Functional analyses of *BRCA1* variants of unknown significance found in hereditary breast or ovarian cancer families in Norway: A tool for improved diagnosis

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Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

The work presented in this thesis was carried out between 2019 and 2022 at the Western Norway Familial Cancer Center, Department of Medical Genetics, at Haukeland University Hospital, with Dr. Elisabet Ognedal as main supervisor and Dr. Ingvild Aukrust, Dr. Bjørn Ivar Haukanes and Dr. Per Morten Knappskog as co-supervisors. Financial support was provided by the Western Norway Regional Health Authority (Grant Number F-10199/4800001941).

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Last but not least, I want to thank my boyfriend Simen, family and friends for always being supportive and encouraging.

Bergen, December 2022

Henrikke

Contents

Abstract (English)

The tumour suppressor gene *BRCA1* plays multiple roles in preventing tumour development, and alterations in the *BRCA1* gene are one of the main causes of hereditary breast and ovarian cancer (HBOC). In paper I, an overview of the *BRCA1* variant spectrum found in families with suspected HBOC at the four diagnostic genetic laboratories in Norway was made. The internal variant classifications were compared, which revealed discrepancies in 30% of the classification between the laboratories. The discrepancies were reduced to 10% through a series of digital meetings, which illustrates that variant interpretation needs to be regularly updated, and that data sharing and inter-laboratory collaboration improves the accuracy of variant interpretation.

In paper II, 14 rare missense *BRCA1* variants from Paper II, all of uncertain clinical significance (VUSs) and distributed throughout the gene, were assessed by multiple functional analyses, i.e. protein expression levels and stability, subcellular localisation, and protein interactions with BARD1 and PALB2. In contrast to several previous studies focusing on separate domains, the full-length protein was utilised to better mimic the native state of the protein, and we aimed to investigate the hypothesis stating that *BRCA1* missense variants located outside protein domains with known function are of no functional importance. In total, four variants located outside the known domains were found to make the BRCA1 protein more prone to proteasome-mediated degradation, or showed reduced protein stability compared to the wild type (WT) protein. These findings indicate that also variants located outside the RING, BRCT and coiled-coiled domains could affect the BRCA1 protein function.

In paper III, we investigated the effect of 11 rare *BRCA1* VUSs selected from paper I, located either within or in close proximity to the BRCT domain, with respect to homologous recombination repair (HRR) of double stranded DNA breaks and transcriptional activation (TA). Only one variant exhibited HRR activity comparable to the WT protein, whereas all other variants showed a significantly lower activity. Two of the variants exhibited TA activity similar to the pathogenic controls. Our results thus indicate that several of the variants of interest could potentially impair BRCA1 protein

function, but further studies are needed to clarify their pathogenicity. We highlight the importance of comparing results obtained from several functional assays for multifunctional proteins such as BRCA1, as a variant could potentially affect only one or some of the proteins' multiple activities.

The functional assays performed in paper II and III provided new knowledge, which contributed to reclassification of seven of the *BRCA1* variants from VUS to likely benign, and one variant to likely pathogenic.

Abstrakt (norsk)

Tumorsuppressorgenet *BRCA1* speler mange viktige roller for å hindre tumorutvikling, og endringar i *BRCA1*-genet er ei av hovudårsakene til arveleg bryst- og eggstokk-kreft (HBOC). I artikkel I vart det laga ei oversikt over *BRCA1*-variantar påvist i familiar der ein mistenkjer HBOC ved dei fire medisinsk genetiske avdelingane i Noreg. Dei interne variantklassifiseringane vart samanlikna, og resultatet viste diskrepans i 30% av klassifiseringa mellom dei ulike laboratoria. Etter fleire digitale møter vart diskrepansen redusert til 10%, noko som viser at varianttolking bør bli oppdatert jamleg og at datadeling og nært samarbeid mellom laboratoria gir meir nøyaktige tolkingar.

I artikkel II vart 14 sjeldne *BRCA1*-missensvariantar frå artikkel I, alle av usikker klinisk betyding (VUSar) og fordelt langs heile genet, analysert med fleire ulike funksjonelle analyser, inkludert undersøking av proteinuttrykking og proteinstabilitet, subcellulær lokasjon og proteininteraksjon med BARD1 og PALB2. I motsetning til fleire tidlegare studiar som berre har studert separate proteindomener av BRCA1, har vi her brukt full-lengdeprotein for å betre kunne gjenskape heile den naturlege tilstanden til proteinet. Målet var å undersøke hypotesa som seier at *BRCA1*-missensvariantar lokalisert utanfor proteindomener med kjend funksjon ikkje er funksjonelt viktige. Totalt fire variantar lokalisert utanfor kjende domener gjorde BRCA1 proteinet meir utsett for proteasom-mediert degradering, eller ga lågare proteinstabilitet samanlikna med villtypeproteinet. Desse funna indikerer at også variantar utanfor dei kjende domenene RING, BRCT og coiled-coil kan påverke BRCA1-proteinfunksjon.

I artikkel III undersøkte vi effekten av 11 sjeldne *BRCA1* VUSar frå artikkel I som var lokalisert enten i eller nær BRCT-domenet med hensyn til homologi-retta reparasjon (HRR) av dobbeltråda DNA-brot og transkripsjonell aktivering (TA). Berre ein av dei analyserte variantane viste same HRR-aktivitet som villtypeproteinet, medan alle dei andre viste signifikant redusert aktivitet. To av dei analyserte variantane viste redusert TA-aktivitet lik dei patogene kontrollane. Resultatet indikerer at fleire av variantane potensielt kan påverke BRCA1-proteinfunksjonen, men fleire studiar er nødvendige for å avklare patogeniteten til variantane. Vi framhevar også viktigheita av å samanlikne

resultat frå fleire ulike funksjonelle analysemetodar. Sidan ein variant potensielt kan påverke berre ein eller nokre av funksjonane til eit proteinet er dette spesielt viktig for multifunksjonelle protein slik som BRCA1.

Dei funksjonelle analysane i artikkel II og III ga ny kunnskap, noko som bidrog til reklassifisering av sju variantar frå VUS til truleg benign, og ein variant til truleg patogen.

List of publications

Paper I

Hovland, H. N., Al-Adhami, R., Ariansen, S. L., Van Ghelue, M., Sjursen, W., Lima, S., Bolstad, M., Berger, A. H., Høberg-Vetti, H., Knappskog, P. M., Haukanes, B. I., Aukrust, I., Ognedal, E. (2022). *BRCA1* Norway: comparison of classification for *BRCA1* germline variants detected in families with suspected hereditary breast and ovarian cancer between different laboratories. Familial Cancer. https://doi.org/10.1007/s10689-021-00286-6 (PMID: 34981296)

Paper II

Hovland, H. N., Mchaina, E. M., Høberg-Vetti, H., Ariansen, S. L., Sjursen, W., Van Ghelue, M., Haukanes, B. I., Knappskog, P. M., Aukrust, I.*, Ognedal, E*.

Functional analyses of rare germline missense *BRCA1* variants located within and outside protein domains with known functions

Manuscript submitted to Genes.

Paper III

Bassi, N.*, Hovland, H. N.*, Rasheed, K., Jarhelle, E., Pedersen, N., Mchaina, E. K., Bakkan, S. M. E., Iversen, N., Høberg-Vetti, H., Haukanes, B. I., Knappskog, P. N. Aukrust, I., Ognedal, E.**,Van Ghelue1, M**.

Functional analyses of rare germline BRCA1 variants by transcriptional activation and homologous recombination repair assays

Manuscript.

* These authors contributed equally

** These authors contributed equally

Abbreviations

Aa	Amino acid
ACMG	American College of Medical Genetics and Genomics
AMP	Association for Molecular Pathology
B	Benign
BA	Benign stand alone
BARD1	BRCA1-associated RING domain 1
BP	Benign supporting
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
BRCT	BRCA1 C-terminal domain
BS	Benign strong
CanVIG-UK	Cancer Variant Interpretation Group UK
Co-IP	Co-immunoprecipitation
DBD	DNA binding domain
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSB	Double-stranded breaks
ENIGMA	Evidence-based Network for the Interpretation of Germline Mutant Alleles
ExAC	Exome Aggregation Consortium
GAL4	Galactose-responsive transcription factor
GnomAD	Genome Aggregation Database
HBOC	Hereditary breast and ovarian cancer
HDR	Homology-directed recombination repair
HRR	Homologous recombination repair
HUH	Haukeland University Hospital
LB	Likely benign
LP	Likely pathogenic
MMR	Mismatch repair
NES	Nuclear export signal

NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NLS	Nuclear localisation sequence
P	Pathogenic
PALB2	Partner and localiser of BRCA2
PARP	Poly (adenosine diphosphate–ribose) polymerase
PM	Pathogenic moderate
PP	Pathogenic supporting
PS	Pathogenic strong
PVS	Pathogenic very strong
RING	Really interesting new gene
TA	Transcriptional activation
TNBC	Triple-negative breast cancer
VUS	Variant of uncertain significance
WT	Wild type

1. Introduction

1.1 The genetics of cancer

Cancer is a genetic disorder which can be caused by environmental risk factors such as chemicals, radiation, and viruses, but it can also be due to inherited germline alterations in the genome. A malignant tumour results from the expansion of one single progenitor cell harbouring genetic alterations which provide growth advantages compared to the normal cells. As tumourigenesis proceeds, multiple alterations in several genes induced by genomic instability accumulate and contribute to the formation of multiple cancer cells. As this is often a time-consuming process, the incidence of most cancers increases with age. The uncontrolled growth and survival advantages in tumour cells are summarised by the properties commonly known as "The Hallmarks of Cancer", as illustrated in Figure 1 [1-3].

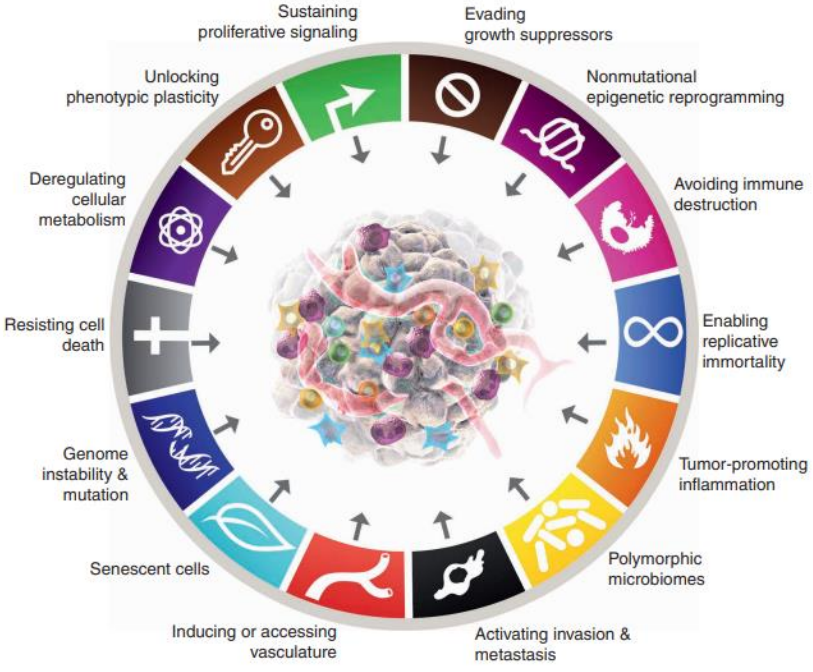

Figure 1 – The Hallmarks of Cancer. Reprinted from [3] with permission from American Association for Cancer Research.

1.2 Breast and ovarian cancer in the population

Worldwide, breast cancer constituted 11.7% of all new cancer cases in 2020, corresponding to 2 261 419 new cases (Figure 2) [4, 5]. This makes breast cancer the most common form of cancer, followed by lung (11.4%) and colorectum (10%) cancer [4, 6]. In 2021, 3726 Norwegian women were diagnosed with breast cancer, and the relative five year-survival (2017-2021) was 92.3% [7]. The cumulative risk of developing breast cancer by the age of 80 was 10.5% [7].

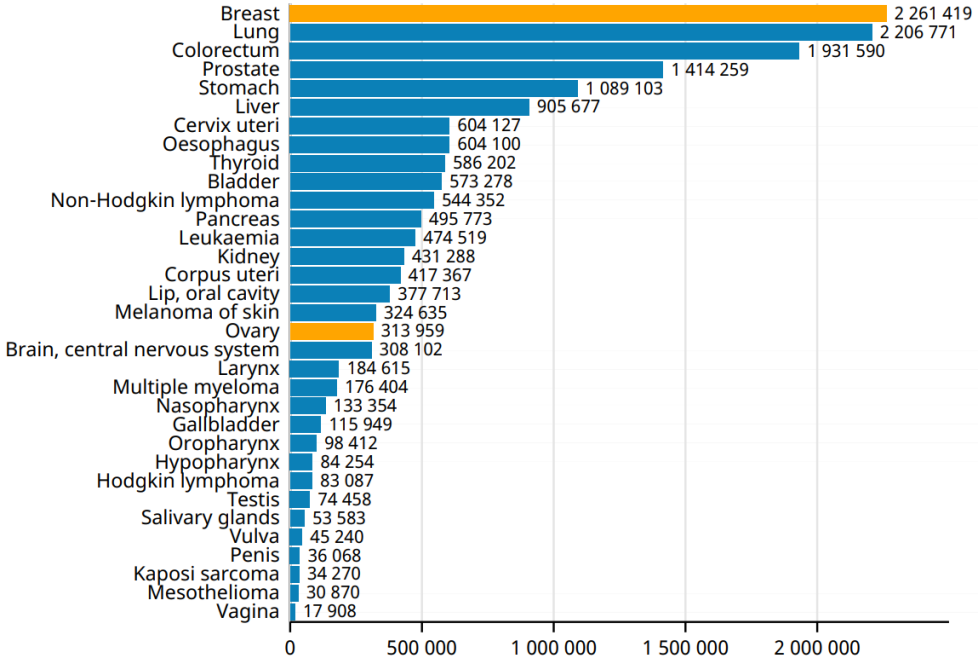

Figure 2 – Number of new cancer cases worldwide in 2020. The numbers include both sexes and all ages. Breast and ovarian cancer are highlighted in yellow. Figure adapted from [5].

