballif BA, et al. Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response. *Science*. 2007;316(5828):1194-1198.
103. Kim H, Chen J, Yu X. Ubiquitin-binding protein RAP80 mediates BRCA1-dependent DNA damage response. *Science*. 2007;316(5828):1202-1205.
104. Liu Z, Wu J, Yu X. CCDC98 targets BRCA1 to DNA damage sites. *Nat Struct Mol Biol*. 2007;14(8):716-720.
105. Sobhian B, Shao G, Lilli DR, et al. RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites. *Science*. 2007;316(5828):1198-1202.
106. Cantor SB, Bell DW, Ganesan S, et al. BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. *Cell*. 2001;105(1):149-160.
107. Sharan SK, Bradley A. Murine Brca2: sequence, map position, and expression pattern. *Genomics*. 1997;40(2):234-241.
108. Tutt A, Gabriel A, Bertwistle D, et al. Absence of Brca2 causes genome instability by chromosome breakage and loss associated with centrosome amplification. *Curr Biol*. 1999;9(19):1107-1110.
109. Marmorstein LY, Kinev AV, Chan GK, et al. A human BRCA2 complex containing a structural DNA binding component influences cell cycle progression. *Cell*. 2001;104(2):247-257.
110. Daniels MJ, Wang Y, Lee M, Venkitaraman AR. Abnormal cytokinesis in cells deficient in the breast cancer susceptibility protein BRCA2. *Science*. 2004;306(5697):876-879.
111. Mondal G, Rowley M, Guidugli L, et al. BRCA2 localization to the midbody by filamin A regulates cep55 signaling and completion of cytokinesis. *Dev Cell*. 2012;23(1):137-152.
112. Oliver AW, Swift S, Lord CJ, Ashworth A, Pearl LH. Structural basis for recruitment of BRCA2 by PALB2. *EMBO Rep*. 2009;10(9):990-996.
113. Yang H, Li Q, Fan J, Holloman WK, Pavletich NP. The BRCA2 homologue Brh2 nucleates RAD51 filament formation at a dsDNA-ssDNA junction. *Nature*. 2005;433(7026):653-657.
114. Esashi F, Christ N, Gannon J, et al. CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair. *Nature*. 2005;434(7033):598-604.
115. Guidugli L, Carreira A, Caputo SM, et al. Functional assays for analysis of variants of uncertain significance in BRCA2. *Hum Mutat*. 2014;35(2):151-164.

116. Goldgar DE, Easton DF, Byrnes GB, et al. Genetic evidence and integration of various data sources for classifying uncertain variants into a single model. *Hum Mutat*. 2008;29(11):1265-1272.

117. Tavtigian SV, Greenblatt MS, Lesueur F, Byrnes GB. In silico analysis of missense substitutions using sequence-alignment based methods. *Hum Mutat*. 2008;29(11):1327-1336.

118. McGee S. Simplifying likelihood ratios. *J Gen Intern Med*. 2002;17(8):646-649.

119. Lindor NM, Guidugli L, Wang X, et al. A review of a multifactorial probability-based model for classification of BRCA1 and BRCA2 variants of uncertain significance (VUS). *Hum mutat*. 2012;33(1):8-21.

120. Richards CS, Bale S, Bellissimo DB, et al. ACMG recommendations for standards for interpretation and reporting of sequence variations: Revisions 2007. *Genet Med*. 2008;10(4):294-300.

121. Plon SE, Eccles DM, Easton D, et al. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. *Hum Mutat*. 2008;29(11):1282-1291.

122. Moghadasi S, Hofland N, Wouts JN, et al. Variants of uncertain significance in BRCA1 and BRCA2 assessment of in silico analysis and a proposal for communication in genetic counselling. *J Med Genet*. 2013;50(2):74-79.

123. Moghadasi S, Grundeken V, Janssen LAM, et al. Performance of BRCA1/2 mutation prediction models in male breast cancer patients. *Clin Genet*. 2017.

124. Moghadasi S, Eccles DM, Devilee P, Vreeswijk MP, van Asperen CJ. Classification and Clinical Management of Variants of Uncertain Significance in High Penetrance Cancer Predisposition Genes. *Hum Mutat*. 2016;37(4):331-336.