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

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Discussion

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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

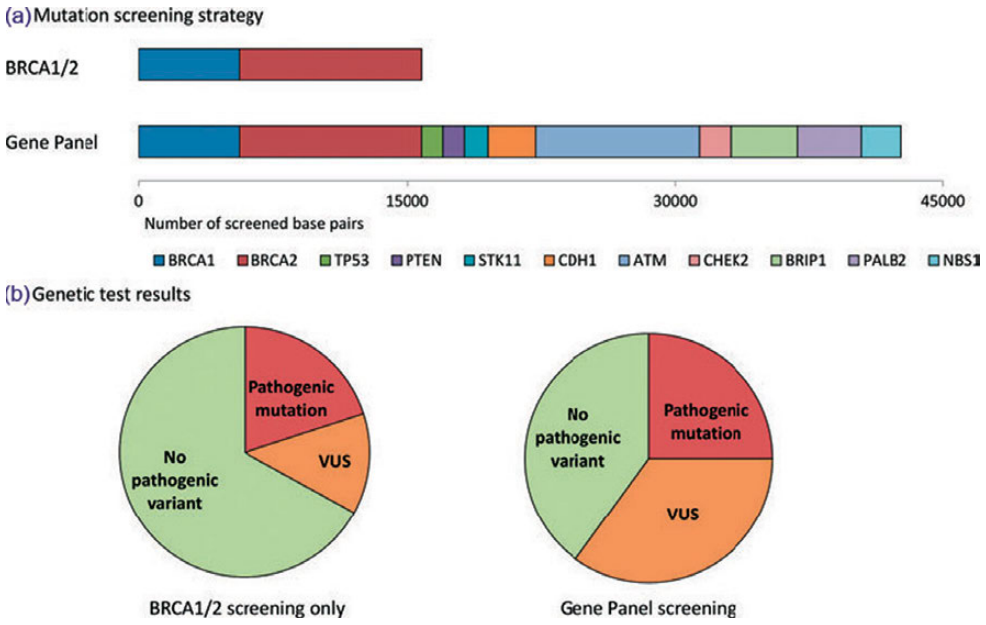

Figure 1. Test results from different genetic screening strategies in the clinic.(a) The screened genes for the different genetic screening strategies and the corresponding number of screened coding base pairs. (b) The distribution of test results for *BRCA1/2* screening based on Frank et al[24] The 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*,

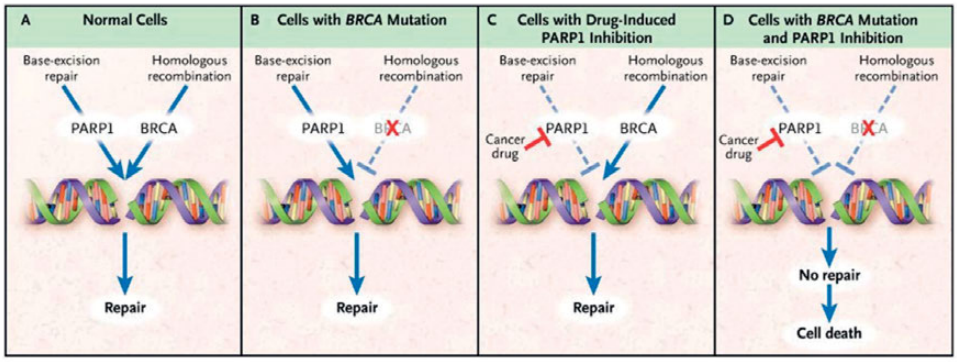

Figure 2. Mechanism of Cell Death from Synthetic Lethality, as Induced by Inhibition of Poly Adenosine Diphosphate [ADP]–Ribose) Polymerase 1 (PARP1). Reused with permission from Iglehart et al. N Engl J Med 2009; 361:189-191,[45] Copyright Massachusetts Medical Society.

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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