Being more rare than breast cancer, ovarian cancer constituted 1.6% of all new cancer cases in 2020, corresponding to 313 959 new cases worldwide (Figure 2) [5, 6]. In 2021, 531 Norwegian women were diagnosed with ovarian cancer. The relative five year-survival (2017-2021) was 51.1%, thus significantly lower than for patients with breast cancer [7]. The cumulative risk of developing ovarian cancer by the age of 80 was 1.6% [7].

1.3 Hereditary breast and ovarian cancer

While most cancers are sporadic and initiated by genetic alterations in a single somatic cell, an important minority of cases are of hereditary origin and caused by damaging germline alterations in cancer predisposing genes present in all cells. In most cases, the inherited pattern is autosomal dominant, exhibiting 50% chance of passing the germline variant to the offspring. Consequently, carriers of such alterations often develop cancer at an earlier age compared to individuals affected with sporadic cancer, and may also develop multiple, related, primary tumours like breast and ovarian. In addition, they also often have relatives with related cancer forms [8-10]. Hereditary breast and ovarian cancer (HBOC) is one of the most common inherited cancer syndromes [11]. HBOC is characterised by an increased risk of breast cancer in both men and women, and ovarian cancer in women. HBOC can also lead to increased risk of other cancers like prostate cancer, pancreatic cancer, and melanoma to a lesser extent, with the exact risk depending on the gene causing the disease [12]. The two primary genes causing HBOC are *BRCA1* (Breast cancer susceptibility gene 1) and *BRCA2* (Breast cancer susceptibility gene 2) [13]. Other high risk, but less prevalent, genes causing breast cancer are *PALB2* and *TP53* [13-19]. Examples of moderate breast cancer risk genes are *PTEN, CHEK2, ATM* and *BARD1* [13, 20-22]. Ovarian cancer can also be caused by genetic alterations in the mismatch repair (MMR) genes (*MLH1*, *MSH2* and *MSH6*), *RAD51C*, *RAD51D* and *BRIP1* [23-31].

1.3.1 *BRCA1* and cancer risk

The main focus of this thesis is the *BRCA1* gene, which was first discovered and linked to increased risk of breast cancer in 1994 [32-34]. The following year, the *BRCA2* gene was discovered [35]. Carriers of disease-causing variants of *BRCA1* have a risk of 65-79% for breast cancer and 36-53% for ovarian cancer by the age of 80 [36]. The risk of a secondary primary breast malignancy is 35-45% within 20 years, and 83% by the age of 70 [36-38]. In male carriers, the lifetime risk of breast cancer is 1-17% (0.1% in the general population) [39]. In addition, *BRCA1* is associated with a slight increased risk of pancreatic (1.5-3.7%) and stomach cancers (1.3-2.8%) [39]. Unlike *BRCA2*, *BRCA1*

is not associated with increased risk of prostate cancer compared to the general population [39].

Breast cancer occurring among *BRCA1* carriers are often characterised as triple-negative breast cancer (TNBC), meaning they have low or no expression of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 [40-42]. Compared to other types of breast cancer, TNBC is associated with younger age at diagnosis, poor prognosis, high proliferation, and higher tumour grade [40, 43]. *BRCA1* ovarian cancers are typically high-grade serous carcinomas [38].

1.3.2 Surveillance and risk-reduction

Carriers of disease-causing *BRCA1* variants have a high risk of developing breast and ovarian cancer, and are therefore offered follow-up consisting of genetic counselling, screening for early detection and better diagnostics (MRI and mammography), and prophylactic risk-reducing strategies like surgical removal of breasts (mastectomy) and ovaries (salpingo-oophorectomy) according to national guidelines [44]. The surveillance and risk-reducing strategies are offered to healthy carriers, as well as to cancer patients harbouring disease-causing gene variants in *BRCA1* for secondary cancer prevention and to guide treatment choices.

1.3.3 Cancer treatment

In 2014, Olaparib was approved as treatment for patients with advanced ovarian cancer harbouring deleterious germline BRCA variants [45, 46]. Olaparib is an inhibitor of the enzyme poly (adenosine diphosphate–ribose) polymerase (PARP), which together with the BRCA proteins are key components of the DNA damage repair pathway (DDR) [45, 47]. PARP enzymes are involved in repair of DNA damage in both normal and cancer cells by base-excision repair of single stranded DNA (ssDNA). PARP inhibitors like Olaparib inhibit the base-excision repair pathway, which results in accumulation of unrepaired ssDNA breaks. These breaks cause inflated replication forks in the S phase of the cell cycle, which subsequently leads to development of DNA double-stranded breaks (DSBs) [48]. In normal cells, these dsDNA breaks will be repaired by alternative

mechanisms involving the BRCA proteins. However, cancer cells deficient for homologous recombination repair (HRR), which is one of the main DDR pathways, will eventually die due to toxic levels of DSBs. (Figure 3). Such tumours are thus hypersensitive to PARP induced DNA damage. Today, PARP inhibitors are the main treatment of cancer patients with defective double-stranded DNA repair tumours, including tumours associated with germline or somatic variants in *BRCA1* [49, 50]. Consequently, correct *BRCA1* variant interpretation status is extremely important for treatment decisions.

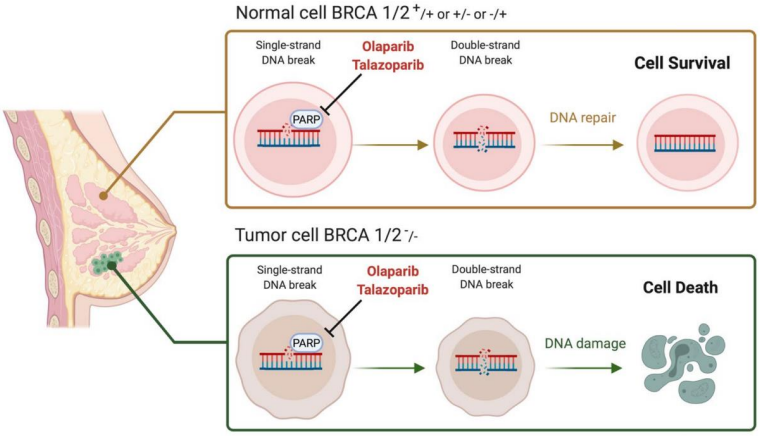

Figure 3 – PARP inhibitors selectively kill cancer cells deficient for homologous recombination repair (HRR). Figure created by BioRender.com, retrieved from [51].

1.4 The tumour suppressor gene *BRCA1*

1.4.1 The *BRCA1* gene

The tumour suppressor gene *BRCA1* is located on chromosome 17q21.3 and consists of 23 coding exons (reported according to the reference sequence NM_007294.3). The gene encodes a large protein of 220 kDa (1863 aa) primarily located in the nucleus, which is involved in numerous important cellular processes. As illustrated in Figure 4, the BRCA1 protein consists of several functional domains. The N-terminal Really Interesting New Gene (RING) domain (aa 22-64) is composed of a series of eight conserved C3HC4 type zinc-finger motif repeats [52]. This RING domain binds to BRCA1-associated RING domain 1 protein (BARD1), forming a heterodimer with E3 ubiquitin ligase activity [53-55]. Two nuclear localisation sequences (NLS, aa 503-508 and 607-614) allocate the BRCA1 protein to the nucleus where it exerts its functions [56]. A nuclear export signal (NES, aa 81-99) is involved in nuclear cytoplasmic shuttling [57]. The coiled-coil domain (aa 1364-1437) located towards the C-terminal is involved in binding to the interaction Partner And Localiser of BRCA2 (PALB2). Through the BRCA1 C-terminal (BRCT) domain consisting of two tandem BRCT repeats (aa 1646-1736 and 1760-1855) connected by a linker region, BRCA1 interacts with multiple proteins involved in transcription and DNA damage response [58-61]. No known protein domains are located in the central region of BRCA1. Such disordered regions flanked by structural domains are typical for proteins serving as scaffold proteins, and are thought to allow structural and conformational flexibility to facilitate the formation of multiple protein complexes [62-64].

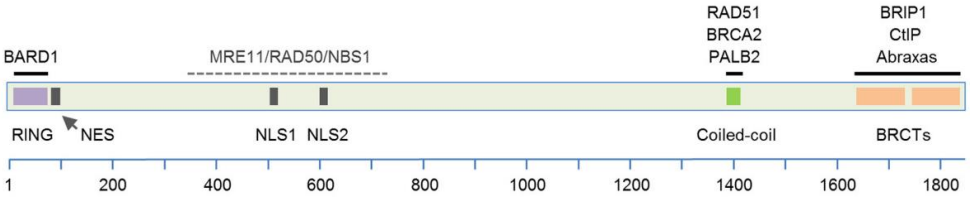

Figure 4 – Schematic presentation of BRCA1: Protein domains are indicated by coloured boxes, and known interaction partners of BRCA1 are listed on the top. RING = Really Interesting New Gene, NES = Nuclear Export Signal, NLS = Nuclear Localisation Signal, BRCT = BRCA1 C-terminal. The numbered scale indicates amino acid residue positions. Figure adapted from [65].

1.4.2 BRCA1 protein function

BRCA1 plays a pivotal role in maintaining genomic stability and prevention of tumourigenesis. The protein is one of the main actors in the DDR pathway, and functions as a scaffolding protein that facilitates and coordinates the assembly of multiple protein complexes involved in DDR. The formation of such complexes is thought to be regulated by DNA damage induced phosphorylation of BRCA1 by ATM, ATR and CHK2 kinases [66]. Among others, BRCA1 also maintains genomic stability through cell-cycle and centrosome regulation during mitosis, HRR of DSBs, chromatin remodelling, transcriptional regulation through association with multiple transcriptional factors, and regulation of apoptosis (Figure 5) [66-68]. Unrelated to DNA repair, BRCA1 is also involved in monoubiquitylation of histone H2A, where it acts as an adaptor protein in the ubiquitin ligase complex and leads to the formation of heterochromatin [67]. In the following sections, two of the main BRCA1 protein functions will be further described, namely HRR and transcriptional regulation.

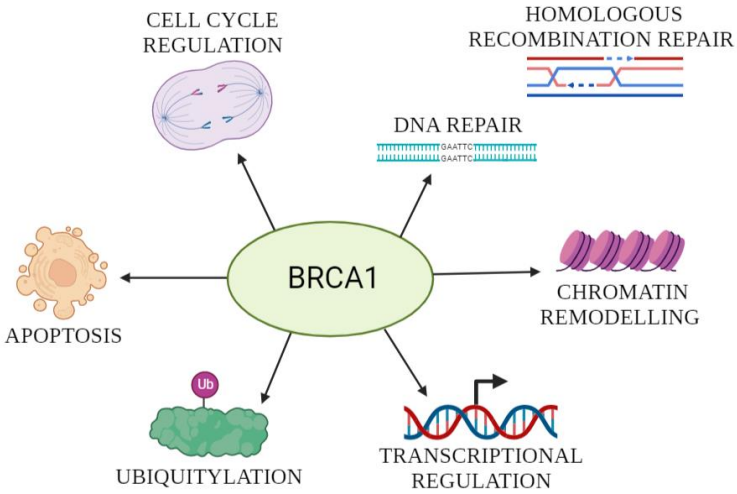

Figure 5 – Overview of BRCA1 protein functions. The figure was created in BioRender and inspired by [69].

1.4.2.1 Homology-directed repair of double stranded DNA breaks

DSBs pose a great danger for the DNA integrity, thus several different cellular pathways have evolved to repair DSBs. The two most studied pathways are non-homologous end joining (NHEJ) and HRR [70]. In the G0/G1 phase of the cell cycle, NHEJ is the predominant repair pathway. NHEJ is considered as a rapid response pathway, which introduces some risks of genomic rearrangements and increased genomic instability. During HRR, the homologous DNA sequence in the sister chromatid is used as a template to repair the broken DNA double helix, and the DSB can be fully restored without incorporation of alterations. Thus, HRR constitutes a smaller source of error and renders higher genomic stability [64].

HRR occurs during late S and G_2 phases of the cell cycle, and is suppressed in the G_1 phase to ensure that the recombination occurs solely between sister chromatids [67, 71, 72]. In HRR, DSBs are first sensed by the MRE11-RAD50-NBN (MRN) complex, which is responsible for loading helicase and exonucleases onto the DNA breaks to start 5'-3' double-stranded DNA resection. The ATR-dependent checkpoint is then switched on by ATR localised to the ssDNA ends, which results in arrest of the cell cycle for HRR to proceed [73]. DNA damage response kinases such as ATM, ATR and CHK2 phosphorylate BRCA1 in response to DNA damage, which enables the cell to repair DNA before entering mitosis [73-77]. In the S and G_2 phases, BRCA1 accumulates on chromatin flanking the DSB sites, and promotes DNA-end resection to produce the ssDNA necessary for homology search and strand invasion [70]. BRCA1 also promotes recruitment of BRCA2 and RAD51 to the DBSs through interaction with PALB2, and BRCA1 mediates loading of RAD51 onto the resected ssDNA ends at the site of the DSB [78-80]. This promotes invasion of the sister chromatid and the formation of Holliday junction, which allows DNA polymerase to repair the DSB [64]. A schematic overview of the role of BRCA1 in the DSB repair pathway is shown in Figure 6.

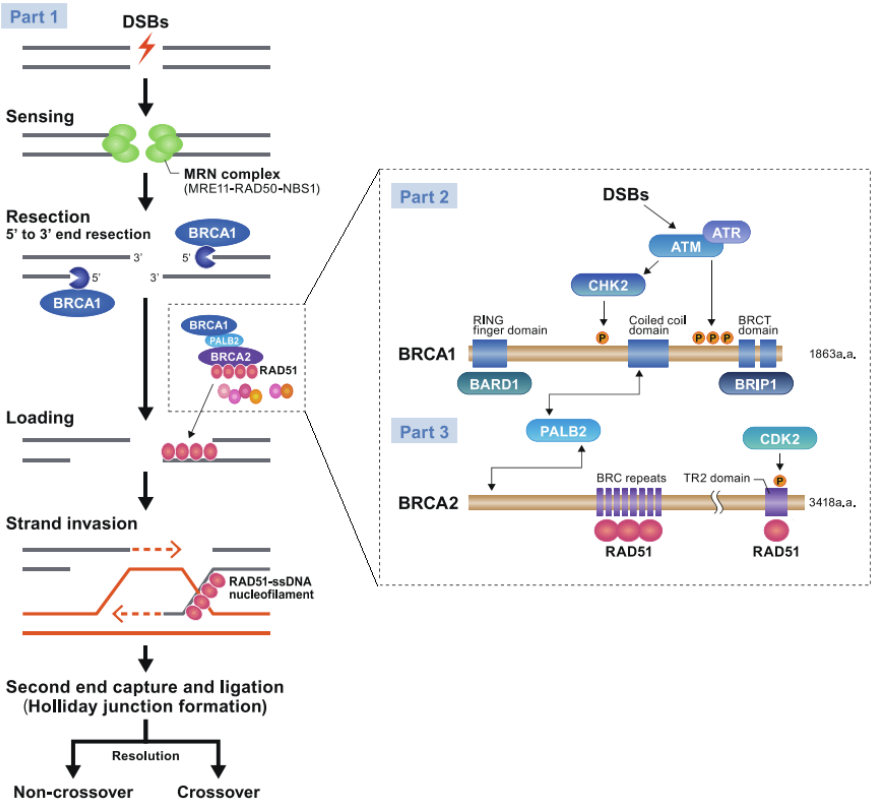

Figure 6 – Overview of the role of BRCA1 in DSB repair. Figure adapted from [81].

1.4.2.2 Transcriptional regulation

The two C-terminal BRCT repeats of BRCA1 contain several conserved hydrophobic/acidic patches, which have been shown to have transcriptional activation (TA) activity [68, 82]. Compared to many classical transcription factors, BRCA1 does not regulate transcription through direct binding to specific DNA sequences, but through binding to, and regulation of, other transcription factors [83]. Through formation of protein complexes, BRCA1 affects the transcriptional regulation of multiple genes with the ability to both co-activate and co-repress genes involved in different cellular processes [64]. Examples of transcription factors interacting with BRCA1 leading to either activation or repression of downstream genes involved in different functions (DNA repair and growth promotion, among others) are shown in Figure 7.

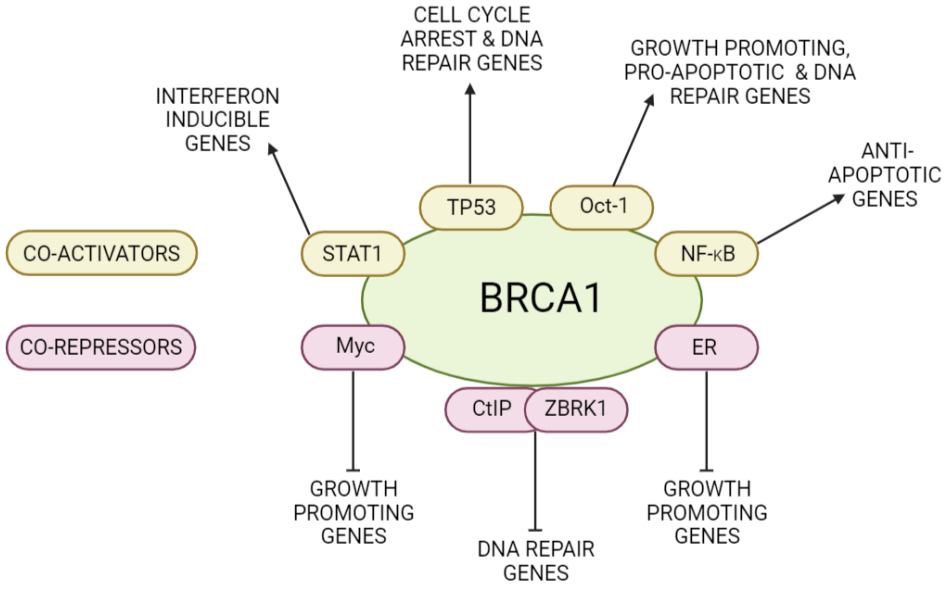

Figure 7 – Transcriptional regulation by BRCA1: Proteins which BRCA1 binds to and form transcriptional activation complexes with are shown in yellow. Proteins which BRCA1 binds to and form transcriptional repression complexes with are shown in red. The genes regulated by these complexes are involved in several different functions, as indicated by the arrows. The figure was created in BioRender and inspired by [64].

1.5 Genetic testing

Genetic testing is used to identify individuals at high risk of developing hereditary cancer, and the *BRCA1/2* guidelines for who should be tested vary between countries [84-86]. In Norway, individuals are referred to genetic testing at one of the departments of medical genetics if HBOC is suspected and testing criteria assigned by national health authorities is fulfilled [44]. Samples are mainly analysed by Sanger sequencing, next-generation sequencing (NGS) or multiplex ligation-dependent probe amplification (MLPA). Previously, one single gene was often analysed separately, but in recent years it has become common to analyse the BRCA genes in cancer panels together with other genes associated with increased risk of breast and/or ovarian cancer (e.g. *ATM, CHEK2, MLH1, MSH2, MSH6, PALB2, PTEN, STK11, TP53, BRIP1, RAD51C, RAD51D*).

For a cancer patient to get a diagnostic test of the *BRCA1* and *BRCA2* genes in Norway, one of the following criteria must be fulfilled; women with breast cancer ≤60 years, women with breast cancer >60 years and strong family history indicating a BRCA disease-causing variant, men with breast cancer, and women with ovarian cancer [44]. When considering predictive testing, assessment of the family history is crucial, and one of their first-degree relatives must fulfil the following criteria: women with breast cancer ≤50 years, man with breast cancer, women with TNBC ≤60 years, women with bilateral breast cancer ≤60 years, women with ovarian cancer, or women with breast cancer and strong family history as specified in the national guidelines [44].

1.6 *BRCA1* gene variants

BRCA1 is a large gene, and more than 13 000 different *BRCA1* variants are registered in the ClinVar database to date (https://www.ncbi.nlm.nih.gov/clinvar/), which is a public archive of suggested variant classifications submitted from different clinical and research laboratories [87]. Several different types of variants have been described in this high penetrant gene, including missense (5286 variants), deletions (2276 variants), insertions (1254 variants), nonsense (836 variants) and splice-site (386 variants) variants (as of ClinVar, 01.12.2022) [87]. In some populations, disease-causing founder variants contribute to a majority of the HBOC cases, and this is also the case in Norway where

founder variants are estimated to account for approximately half of the HBOC cases [88, 89]. Many disease-causing nonsense variants are causing premature stop codons in the gene sequence, which either cause nonsense mediated mRNA decay (NMD) leading to degradation of the mRNA, or truncate the protein and disrupt the function. Deletion, insertion and splice site variants causing frameshifts (2081 variants in ClinVar) and introduction of an early stop codon also constitute a major part of the disease-causing variants [90]. *BRCA1* missense variants, which introduce an amino acid substitution at a certain position, also constitute a significant portion of the germline variants. The cancer risk of *BRCA1* missense variants are often more complicated to assess, and they therefore present a major clinical challenge for clinicians and patients [19]. Although *BRCA1* missense variants are scattered throughout the gene, most of the known disease-causing missense variants are located in the RING and BRCT domains [90]. Disease-causing variants affecting the BRCT domain can disrupt both the DNA damage repair function and the transactivation activity of BRCA1, and exon 17 (BRCT1 repeat) and 20 (BRCT2 repeat) have been shown to contain the highest number of such variants [90, 91]. The RING domain is important for interaction with the BARD1 protein, and a disease-causing variant in this domain will disrupt the ubiquitin ligase activity of BRCA1 [92]. The most vulnerable area of the RING domain, with respect to variation, is encoded by exon 4 [90].

1.7 Interpretation of *BRCA1* gene variants

A gene variant is classified according to a five-tier score system with the following designations: benign (B, class 1), likely benign (LB, class 2), uncertain significance (VUS, class 3), likely pathogenic (LP, class 4) and pathogenic (P, class 5, previously referred to as disease-causing in this thesis) [93]. The American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) have made a joint recommendation of standards and guidelines for the interpretation of sequence variants into these five categories [94]. The recommendations contain different evidence categorised according to different strength and weighting, pointing either in the benign or pathogenic direction, as detailed in Figure 8. In addition, some criteria listed as one weight can be moved to another weight using professional judgement depending on the evidence collected, and weight assigned to certain criteria may vary by gene and disease [94]. The evidence is then combined to determine the final variant classification.

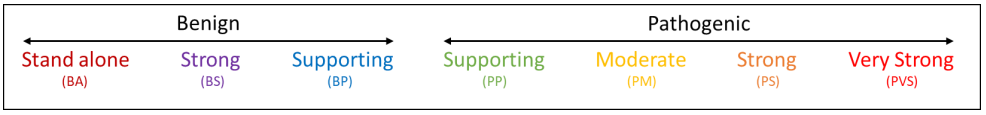

Figure 8 – Evidence strength of the ACMG-AMP criteria. Criteria of different strength in the benign (left) or pathogenic (right) direction are combined to decide the final classification of a variant. BA = benign stand alone, BS = benign strong, BP = benign supporting, PP = pathogenic supporting, PM = pathogenic moderate, PS = pathogenic strong, PVS = pathogenic very strong.

In addition to the general recommendations, several expert groups have developed classification recommendations for specific cancer genes. The consortium Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) has provided expert opinions of the interpretation of breast cancer genes, and the Cancer Variant Interpretation Group UK (CanVIG-UK) has developed detailed specifications for germline cancer variant interpretation (both in general and for the *BRCA1/BRCA2* genes) [95-97].

Several factors are evaluated during variant interpretation, including the type of variant (e.g. missense variants, splice variants and nonsense variants), the conservation of the affected residue(s), physicochemical properties of the original versus new amino acid residue (for missense variants), computational data from *in silico* tools, clinical information like tumour type and origin, family history as well as segregation, and functional analysis assessing the effect of the variant on protein functions and/or structure described in the literature (available in PubMed and the Human Gene Mutation Database (HGMD)). HGMD contains a collection of published literature on human germline variants responsible for inherited diseases [98]. Also crucial are variant allele frequencies reported by the Genome Aggregation Database (gnomAD), which is an international online reference database containing information on almost 230 million genome variants and allele frequency data from over 140 000 unrelated individuals of different ethnicities [99]. Rare diseases are generally not caused by common variants (>5% in the general population), and the minor allele frequency can therefore be used to exclude such variants as the cause of hereditary cancer. In addition, useful information can be found in the ClinVar database, which is a public archive of submitted classification reports from different laboratories [87]. The BRCA Exchange contains *BRCA1/BRCA2* variant interpretations that have been reviewed by the ENIGMA consortium [100].

BRCA1 germline variants classified as likely pathogenic or pathogenic increase the risk of cancer by impairing protein structure and/or function. Carriers of such variants are therefore offered regular surveillance and prophylactic surgery according to national guidelines, as described in section 1.3.2 and 1.3.3. Likely pathogenic and pathogenic variants are thus both clinically actionable and are managed similarly in the clinic. *BRCA1* germline variants classified as either likely benign or benign are not associated with increased risk of cancer, and is assumed not to be the explanation for the cancer in the family. However, for a large number of *BRCA1* variants, the knowledge is either very limited or conflicting, and accordingly these are classified as variants of uncertain significance (VUS).

1.7.1 The challenging *BRCA1* VUSs

Even though *BRCA1* is a well-characterised gene, interpretation of *BRCA1* variants is still challenging for the clinical laboratories. Lower threshold for genetic testing and reduced costs for sequencing has significantly increased the finding of novel *BRCA*1 variants including VUSs, which are variants where it is not known whether the variant has any effect on protein structure and/or function that might confer an increased cancer risk. Studies have shown that one or more VUSs are found in 33-54% of breast cancer patients, and to date, more than 3500 *BRCA1* VUSs are registered in the ClinVar database [101-105]. When a VUS is detected, no clinical management decisions can therefore be made based on the finding [94]. This makes the surveillance and management of VUS carriers particularly challenging, and the classification of a VUS is a significant barrier for the clinicians [106].

Further complicating the interpretation of VUSs, is the width of the class 3. To classify a variant as likely pathogenic (class 4) is has to be >90% certainty that the variant is pathogenic. Likewise, to classify a variant as likely benign (class 2), it has to be >90% certainty that the variant is benign. The likelihood of a VUS being pathogenic are thus ranging from 10-90% [94, 107]. Consequently, some laboratories do further sub-classifications of VUSs using a temperature gradient, ranging them from "cold" to "hot" according to the likelihood of pathogenicity [107]. In addition, there are differences in how laboratories chose to report the finding of VUSs to the patients [108]. Some laboratories report only "hot VUSs" as there is a high level of supporting evidence that the variant is pathogenic. Other laboratories chose to report all VUSs to the patients [107]. Consequently, if reported back to the patient, the meaning of a VUS might be misinterpreted by the receiver and cause significant anxiety among the carriers.

In addition, misclassification of a VUS as (likely) pathogenic can have tragic consequences for both the patient and relatives carrying the same variant. Recently, an example of misinterpretation was unveiled in a hospital in Norway, where 21 female carriers had their breast and/or ovaries removed by prophylactic surgery without sufficient evidence that their variant was pathogenic [109, 110]. The other Norwegian

hospitals did not classify this variant as pathogenic, but this was unknown at the time, as there was no general practice for data sharing or a common national variant database.

To assist in the correct classification of *BRCA1* VUSs, there is a major need for additional evidence to clarify the pathogenicity. In cases where there is a strong history of cancer in a family it can help to assess the cancer risk of a VUS, as the findings of several related tumours in the family, cancer at young age and segregation data, indicates the presence of a hereditary predisposition. However, as many missense variants are very rare and present in the general population at a low frequency, there are often not enough available family members to perform segregation analysis of a given variant. As indicated in section 1.7, functional analysis can be used as a tool to gain more information about the clinical significance of a gene variant. According to the ACMG-AMP interpretation guidelines, functional analyses can be used as evidence of supporting to strong strength during the interpretation of variants [94].

2. Aims of the project

The main focus of this thesis was variants in the *BRCA1* gene, aiming to gather and harmonise the classification of all *BRCA*1 variants detected at Norwegian diagnostic genetic laboratories, and to generate a better understanding of the BRCA1 protein through multiple functional analyses of rare missense variants classified as VUSs.

Specific aims were as follows:

Paper I:

- To gather and generate an overview of all *BRCA1* variants of class 2-5 (likely benign to pathogenic) detected in diagnostic genetic laboratories in Norway ("*BRCA1* Norway").
- To unveil potential discrepancies in *BRCA1* variant classification between the laboratories in Norway, serving as a quality control at the national level.
- To examine the causes of disagreements for the variants with conflicting interpretations.
- To improve national inter-laboratory collaboration and increase the consensus regarding *BRCA1* variant classification.

Paper II:

- To functionally investigate the effect of 14 rare *BRCA1* missense variants classified as VUSs (from paper I) distributed throughout the gene, including regions without known function.
- To establish multiple functional protein assays including protein expression and protein stability, subcellular localisation, and protein interactions with BARD1 and PALB2, using the full-length BRCA1 protein.
- To investigate the hypothesis stating that most *BRCA1* variants located outside protein domains with known function are benign and of no functional importance.

- To use the data from the different functional assays in this study, in combination with other available information, as a tool to clarify the pathogenicity of these variants.

Paper III:

- To functionally investigate the effect of 11 rare *BRCA1* missense VUSs (from paper I) located in or in close proximity to the BRCT domain with respect to repair of double stranded DNA breaks using a homology-directed recombination repair assay.
- To investigate the effect of the same variants on transcriptional activation using the Dual-Luciferase Reporter Assay.
- To use the data generated in the different functional assays in this study, in combination with other available information, as a tool to clarify the pathogenicity of these variants.

3. Methodology

In this section, the most central parts of the methodology used in this thesis is described. A detailed description of the materials and methods can be found in the respective papers. The study was approved by the Regional Committee for Medical and Health Research Ethics of Haukeland University Hospital (2018/2467).

Paper I

In paper I, a systematic overview of all *BRCA1* variants of class 2-5 (likely benign to pathogenic) detected in Norway was generated. *BRCA1* variants were collected from the four diagnostic genetic laboratories in Norway: Haukeland University Hospital (Bergen), Oslo University Hospital (Oslo), the University Hospital of North Norway (Tromsø) and St. Olav's University Hospital (Trondheim). All *BRCA1* variants were detected by genetic testing of patients with suspected HBOC or healthy family members in the years from late 1990s to July 2019. For each variant, available information regarding criteria of classification, a limited amount of clinical information regarding cancer diagnosis and age of onset, and family history were requested. Each variant was classified locally at the hospitals according to ACMG-AMP guidelines or equivalent procedures. For VUSs observed in only one hospital and with a classification report older than three years in 2019, a reassessment of the variants was performed by Haukeland University Hospital (HUH). For variants detected in more than one hospital, the internal classifications were compared. In cases of conflicting classifications, the individual laboratories were asked to do a second assessment of the variant. Subsequently, a series of digital meetings between all laboratories were arranged to disclose the cause of disagreement and to try to reach consensus. In addition, for a subset of variants, the changes in classifications at HUH and in ClinVar over time were compared and presented by a heatmap.

Paper II

In paper II, multiple functional analyses were performed in order to investigate the impact of 14 rare *BRCA1* missense variants from paper I with respect to protein expression and stability, subcellular localisation, and interaction with the two protein partners BARD1 and PALB2. This was achieved using the full-length BRCA1 protein expressed with a N-terminal mCherry-tag, and by comparing the effect of the variants of interest to the wild type (WT) BRCA1 protein and to known benign and pathogenic control variants. The chosen variants were classified as VUSs at the time of selection and distributed throughout the entire protein, mainly outside domains with known functions. As the protein expression level of a variant is known to correlate with its pathogenicity for many genes, western blot analysis was performed to investigate the effect of the variants on BRCA1 protein expression [111, 112]. The western blot analyses were performed in both HEK293FT cells and in the breast cancer cell line MDA-MB-231, while the remaining assays were performed in HEK293FT cells only. Following western blot analysis, qPCR was performed for variants showing severely reduced protein expression in HEK293FT cells (<20% protein levels compared to the WT, 100%) in order to investigate if the low expression was caused by reduction of the mRNA levels. In addition, a MG132 assay was performed on the same variants to investigate if the low protein levels were due to degradation by the ubiquitin-proteasome system. MG132 is a proteasome inhibitor which reduces the degradation of ubiquitin-conjugated proteins when added to the cells. For the BRCA1 variants which showed protein expression levels above 20% compared to the BRCA1 WT protein, a cycloheximide chase assay that follows protein degradation over time was performed. The protein synthesis inhibitor cycloheximide was added to transfected HEK293FT cells, and the amount of BRCA1 protein at different time points (0, 2 and 8 hours) were compared to transfected cells without cycloheximide. The BRCA1 protein is mainly located in the nucleus, and a correct localisation is crucial for maintaining its protein functions. Thus, subcellular localisation of the same variants of interest was tested by a fractionation assay separating the cytosolic and nuclear cell fractions. The fractions were analysed by western blotting, and the percentage of BRCA1 protein in the nucleus was compared to the cytosolic amount. Lastly, to test the potential effect of the investigated

BRCA1 variants on binding to other protein partners, a co-immunoprecipitation (Co-IP) assay was performed with BARD1 and PALB2. BARD1 interacts with the RING domain of BRCA1 which is important for the stability of both proteins, while PALB2 interacts with the coiled-coil domain and is involved in the DNA damage response pathway. The Co-IP was performed using the DynabeadsTM Protein G Immunoprecipitation Kit (Invitrogen) according to the manufacturer's protocol. After finalising the functional assays, a reinterpretation of all 14 *BRCA1* variants were suggested according to the *BRCA1/BRCA2* gene-specific variant interpretation guidelines from CanVIG-UK [96].

Paper III

In paper III, we investigated the effect of 11 *BRCA1* variants located within or in close proximity to the BRCT domain of BRCA1 on protein expression, repair of double stranded DNA breaks and TA activity. The variants were selected from our "*BRCA1* Norway" study (paper I). Protein expression levels of the variants were investigated in both the full-length His-BRCA1 protein and the fusion protein DBD-BRCT which includes the C-terminal part of BRCA1 (aa 1396–1863) fused to the GAL4 DNA Binding Domain (DBD). The western blot analyses were performed on HEK293FT cells.

To investigate if the variants affected the capacity of BRCA1 to repair double stranded DNA breaks by HRR, a homology-directed recombination repair (HDR) assay was performed as described by Ransburg *et al*. 2010 [113]. In the HDR assay, which involves the use of a green fluorescence protein (GFP) reporter and flow cytometry-based detection, a plasmid encoding full-length *BRCA1* WT or variant is transfected into HeLa-DR-GFP cells. The HeLa-DR-GFP cells are characterised by the presence of two inactive GFP copies integrated in the genome. The first copy (Sce-GFP) is inactive due to the presence of a I-SceI cleavage site, and the second copy (iGFP) is truncated at both ends. A second transfection of a plasmid expressing I-SceI endonuclease will result in a double-stranded DNA break in the first GFP copy, as illustrated in Figure 9. If the investigated *BRCA1* variant is capable of double stranded DNA repair, this break will

be repaired by HRR using the second GFP as a donor sequence [113]. Cells that are subjected to HRR will turn green and can be sorted and quantified using flow cytometry. In addition, the results were confirmed by fluorescence microscopy analyses.

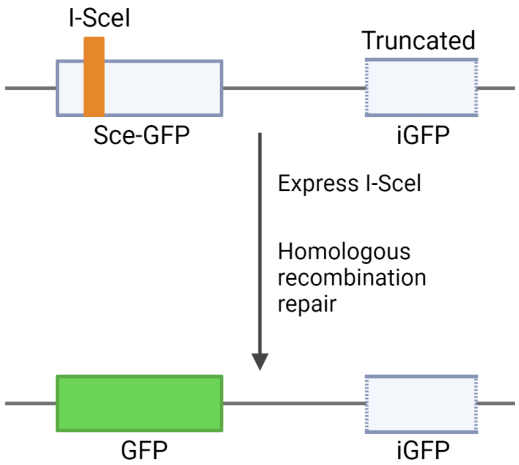

Figure 9 – Overview of the HDR assay: The HeLa-DR-GFP cells contain two inactive copies of the *GFP* gene. The first copy (Sce-GFP) is inactive due to the presence of a I-SceI cleavage site, and the second copy (iGFP) is truncated at both ends. Transfection of a plasmid encoding the I-SceI endonuclease will result in a double-stranded DNA break in the Sce-*GFP* copy. During HRR, BRCA1 will use the iGFP copy as a sequence donor to repair the break, and GFP will subsequently be expressed. Figure inspired by [113].

BRCA1 variants in the BRCT domain causing destabilisation of the structure are known to impair TA and thus increase the risk of cancer [82, 114-116]. In the TA assay, a functional *BRCA1* variant will have the ability to activate transcription of a reporter gene when it is fused to a heterologous DNA binding domain. In this study, a fusion protein of yeast GAL4 DNA binding domain and the human BRCA1 BRCT domain, hereafter called DBD-BRCT, was used. The assay was performed using the Dual-Luciferase Reporter Assay System kit from Promega Corporation. The assay is based on measuring the expression of a firefly luciferase plasmid, which is normalised by the activity of the *Renilla* luciferase. Both reporter plasmids encode enzymes that produce light as a by-product of catalysis. The two firefly and *Renilla* reporter plasmids are co-transfected to

HEK293 cells together with DBD-BRCT. The DBD-BRCT protein will bind to the upstream *GAL4* promoter of firefly luciferase through DBD (Figure 10), resulting in transactivation of firefly. The bioluminescent signal created when firefly oxidases its' substrate is then measured by a luminometer, and this signal correlates to the amount of DBD-BRCT protein that binds to the *GAL4* promoter. While the firefly signal is dependent on transactivation, the *Renilla* expression is independent of the DBD-BRCT protein. The signal created by *Renilla* luciferase is therefore used as an internal control to normalise against differences in cell number and transfection efficiency.

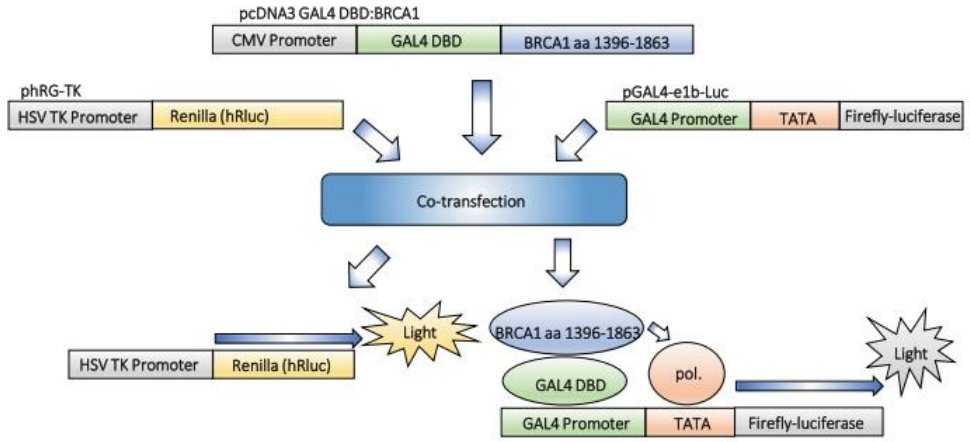

Figure 10 – Overview of the TA assay: The plasmid *DBD-BRCT* is co-transfected into HEK293 cells together with the reporter plasmids expressing Firefly and *Renill*a. DBD-BRCT will activate the *GAL4* promoter of firefly, resulting in submission of light. The signal is normalised to the *Renilla* signal, which accounts for differences in cell number and transfection efficiency. Figure from [117].

4. Summary of results

Paper I: *BRCA1 Norway: comparison of classification for BRCA1 germline variants detected in families with suspected hereditary breast and ovarian cancer between different laboratories*

In total, 652 *BRCA1* germline variants were submitted from the four diagnostic genetic laboratories in Norway: 303 variants from Oslo University Hospital, 177 from Haukeland University Hospital (HUH) in Bergen, 88 from the University Hospital of North Norway in Tromsø and 84 from St. Olav's University Hospital in Trondheim. After removing overlapping variants, 463 variants were shown to be unique. Among the unique *BRCA1* variants, 126 variants (27%) were detected in more than one hospital. The remaining 337 variants were observed in one hospital only. For 30% (38/126) of the variants detected in more than one hospital, there were discrepancies in classifications between the hospitals. The differences in interpretation were mainly by one pathogenicity class (class 2/3 or 4/5), except for one major discrepancy (class 3/5) which could potentially affect the clinical management of patients. This class 3/5 discrepancy was detected for the *BRCA1* variant c.457_458ins21. The variant was observed in three hospitals; one of the hospitals reported it as a VUS (class 3), while the two other hospitals reported it as pathogenic (class 5). In the class 5 reports, an insertion of 21 nucleotides leading to a premature stop codon was described. This did not correspond with the class 3 report, where the sequence did not contain a stop codon. Investigations revealed that a stop codon was indeed present in the insertion, and the discrepancy was found to be caused by misreading of the inserted sequence due to software weakness. The mistake was corrected, and all laboratories now classify this variant as pathogenic. The affected family was revised for further genetic testing and switched to correct clinical management, and luckily no new cancer cases had occurred in the family during the period of misclassification (2016-2019). For the remaining 37 variants with conflicting classifications between the hospitals, each laboratory was asked to reassess the variants. After discussing the causes of disagreement, the

discrepancy rate was reduced to 10% (13/126). For VUSs with only one classification report older than three years (n=45), the variant was reassessed by HUH. Eleven variants were reclassified to likely benign variants, while one variant was reclassified as a benign variant. The remaining variants were still assessed as VUSs. Furthermore, for a sub-cohort of the variants (detected at HUH in the period from 2007 to 2017) a heat map presentation of their classification over time at the hospital, as well as in ClinVar, was made. The heat map showed that the concordance in classifications between HUH and ClinVar was relatively high, and that the majority of variants that changed classification over time were VUSs reclassified to likely benign. In addition, comparison of all the Norwegian *BRCA1* variants gathered in this study with previously published *BRCA1* variants found in certain regions of Norway [88, 118, 119] revealed that the two variants *BRCA1* c.5123C>T p.(Ala1708Val) (VUS) and c.4883T>C p.(Met1628Thr) (likely benign) had previously been incorrectly classified as pathogenic in a recent publication [119]. This was later corrected by the authors.

Paper II: *Functional analyses of rare germline missense BRCA1 variants located within and outside protein domains with known functions*

In paper II, 14 *BRCA1* missense VUSs from paper I were functionally analysed using the full-length protein. When transfecting plasmids encoding the *BRCA1* missense variants of interest into HEK293FT cells and analysing the cell lysates by western blotting, four of the protein variants (p.Leu52Phe, p.Met297Val, p.Asp1152Asn and p.Leu1439Phe) displayed severely reduced protein expression levels (below 20% of the WT protein), similarly to the included pathogenic controls. In MDA-MB-231 cells, a similar trend for protein expression was seen. The remaining variants showed protein expression levels similar to the WT and/or benign controls. The four variants found to be expressed at levels similar to the included pathogenic controls were subsequently analysed by qPCR to investigate if the low protein expression was caused by a reduction of the mRNA levels. The results showed that all four plasmids produced similar amounts of mRNA as the WT plasmid. To check if the low protein levels of the aforementioned four variants were due to degradation by the ubiquitin-proteasome system, transfected

HEK293FT cells were treated with the proteasome inhibitor MG132. Western blot analysis of the cell lysates showed that the protein expression clearly increased for three of the variants (p.Leu52Phe, p.Met297Val and p.Asp1152Asn) after treatment with MG132 compared to control samples treated with DMSO only. For the variant p.Leu1439Phe however, equal amounts of protein were observed in the MG132 sample as in the control sample. To further investigate protein stability, all BRCA1 variants which showed protein expression levels above 20% compared to the BRCA1 WT protein in the initial western blot analysis, in addition to the p.Leu1439Phe variant showing equal amount of protein in the MG132 assay, were analysed by a cycloheximide chase assay to follow protein degradation over time. The analyses showed that the protein levels of the two BRCA1 VUSs p.Gly890Arg and p.Leu1439Phe were reduced to 11% and 10%, respectively, after 8 hours treatment with cycloheximide, similar to the pathogenic controls. For the remaining variants, the reduction in protein levels were comparable to the WT and/or benign controls. When investigating the potential effect of the variants of interest on the nuclear localisation of the protein by a fractionation assay, the variants were found to be mainly expressed in the nucleus, similar to the WT protein. In addition, Co-IP assays were performed to test the potential effect of the VUSs on the binding of BRCA1 protein to the interaction partners BARD1 and PALB2. None of the protein variants of interest showed significantly reduced binding to either BARD1 or PALB2. When combining the newly achieved functional evidence with other available information for the variants, reclassification of seven variants from VUS to likely benign was suggested.

Paper III: *Functional analyses of rare germline BRCA1 variants by transcriptional activation and homologous recombination repair assays*

The effect of 11 *BRCA1* missense variants located within or in close proximity to the BRCT domain of BRCA1 were investigated by protein expression, HRR and TA. The protein expression of both the full-length His-BRCA1 protein and the DBD-BRCT version, expressing a fusion protein of DBD and the BRCT domain of BRCA1, were investigated in HEK293FT cells. Four variants (p.Ala1708Val, p.Gly1709Arg,

p.Lys1711Gln and p.Trp1718Ser) were found to be expressed at levels similar to the pathogenic controls and below 20% of the WT protein for both the fusion protein DBD-BRCT and the full-length BRCA1. The variant p.Phe1668Leu was expressed at low levels in DBD-BRCT, and intermediate levels in His-BRCA1. The six remaining variants were expressed at levels similar to the WT and/or benign controls in both DBD-BRCT and full-length BRCA1. When evaluating the potential effect of the various BRCA1 VUSs on HRR, only one variant (p.Pro1749Ala) exhibited HRR activity comparable to the WT BRCA1 and the benign controls. All other variants displayed HRR activity similarly to the pathogenic control variants. When assessing the variants' effect on transactivation, two variants (p.Ala1708Val and p.Trp1718Ser) showed TA activity levels similar to the pathogenic controls, while two variants (p.Gly1709Arg and p.Lys1711Gln) displayed reduced TA activity levels compared to the WT, but still similar to the benign controls. The remaining variants exhibited TA activity comparable to or even surpassing the WT. When combining the newly achieved functional evidence with other available information for the variants, all variants except one (p.Trp1718Ser), were still classified as VUSs.

5. Discussion

5.1 *BRCA1* Norway

Sequencing of the *BRCA1* gene in families with suspected HBOC has been performed in Norway since the late 1990s. However, previous studies characterising *BRCA1* variants in Norway have included only specific parts of the country [88, 118-120]. In paper I, a complete overview of all *BRCA1* variants classified as likely benign to pathogenic identified at the four diagnostic genetic laboratories in Norway until 2019 was generated ("*BRCA1* Norway"), consisting of 463 unique *BRCA1* variants. Comparison of the variant classifications revealed some discrepancies, which was reduced by collaboration between the laboratories. Sharing and combining information from the four different Norwegian laboratories were found to increase the amount of evidence, and comparing independent classifications will thus ensure more trustworthy and updated classifications. This is especially important for rare missense variants, for which information is sparse, and crucial to reduce the number of VUSs. As the "*BRCA1* Norway" study revealed discrepancies in the variant interpretation and classification among the different laboratories, as outlined below, the collaboration will also help to ensure that family members harbouring the same genetic variant, but living in different parts of the country, will receive the same medical advice. In addition, sharing data will reduce the time and effort spent on variant interpretation in the future. Prior to initiating this study, the scientific community in Norway had expressed a desire for increased sharing of data regarding variant interpretation. To date, the four diagnostic genetic laboratories in Norway use different software for storage of variant interpretations, which further complicates the sharing of variant data. Previously, variant data sharing in Norway has been limited, mostly due to the strict laws regarding the privacy of patients, as rare variants may be used for identification of an individual. However, it was recently decided to change this law, which will make it possible to share such information through a common platform, given that the important information about the variants can be exchanged in accordance with the guidelines for patient privacy policies [121]. Therefore, it is now a mutual goal to use a common software and establish a

database for registration of variant classifications that will make it easier to share variant interpretations between the different genetic laboratories in Norway.

5.2 The importance of revealing conflicting variant classifications

As the *BRCA1* status may affect both the surveillance of healthy carriers and the personalised management of cancer patients, unveiling potential conflicting interpretations between different diagnostic genetic laboratories is crucial. One of the main aims of paper I was therefore to compare internal classifications at the different laboratories to explore the national consistency of *BRCA1* variant interpretation. Due to the complexity of the interpretation process, some discrepancies were expected. The finding of a discrepancy rate of 30% among the Norwegian laboratories as described in paper I is within the range of previous findings in similar studies in countries like Canada and USA [122-126]. Alarmingly, one VUS/pathogenic (class 3/5) discrepancy was detected for the variant *BRCA1* c.457_458ins21 (insertion of ATTAGCAGGAAACCAGTCTCA). There is a major difference in the clinical management of patients harbouring a VUS and a pathogenic *BRCA1* variant, and misclassification of this pathogenic variant as a VUS has potentially serious consequences by depriving the affected family of appropriate treatment. This is an example of how misclassification could potentially be very harmful for the affected individuals, similar to the recent case in Norway where 21 female carriers had their breasts and/or ovaries removed without sufficient evidence that their variant was pathogenic [109, 110]. The remaining discrepancies in interpretation were by one pathogenicity class only (likely benign/VUS or likely pathogenic/ pathogenic). These discrepancies did not affect the clinical management of the carriers as likely pathogenic/pathogenic variants are both clinically actionable and managed the same way in the clinic. Likewise, likely benign/VUS variants are not clinically actionable.

5.3 Harmonising *BRCA1* variant classification among the Norwegian laboratories

In paper I, a second aim of the Norwegian collaboration was to examine the causes of disagreements for the variants with conflicting interpretations. We wanted both to increase the national consensus regarding *BRCA1* variant classification, and to create a

forum for future discussions on variant interpretation in general. All variants with conflicting classifications were therefore discussed among the participating laboratories through multiple digital meetings, after which 66% of the original conflicting classifications reached consensus. All discrepancies of likely pathogenic/pathogenic (class 4/5) were resolved, while ten likely benign/VUS (class 2/3) and three benign/likely benign (class 1/2) discrepancies remained. This illustrates that combining evidence and experience across multiple laboratories through establishment of a national network results in harmonisation of the classifications, and could give more accurate classifications compared to individual interpretations locally at the laboratories.

Two main reasons for the remaining conflicting classifications between likely benign/VUS were found to be differences in how strictly the different laboratories followed the ACMG-AMP classification guidelines, in addition to different understandings of some of the guidelines [94]. Different usage of the BP1 evidence ("Missense variant in a gene for which primarily truncating variants are known to cause disease") in the ACMG-AMP guidelines caused the majority of the likely benign/VUS discrepancies. As only two supporting benign evidences are enough to classify a variant as likely benign according to these guidelines, frequent usage of the BP1 criteria would more easily lead to classification of non-conserved *BRCA1* missense variants outside the RING, coiled-coil and BRCT domains as likely benign. Several publications have suggested that most *BRCA1* missense substitutions located outside these well-established protein domains could be classified as likely benign, arguing that pathogenic missense variants are infrequent in these regions which are thought to be without essential functions and tolerate variation [127-129]. It has also been suggested to incorporate a criteria regarding "coldspots" as a counterweight to hotspots (PM1 evidence, "Located in a mutational hot spot and/or critical and well-established functional domain") to improve the ACMG-AMP variant interpretation guidelines. A coldspot criteria is already covered by the BP1 evidence criteria in the *BRCA1/BRCA2* gene-specific guidelines for variant interpretation from CanVIG-UK [96, 97]. This evidence suggests a benign effect for missense variants at non-conserved residues outside the RING, coiled-coil and BRCT domains. While this coldspot BP1 criteria from

the updated CanVIG-UK guidelines had already been used for some time at one of the laboratories, this was not yet the case at the three other laboratories. As one of only two supporting benign criteria needed to achieve likely benign according to ACMG-AMP, it was argued against extensive use of BP1. This was particularly pointed out when using BP1 in combination with BP4 ("Multiple lines of computational evidence suggest no impact on gene or gene product") as *in silico* predictions are not accurate, and there was little consensus among laboratories on which algorithms to use [130-136]. There are several reasons for using BP1 with caution. During the folding of proteins, amino acid residues located outside well-established domains in the primary structure of the polypeptide chain can potentially interact with or become part of important structural and functional elements in the native folded three-dimensional structure of the BRCA1 protein. Replacement of amino acid residues originally located outside an important domain in the primary structure could therefore potentially affect both the structure and function of the protein. Although information on the three-dimensional structure of BRCA1 other than the RING and BRCT domains is not known, it is possible that the consequence of introducing a missense variant involving an amino acid with major differences in size, polarity and physiochemical properties compared to the original residue could be fatal, also for residues located outside well-established domains. Furthermore, the approximately 1500 residue non-conserved central region of BRCA1 has been suggested to act as a long flexible scaffold for intermolecular interactions which obtains a more ordered structure upon binding to protein partners, and may thus still be functionally important in the DNA damage response [137-141]. It was also argued that although the ClinVar dataset contains hardly any (likely) pathogenic *BRCA1* missense variants located outside the critical domains, it does not necessarily mean that it does not exist, and more knowledge about the regions outside these protein domains are needed. After the discussions, an agreement was made to use BP1 with caution.

Other factors that were found to cause discrepancies during variant interpretations were the use of the ACMG-AMP BS1 evidence ("Allele frequency is greater than expected for disorder"), as different cut-off values regarding allele frequencies were used among the different hospitals, and the degree of emphasis on classifications performed by the

expert consortium ENIGMA (BP6 evidence, "Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation"). In addition, some discrepancies were caused by differences in the clinical data and family history of carriers available between the different laboratories, which is an important aspect of the variant interpretations, and if the variant of interest was found together with an additional pathogenic variant. Out-dated information and availability of new evidence (new publications etc.) were also a reason for conflicting classifications.

5.4 Variant classification changes over time

Driven by emerging new evidence or changes in weighing or combination of evidence, variant classifications may change over time [142]. In paper I, a third aim was to assess the change in *BRCA1* variant classifications over time with increasing information available. Knowledge about variant allele frequencies in the general population was made available through the Exome Aggregation Consortium (ExAC) in 2014, and its successor Genome Aggregation Database (gnomAD) in 2017. The open access database ClinVar was made available in 2013. The ACMG-AMP interpretation guidelines were published in 2015, and the gene specific ENIGMA criteria for *BRCA1* variants were published in 2009 (lastly updated in 2017). The CanVIG-UK *BRCA1/BRCA2* gene-specific interpretation guidelines were released in 2021. Given this background, 45 *BRCA1* VUSs with classification reports older than three years were therefore reassessed in paper I, resulting in reclassification of 11 variants to likely benign and one variant to benign. This demonstrates the importance of updating variant interpretations regularly, and shows that regular reassessment can decrease the number of VUSs. The classification of a variant is dependent on the information available at the local hospital at the time of interpretation, but the available information on VUSs are often sparse or conflicting. Thus, a given classification is most correct at the specific time of interpretation based on available information and the applicable variant interpretation guidelines, but can be outdated and should ideally be reassessed when new information is available. However, in Norway a variant is in most cases only reassessed if it is identified in a new individual, but due to the rarity of many missense variants in the

population, certain variants might never be reinterpreted. Consequently, a more regular system for reassessment would be beneficial, but would require an substantial increase in resources [142]. This will however be more achievable upon introduction of a common national software for registration of variant classifications, as discussed in section 5.1. When reassessing variants in paper I, only variants with one single interpretation report older than three years were reclassified. Thus, variants with older reports observed at several laboratories might still contain outdated interpretations, and one might speculate that some of the oldest registrations of variant classification are incomplete or insufficient, as the guidelines for interpretation have improved since the beginning of the BRCA1 sequencing in the 1990s. In addition, during the years the samples were analysed (late 1990s to 2019), both the criteria for eligibility of having a genetic test and the criteria used to classify a variant have changed.

To investigate how *BRCA1* variant classification has changed over time in general, the classification history at HUH and in ClinVar for a subset of *BRCA1* variants reported between 2007 and 2017 was evaluated. At HUH and in ClinVar the majority of reclassified variants were downgraded from VUS to likely benign, in concordance with other studies [143-149]. This is probably due to the gradually increasing knowledge, primarily about variant allele frequencies from the large population databases like ExAC and gnomAD, which has revealed that several variants are too abundant in the normal population to be disease-causing. Some variants were upgraded from VUS to (likely) pathogenic, probably due to increased knowledge from functional studies, discovery of the variants in more HBOC individuals, and family segregation data.

5.5 Functional assays as a tool to assess *BRCA1* VUSs

To date, the pathogenicity associated with rare missense variants in breast cancer predisposition genes have been largely unclear [19]. In fact, a majority of the *BRCA1* missense variants identified by clinical genetic testing reported in ClinVar are reported as VUSs [150]. In paper I, 25% of the variants from the Norwegian HBOC variant overview were initially classified as VUSs. In addition, there were variants classified as both VUSs and likely benign among the different hospitals (6% of the variants). These

variants are highly challenging for both clinicians and patients, and measures are therefore needed to clarify their pathogenicity and reduce the number of *BRCA1* VUSs. Several computational tools have been developed to assist missense variant interpretation, but due to variable performance and reliability, *in silico* tools should be used with caution, and are insufficient to be applied as stand-alone evidence in clinical diagnostics [131, 150, 151]. According to the ACMG-AMP and CanVIG-UK guidelines, the *in silico* criteria (PP3/BP4 evidence) should be used as supporting evidence only [94, 97]. The finding of missense variants will therefore often need further analysis like functional analysis, and reassessment of the pathogenicity when new knowledge is unveiled. In the ACMG-AMP and CanVIG-UK guidelines, functional assays are used as evidence of supporting to strong strength (depending on the validity of the assay and the number of control variants included) when determining the pathogenicity of a variant (BS3 or PS3 evidence, "Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product / Well-established *in vitro* or *in vivo* functional studies show no damaging effect on protein function or splicing") [94, 97]. Functional assays can therefore be used as a robust tool to generate new information about a variant, which can contribute to the clarification of its classification.

5.5.1 Previous functional studies of the BRCA1 protein

Several functional assays have been utilised as a method to generate new knowledge supporting the interpretation of *BRCA1* variants. Among others, functional assays assessing TA [114, 117, 118, 152-154], protease sensitivity [155, 156], HRR [113, 157], centrosome amplification [158-161], E3 ubiquitin ligase activity [92, 162], as well as protein-protein interactions [163] have been performed. According to the *BRCA1* gene-specific CanVIG-UK guidelines, five published functional studies of the BRCA1 protein are suggested with specific recommendations regarding the strength of their respective functional evidence [96, 157, 164-167]. However, several of the functional assays on BRCA1 published to date focus mostly on variants located in the known protein domains, using plasmid constructs expressing only parts of the full-length protein [117, 162, 168-171]. This is also the case for some of the CanVIG-UK

recommended studies, where three out of five studies examine only specific domains [157, 165, 166]. As *BRCA1* VUSs are distributed throughout the entire protein including regions outside well-established protein domains, examining only known domains may be misleading [172]. The two full-length functional studies (Findlay *et al*. 2018 [164] and Bouwman *et al*. 2020 [167]) recommended by CanVIG-UK are considered as PS3/BS3 evidence weighted as strong [96]. In Findley *et al*. [164], saturation genome editing was used to assess 96.5% of all possible single-nucleotide variants (SNVs) in 13 exons that encode functional domains of BRCA1 [164]. Although utilising the full-length protein, the study did however not include any variants outside of known protein domains. In Bouwman *et al*. [167], 238 *BRCA1* VUSs distributed along the entire length of the protein were functionally characterised using three different HRR-related assays. Among the three remaining functional studies of BRCA1 recommended by CanVIG-UK, the work from Starita *et al*. 2018 [157] is weighted as strong (PS3) and moderate (BS3) evidence, while Fernandes *et al.* 2019 [165] and Petitalot *et al.* 2019 [166] are weighted as supporting evidence (both PS3 and BS3) [141]. In the wide-scale experiment performed by Starita *et al*. [157], 1056 BRCA1 missense variants in the first 192 residues of BRCA1 (including the RING domain) were assessed by a multiplex HDR assay. In Fernandes *et al.* [165], 99 missense variants located in the BRCT domain were assessed in a TA assay. In Petitalot *et al.* [166], variants located in the BRCT domain were investigated by a combination of assays measuring HRR, BRCT solubility, and phosphopeptide binding.

To supplement the already published *BRCA1* studies discussed above, and to clarify how variants in the more non-conserved parts of the protein can affect its function, there is a need for functional assays utilising the full-length protein to mimic the more native state of the BRCA1 protein. Furthermore, in addition to the limitations of not using the full-length protein and only analysing variants located in the known domains, several of the previously published studies perform assays characterising only one of the multiple functions of the BRCA1 protein. However, as the BRCA1 protein is involved in a myriad of cellular functions, performing multiple assays covering different activities of the protein are highly beneficial. In addition, some of the protein functions of BRCA1

involve several domains of the protein. As an example, HRR involves both the RING, BRCT and coiled-coiled domains [65]. Consequently, there is a need for multiple functional assays covering different activities using the full-length protein.

5.6 *BRCA1* variants selected for functional analysis in this thesis

The variants analysed in paper II and III were selected from the "*BRCA1* Norway" study (paper I). All variants were missense variants previously classified as VUSs by one or more of the Norwegian hospitals and/or reported as VUSs in the ClinVar database.

In this thesis, both the full-length BRCA1 protein and a fusion protein consisting of DBD and the BRCT domain of BRCA1 were used to functionally characterise a selection of rare germline *BRCA1* missense variants. In paper II, variants distributed along the entire *BRCA1* gene were chosen, including variants located both within and outside known functional protein domains. As discussed in section 5.5.1, several of the published functional studies of *BRCA1* have analysed only a single characteristic using an isolated domain [117, 118, 162, 169-171]. Consequently, the potential influence of intramolecular interactions in BRCA1 are not taken into account. To avoid this limitation, we therefore expressed the full-length BRCA1 protein in human cell lines. Characterising the full-length protein and not only selected domains will also generate a better molecular understanding of the whole WT BRCA1 protein, and will better mimic the native state of the protein. In paper III, the selected variants were located within or in close proximity to the BRCT domain. The reason for this was the use of a previously established TA assay, where a plasmid expressing a DBD domain fused to the BRCT domain of BRCA1 is used. In the HDR assay, the same variants were analysed using the full-length BRCA1 protein. A schematic presentation of the whole BRCA1 protein including the variants studied in paper II and the BRCT domain including the variants studied in paper III are shown in Figure 11A and B, respectively.

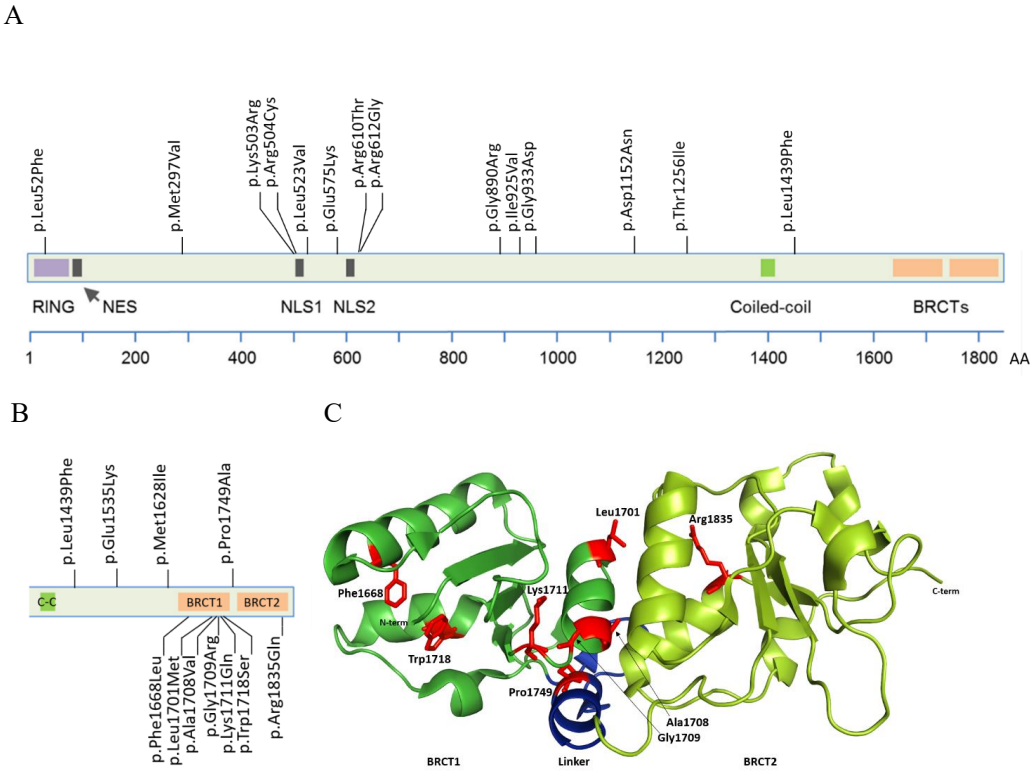

Figure 11 – Location of the studied *BRCA1* missense variants. A) Schematic presentation of BRCA1 and location of the variants analysed in paper II. Figure adapted from [65]. B) Schematic presentation of the BRCT domain and the position of the BRCA1 variants analysed in paper III. C) As in B, but shown in the three-dimensional structure of the protein. Figure created using PyMOL v1.8.4.0 (Schrödinger, LLC) according to structure data from the RCSB Protein Data Bank, PDB ID 1T15.

5.7 Specific findings of paper II

In paper II, we investigated the effect of 14 *BRCA1* missense VUSs on the full-length BRCA1 protein through multiple functional protein assays. The assays included both general protein studies analysing the protein expression levels, protein stability, and subcellular localisation, and more BRCA1 specific analysis investigating protein interactions with BARD1 and PALB2. We were especially interested in investigating how the variants outside known domains affected the protein function.

For many genes where loss-of-function is a known mechanism for disease, the protein expression level of a variant correlates to pathogenicity [111, 112]. In contrast, it has been demonstrated that BRCA1 protein variants displaying low protein levels may still sustain structure/function similar to the WT protein [165, 173]. However, these previous studies were performed by expression of isolated protein domains and not the full-length BRCA1 protein. To our knowledge, the effect of missense changes on protein expression levels of the full-length BRCA1 protein has therefore not previously been thoroughly investigated, particularly for missense variants located outside of the known domains. When analysing the effect of the selected *BRCA1* variants in HEK293FT cells, four variants (p.Leu52Phe, p.Met297Val, p.Asp1152Asn and p.Leu1439Phe) were found to have a severely reduced protein expression compared to the WT protein (<20%). Noteworthy, several of the included benign control variants also showed reduced expression levels, which is in concordance with previous BRCA1 studies and indicates that even significantly reduced BRCA1 expression levels can be sufficient to maintain BRCA1 protein functions [165, 173]. This clearly illustrates the importance of including multiple benign control variants to clarify the lower threshold of protein expression levels compatible with benignity. The expression levels of the benign controls in this study were however considerably higher than the pathogenic control variants. The lower limit of what is a sufficient amount of BRCA1 protein to avoid pathogenicity caused by lack of protein expression is not known, but previous studies have suggested that in general, only 10% of the protein molecules have to be folded for there to be no detrimental impact on the cell [174-176]. In future studies of protein expression levels of BRCA1 missense variants, additional benign and pathogenic control variants should therefore be included to better discriminate the variants and to determine the pathogenicity threshold. The protein expression levels of the variants in MDA-MB-231 cells were found to show similar trends as in the HEK293FT cells, but due to the potential effect of cellular factors in different cell types, it would also be of interest to investigate protein expression levels in other additional tissue specific cell lines. Furthermore, it has been shown that parts of the protein activity of unstable variants can be rescued by environmental factors in the cell like binding partners or

chaperones, enabling a proportion of the altered protein molecule to fold and maintain a correct functional structure [174].

Low protein expression can be caused by low transcription levels, protein instability or increased protein degradation. Although protein expression analysis alone is not adequate to distinguish between benign and pathogenic variants, protein expression analysis can thus still provide important insights regarding the underlying reasons for the loss of protein function. To achieve a better understanding of protein stability and degradation mechanisms for the variants showing deviations in the protein expression levels in the initial western blot analysis, we investigated these four variants further by qPCR and by inhibiting the ubiquitin-proteasome degradation pathway. The mRNA levels for all four variants were found to be in the same range as the WT, indicating that the mechanism underlying the low protein expression is downstream of the transcription. When investigating the four variants by blocking the ubiquitin-proteasome degradation pathway in transfected cells, clearly increased protein levels were observed for three of the four variants (p.Leu52Phe, p.Met297Val, p.Asp1152Asn). This suggests that increased ubiquitin-mediated degradation might explain the reduced expression levels, and that the small amounts of protein still detectable by western blot may represent proteins that managed to obtain a folded state and escape degradation [177]. For the remaining variant (p.Leu1439Phe), equal amounts of protein were observed, indicating that this variant is not removed by the proteasomal system.

Protein instability is an underlying mechanism for several human diseases. To further evaluate the protein stability of the BRCA1 protein variants over time, a cycloheximide chase assay was performed for the BRCA1 variants showing protein expression levels comparable to the benign controls/WT. As the MG132 assay indicated that proteasomal degradation is not the cause of the low protein levels of p.Leu1439Phe detected in HEK293FT cells, this variant was also included in the cycloheximide chase assay. Surprisingly, all benign control variants illustrated reduced protein levels (<35%) after eight hours treatment with cycloheximide, indicating that a variant could harbour pronounced reduction in stability and still be benign. This again highlights the

importance of including multiple benign controls in functional assays [178]. The benign controls were however clearly separated from the pathogenic controls (<9%), and two variants (p.Gly890Arg and p.Leu1439Phe) were found to show severely reduced protein stability similar to the pathogenic controls. This shows that these variants are less stable than the WT protein over time. Four of the VUSs (p.Lys503Arg, p.Ile925Val, p.Gly933Asp and p.Thr1256Ile) demonstrated protein stability at an intermediate level between the pathogenic and benign controls. To increase the capacity of the assay to discriminate between benign and pathogenic variants, more control variants should be included in the future. The remaining five VUSs showed protein stability comparable to the benign controls.

To maintain its normal protein functions, it is essential that the BRCA1 protein is stably present in the nucleus. It has previously been suggested that BRCA1 variants with intact NLS, but lacking a functional BRCT domain, cause the BRCA1 protein to localise primarily to the cytoplasm due to reduced BRCA1 nuclear import by a mechanism consistent with altered protein folding [65, 179]. We therefore wanted to investigate if any of the missense variants of interest in this study could have the same effect, with special interest for the four variants located in the NLS, and the variant located closest to the BRCT domain. However, we did not find evidence of any of the analysed variants affecting the nuclear localisation of the BRCA1 protein in HEK293FT cells.

The BRCA1 protein executes its different roles through interactions with a myriad of other proteins, and to date more than 100 BRCA1 interacting proteins have been reported [64]. As it is reasonable to speculate that variants showing lack of interaction can increase the pathogenicity, we wanted to investigate if any of our variants of interest affected the binding to either BARD1 or PALB2 by a Co-IP assay. BRCA1 interacts with BARD1 though the RING domain, and with PALB2 through the coiled-coil domain. Among the 14 selected VUSs analysed in this study, only one was located in the RING domain, and none in the coiled-coil domain. However, due to potential alterations of the three-dimensional structure in the folded native protein, we wanted to investigate if any of our variants could potentially alter these protein interactions

indirectly. The positive and negative control variants, which were located in the respective domains of the protein (RING and coiled-coil), showed the expected results. In the initial analysis of our study, one variant (p.Lys503Arg) appeared to have a reduced binding to PALB2, but this was shown to be caused by reduced amount of the variant protein in the input sample. Thus, none of the selected variants showed significantly reduced binding to either BARD1 or PALB2.

5.7.1 Reclassification of *BRCA1* variants in paper II

The generation of new functional data on *BRCA1* VUSs can be useful to strengthen the overall evidence needed to classify such variants. Thus, even though the functional protein assays performed in this study are not suggested as per CanVIG-UK guideline standards, our data could still provide useful information regarding the effect of variants located outside of the known protein domains of BRCA1. Following the functional assays performed in paper II, a reinterpretation of the 14 *BRCA1* missense variants of interest were therefore suggested as outlined below.

Five of the variants, of which four are located outside of domains with known protein function, illustrated severely altered properties compared to the WT protein (PS3_supporting evidence). Three of these variants were found to show increased proteasomal degradation (p.Leu52Phe, p.Met297Val and p.Asp1152Asn), while two variants showed severely reduced protein stability over time (p.Gly890Arg and p.Leu1439Phe). These findings indicate that also variants located outside the RING, BRCT and coiled-coiled domains could indeed affect the BRCA1 protein function, and that the BP1 evidence should be used with care. Due to conflicting functional data from literature or lack of evidence, all the five variants showing deviating functional effects were however still classified as VUSs. For the nine remaining VUSs, no significant effect on the BRCA1 protein expression, protein stability, subcellular localisation or BARD1/PALB2 interaction were observed, indicating a benign effect (BS3_supporting evidence). When including information on allele frequency, conservation, literature, and *in silico* predictions, seven of these variants were suggested reclassified to likely benign. For carriers of *BRCA1* VUSs, a reclassification of their variant to likely benign could

reduce unnecessary anxiety. In addition, these patients could diagnostically be tested for variants in other HBOC genes to search for an alternative explanation and underlying genetic mechanism for the suspected hereditary cancer. The functional tests performed in paper II thus added important knowledge to the classification process, resulting in reclassification and the reduction of the number of VUSs.

5.8 Specific findings of paper III

In paper III, we investigated the effect of 11 *BRCA1* variants located within or in close proximity to the BRCT domain with respect to protein expression, HRR and TA. Reduction in TA has been reported to be strongly correlated with the pathogenicity of *BRCA1* variants [152], and alterations in the HRR system are prevalent among several tumour types, like breast-, ovarian-, pancreatic- and prostate cancers [73, 180, 181]. Furthermore, the TA and HDR assays are among the functional studies recommended for the BRCA1 protein by CanVIG-UK for the use of PS3/BS3 evidence [96].

The BRCT domain of BRCA1 is highly conserved and has been shown to be crucial for both the HRR and TA activity [82, 114]. Furthermore, several missense variants in the BRCT domain have previously been shown to destabilise the folding of the protein [155]. Prior to investigating the effect of the BRCA1 variants on HRR and TA, we therefore wanted to assess their effect on protein expression levels in both the DBD-BRCT and full-length versions of the BRCA1 protein. In line with the previous findings in paper II, several of the investigated variants showed protein levels in the lower range compared to the WT protein.

The two variants p.Ala1708Val and p.Trp1718Ser were the only variants found to show both severely reduced HRR and TA activities, as well as reduced protein expression levels, indicating a pathogenic effect. Previous functional studies (TA and HDR among others) of p.Ala1708Val have however revealed several conflicting results, complicating the interpretation of this variant [117, 164, 166, 169, 182]. It has also been suggested that p.Ala1708Val may act as a low or moderate disease risk allele [169, 182, 183]. The p.Trp1718Ser variant has to our knowledge not previously been analysed by

TA or HDR, but has shown altered BRCA1 function in a saturation genome editing assay, supporting our findings [164].

The variant p.Pro1749Ala was the only one among the 11 analysed *BRCA1* variants found to have activity levels similar to the benign controls in both the HDR, TA and protein expression assays. This variant is located in the linker region between the two BRCT repeats of the BRCT domain, which might explain why this variant affects neither the TA activity nor the HRR capacity of BRCA1. Our results are in concordance with previous studies of other variants in the linker region, which might indicate that this region is more tolerable for alterations than the two adjacent BRCT repeats [164, 173].

The eight remaining variants showed severely reduced capacity in HRR, but TA activity in the range of WT/benign controls. This highlights the importance of comparing results obtained from several different functional assays, as a variant can affect either one or several of the protein activities. A potential explanation for variants being functional in TA, but not in HRR, can be that these variants, directly or indirectly through conformational changes, alter the binding sites that are crucial for HRR, but not for TA. Recruitment of multiple proteins to the sites of DNA damage along with BRCA1 is essential for HRR, and this is achieved through cascades of protein interactions and formation of macro-complexes. An alternative explanation could be differences in the folding between the full-length and the DBD-BRCT fusion protein, where the potential structural changes induced by the variant could alter binding sites necessary for only some of the downstream functions of BRCA1 [184-186]. In addition, some variants located in the BRCT domain have been shown to retain the BRCA1 protein in the cytoplasm, hampering its transition to the nucleus and preventing binding to damaged DNA [179, 187]. This could potentially explain why some of our variants resulted in loss of HRR function, even if they showed protein expression levels comparable to the WT protein. As the HDR assay is performed using living cells, the function of the HRR pathway is dependent on the BRCA1 protein being present in the nucleus to access the

damaged DNA. The TA assay however, is performed ex vivo on lysed cells, and is thus in contrast to the HDR assay not dependent on correct subcellular localisation.

Three of the above-mentioned eight variants (p.Phe1668Leu, p.Gly1709Gly and p.Lys1711Gln) showed both a significantly lower protein expression than the WT protein and had significantly reduced HRR capacity, but exhibited TA capacity comparable to WT BRCA1 and/or benign controls. This indicates that even significantly reduced BRCA1 expression levels are sufficient to perform TA at similar levels as the WT protein, and that protein expression levels do not necessarily correlate to the level of protein activity. The findings are in concordance with previous studies [173]. Likewise, one of the benign controls (p.Arg1751Gln) in the TA assay showed considerably reduced TA activity compared to the WT protein (<50%), which indicates that some variants might harbour a clearly reduced TA activity and still be (likely) benign. In contrast, the benign controls in the HDR assay all showed activity levels >93% relative to the WT. Apparently, for benign variants, less deviation from the WT activity level is tolerated in the HDR assay compared to the TA assay.

5.8.1 Reclassification of *BRCA1* variants in paper III

Following the functional assays performed in paper III, reinterpretation of the 11 *BRCA1* missense variants of interest were performed. For one of the variants, p.Pro1749Ala, no significant effect on the BRCA1 protein expression, TA or HRR were observed, indicating a functional benign effect (BS3_supporting evidence). However, p.Pro1749 is a highly conserved amino acid located in a well-established functional domain (PM1_supporting evidence), and *in silico* tools predicted a damaging effect (PP3 evidence). The variant was therefore still classified as a VUS. The remaining ten variants all showed reduced TA and/or HRR compared to the WT protein (PS3_supporting evidence). However, due to either lack of sufficient additional evidence enabling reclassification or conflicting results from functional assays in literature, all but one of the variants (p.Trp1718Ser) were still classified as VUSs. The p.Trp1718Ser were reclassified to likely pathogenic due to additional evidence in the literature combined with our functional data.

Ideally, a higher number of control variants should have been included in all assays of this thesis to better estimate cut-off values to differentiate between benign and pathogenic variants, and to potentially reclassify variants by increasing the strength of the functional evidence. In total, six control variants in the HDR assay and eight control variants in the TA assay were included. However, according to Brnich *et al*. [188], a minimum of 11 control variants in total should be included to reach BS3/PS3 evidence at moderate strength. Including 11 control variants in each assay would be highly time consuming and would require high-throughput analysis methods, and this was therefore not possible to achieve within the time frame of the project. Establishing such high-throughput methods is generally hard to achieve for laboratories of small to medium capacity. In addition, the recommendations from Brnich *et al*. [188] were published in 2019, simultaneously as this project was initiated. Following the four-step process recommended by Brnich *et al*. to determine the strength of the PS3/BS3 evidence during interpretation of variants, we therefore applied PS3/BS3 as supportive strength in both paper II and III. If there were evidence for both pathogenicity and benignity when combining the total CanVIG-UK criteria for a variant, the recommendations from Garrett et al. were applied [189]. In cases with conflicting evidence (both pathogenic and benign direction), increasing the strength of the functional criteria would consequently not necessarily increase the total evidence sufficiently to reclassify a VUS to (likely) benign or (likely) pathogenic, as observed for several variants in paper III.

5.9 Conflicting evidence and challenges of interpreting *BRCA1* VUSs

When comparing the newly achieved functional data in this study with functional data described in the literature, several variants from both paper II and paper III were found to have discordant results. Discordant results can be caused by among others experimental errors in one or several of the assays (e.g. sample swapping or technical errors), differences in threshold values set for benignity and pathogenicity, or that only some of the tested functions of BRCA1 are affected by a given variant [116].

In all cases where there were conflicts between the newly achieved functional data in this study and the data in any of the five functional BRCA1 protein studies suggested by the CanVIG-UK *BRCA1* specific guideline for variant interpretation, we chose not to include the functional evidence criteria (PS3) when classifying the variants [96, 157, 164-167]. In cases where our variants of interest were not investigated in any of the CanVIG-UK recommended studies, we chose to apply the functional criteria PS3 or BS3 as supportive strength, as the assays did not include sufficient control variants to increase the strength of the functional evidence to moderate. When our results were in concordance with the CanVIG-UK recommended studies, we applied the functional criteria as strong strength [96, 157, 164-167].

In addition, discordant results between clinical information and results from functional assays might be an indicator of moderate penetrance [189]. The total cancer risk is also affected by polygenic risk scores (PRS), which are a result of individuals being carriers of multiple common breast cancer susceptibility variants which confer a low cancer risk individually, but have a substantial cancer risk when combined [190].

5.9.1 Challenges of interpreting *BRCA1* variants located outside known protein domain

As the majority of variants in paper II were reclassified to likely benign, one might speculate that the regions outside of the RING and BRCT domains of BRCA1 seems to be less vulnerable to alterations, in line with the BP1 coldspot evidence. However, four of the five variants showing severe deviations from the WT protein (p. Met297Val, Gly890Arg, Asp1152Asn and Leu1439Phe) are located outside protein domains of known functions. Furthermore, in paper III, all variants except the one located in the linker region between the two repeats of the BRCT domain (p.Pro1749Ala) appeared to be non-functional in HRR. This included three variants (p.Leu1439Phe, p.Glu1535Lys and p.Met1628Ile) upstream of the BRCT domain borders (1650-1863 aa) specified for the BP1 evidence in the gene-specific *BRCA1* criteria from CanVIG-UK [96]. These findings show that also variants located outside the RING, BRCT and coiled-coiled domains could affect the BRCA1 protein function and that the BP1 evidence should be used with care.

In contrast to the BP1 evidence that can be assigned to all missense variants located outside known protein domains in BRCA1, the PM1 evidence can be used for all missense variants located within the BRCA1 RING (aa 1-101), BRCT (aa 1650-1863) and coiled-coil (aa 1391-1424) domain according to the *BRCA1* specific criteria from CanVIG-UK [96]. This PM1 criteria is therefore valid for the p.Pro1749Ala variant although it is located in the linker region between the two BRCT repeats in the BRCT domain. Despite fulfilling the BS3_strong functional evidence, p.Pro1749Ala cannot be classified as likely benign among other due to the conflicting PM1 evidence, which is assigned purely on the location within the BRCT domain.

The only *BRCA1* variant investigated in both paper II and III was p.Leu1439Phe. This variant is located close to, but not within, the coiled-coil domain (1391-1424 aa) which is included in the BP1 evidence in the gene specific *BRCA1* criteria from CanVIG-UK [96]. The variant was shown to have a reduced protein stability in paper II and reduced HRR activity in paper III. Noteworthy, the variant was shown to be expressed at low protein expression levels in HEK293FT cells in paper II (7%), but at protein levels comparable to the benign control variants in HEK293FT cells in paper III (64% from full-length protein). The protein expression assays were performed according to the same protocol in both papers, but the BRCA1 protein variant was expressed using different plasmids (*mCherry-BRCA1* and *His-BRCA1*, respectively), which indicates that the choice of protein tags might affect the expression levels. This again highlights the importance of including multiple control variants, and using the same cell line and tag/fusion protein in the control variants as the variants of interest.

Altogether, the findings of missense variants in BRCA1 outside the known protein domains with increased proteasomal degradation (p.Met297Val and p.Asp1152Asn), reduced stability (p.Gly890Arg and p.Leu1439Phe) and impaired HRR capacity (p.Leu1439Phe, p.Glu1535Lys and p.Met1628Ile) suggests that not all missense variants located outside established protein domains of BRCA1 are (likely) benign. Previously, it has been suggested that the central region of BRCA1 acts as a long flexible

scaffold for intermolecular interactions even though it lacks substantial conserved motifs, which might explain how missense variants outside of known protein domains can affect the pathogenicity of BRCA1 [64, 141, 191]. Although being a disordered region in the absence of its binding partners, such intrinsically non-conserved disordered regions are known to obtain a more folded structure upon interaction with its protein partners [137-141]. In addition to BRCA1, many other important proteins such as TP53, MYC and BRCA2 contain highly disordered regions, which further supports the fact that investigating disordered regions is important for understanding the molecular basis of human diseases [192-194]. In the BRCA2 protein, the disordered regions have been shown to recruit kinases, phosphatases and other proteins involved in maintaining genome integrity, and to be involved in BRCA2 oligomerisation, which could potentially regulate several other protein functions indirectly [195].

6. Concluding remarks

Paper I provides an overview of the Norwegian *BRCA1* variant spectrum and has laid the foundation for improved collaboration between the laboratories at a national level. The collaboration has led to an increased consensus regarding variant classification, and establishment of a forum for discussions. This has greatly improved the availability of information for variant interpretation and hence increased the accuracy of the overall cancer risk assessment.

In paper II and III, we have analysed the effect of several *BRCA1* missense variants classified as VUSs. The functional assays performed in this project provided new knowledge which contributed to reclassification of seven *BRCA1* variants from VUS to likely benign, and one variant from VUS to likely pathogenic. Further studies are needed to support the final classification of the remaining VUSs.

Furthermore, our findings indicate that also variants located outside the RING, BRCT and coiled-coiled domains could affect the BRCA1 protein, and that the CanVIG-UK BP1 criteria should be used with care. In addition, we have demonstrated that several of the investigated variants affected only one of the assessed activities of the BRCA1 protein, and we therefore highlight the importance of combining several different functional assays when assessing the effects of rare *BRCA1* variants.

To summarise, we have established a set of assays for functional testing of BRCA1 protein variants. Ideally, functional assays in a diagnostic setting should be relatively rapid and efficient to guide the interpretation of variants within reasonable time. While the protein expression analysis are not optimal, both the TA and HDR assays performed with sufficient number of controls could be considered for use in a diagnostic testing pipeline as supplement during variant interpretation.

7. Future aspects

Paper I focus on sharing *BRCA1* variant interpretation data between the different Norwegian hospitals. The results clearly shows the benefit of national collaboration, and data sharing should continue to be a high priority for the laboratories. Importantly, a future aim is to extend the collaboration to include variant interpretation of several other cancer genes like *BRCA2* and the *MMR* genes in near future. Furthermore, the scientific community in Norway has expressed a desire for establishing a common variant database to increase sharing of data regarding variant interpretation between the diagnostic genetic laboratories, and this is currently in progress.

Although our functional studies in paper II and III contributed to clarifying the pathogenicity of eight rare missense *BRCA1* variants, the clinical significance of the remaining VUSs is still unclear. Therefore, it would be interesting to analyse these VUSs in further studies to gather evidence to further support the classification of these variants. One important future task would be to investigate all remaining VUSs from paper II by the HDR assay. As BRCA1 interacts with a myriad of protein partners, another interesting follow-up study would be to investigate the variants' effect on interaction with additional binding partners of BRCA1 like BRIP1 and CHK2. It would also be of interest to confirm the subcellular localisation results from the fractionation assay in paper II by immunofluorescence imaging, and to perform similar subcellular localisation assays on the variants from paper III to further investigate the causes for differences between the HDR and TA assays.

Another important aspect of this thesis was the use of the full-length BRCA1 protein. The TA assay in paper III was performed using fusion protein consisting of DBD and the BRCA1 BRCT domain only. To better mimic the native state of the protein, it would thus be highly interesting to investigate the TA activity using the full-length BRCA1 protein. Furthermore, by performing TA assay with a plasmid encoding the full-length protein fused to the DBD domain, this opens new possibilities to include variants located

outside of the BRCT domain. This would also enable testing all variants in paper II in the TA assay.

Furthermore, it would be highly interesting to confirm our results using a tissue-relevant (breast or ovarian cancer) cell line. In the initial pilot experiments of paper II, several attempts were made to express BRCA1 in several breast cancer cell lines (MDA-MD-231, MCF7 and T47D). However, HEK293FT cells showed much higher expression levels and was chosen as the main cell line. In the future, it would be relevant to optimise the transfection protocol in order to increase the BRCA1 protein expression in the abovementioned cell lines, and subsequently repeat all experiments in a tissue relevant cell line. This would however be highly time consuming, so alternatively only variants showing deviations from the WT in any of the included functional assays in HEK293FT cells, could be tested in additional cancer cell lines.

References

1. Kumar, V., et al., *Robbins basic pathology*. 9th ed. ed. Basic pathology. 2013, Philadelphia, Pa: Elsevier/Saunders.
2. Hanahan, D. and Robert A. Weinberg, *Hallmarks of Cancer: The Next Generation.* Cell, 2011. **144**(5): p. 646-674.
3. Hanahan, D., *Hallmarks of Cancer: New Dimensions.* Cancer Discov, 2022. **12**(1): p. 31-46.
4. *Cancer today, breast (Globocan 2020)*. Available from: https://gco.iarc.fr/today/data/factsheets/cancers/20-Breast-fact-sheet.pdf.
5. *Cancer today, ovary (Globocan 2020)*. Available from: https://gco.iarc.fr/today/data/factsheets/cancers/25-Ovary-fact-sheet.pdf.
6. Sung, H., et al., *Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries.* CA Cancer J Clin, 2021. **71**(3): p. 209-249.
7. *Cancer in Norway 2021, The Cancer Registry of Norway*. [cited 2022 12]; December]. Available from: https://www.kreftregisteret.no/globalassets/cancer-in-norway/2021/cin_report.pdf.
8. Korf, B.R. and M.B. Irons, *Human genetics and genomics*. 4th ed. ed. 2013, Chichester: Wiley-Blackwell.
9. Brandt, A., et al., *Age of onset in familial cancer.* Ann Oncol, 2008. **19**(12): p. 2084-2088.
10. Nagy, R., K. Sweet, and C. Eng, *Highly penetrant hereditary cancer syndromes.* Oncogene, 2004. **23**(38): p. 6445-6470.
11. Robinson, L.S., et al., *Prediction of Cancer Prevention: From Mammogram Screening to Identification of BRCA1/2 Mutation Carriers in Underserved Populations.* EBioMedicine, 2015. **2**(11): p. 1827-1833.
12. Garber, J.E. and K. Offit, *Hereditary Cancer Predisposition Syndromes.* J Clin Oncol, 2005. **23**(2): p. 276-292.
13. Dorling, L.., et al., *Breast Cancer Risk Genes — Association Analysis in More than 113,000 Women.* N Engl J Med, 2021. **384**(5): p. 428-439.
14. Keller, G., et al., *Diffuse Type Gastric and Lobular Breast Carcinoma in a Familial Gastric Cancer Patient with an E-Cadherin Germline Mutation.* Am J Pathol, 1999. **155**(2): p. 337-342.
15. Liaw, D., et al., *Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome.* Nat Genet, 1997. **16**(1): p. 64-67.
16. Lim, W., et al., *Further observations on LKB1/STK11 status and cancer risk in Peutz-Jeghers syndrome.* Br J Cancer, 2003. **89**(2): p. 308-313.
17. Malkin, D., et al., *Germ Line p53 Mutations in a Familial Syndrome of Breast Cancer, Sarcomas, and Other Neoplasms.* Science, 1990. **250**(4985): p. 1233-1238.
18. Antoniou, A.C., et al., *Breast-Cancer Risk in Families with Mutations in PALB2.* N Engl J Med, 2014. **371**(6): p. 497-506.
19. Dorling, L., et al., *Breast cancer risks associated with missense variants in breast cancer susceptibility genes.* Genome Med, 2022. **14**(1): p. 51-51.

20. Easton, D.F., et al., *Gene-Panel Sequencing and the Prediction of Breast-Cancer Risk.* N Engl J Med, 2015. **372**(23): p. 2243-2257.
21. Couch, F.J., et al., *Associations Between Cancer Predisposition Testing Panel Genes and Breast Cancer.* JAMA Oncol, 2017. **3**(9): p. 1190-1196.
22. Hu, C., et al., *A Population-Based Study of Genes Previously Implicated in Breast Cancer.* N Engl J Med, 2021. **384**(5): p. 440-451.
23. Song, H., et al., *The contribution of deleterious germline mutations in BRCA1, BRCA2 and the mismatch repair genes to ovarian cancer in the population.* Hum Mol Genet, 2014. **23**(17): p. 4703-4709.
24. Aarnio, M., et al., *Cancer risk in mutation carriers of DNA-mismatch-repair genes.* International journal of cancer, 1999. **81**(2): p. 214-218.
25. Walsh, T., et al., *Mutations in 12 genes for inherited ovarian, fallopian tube, and peritoneal carcinoma identified by massively parallel sequencing.* Proc Natl Acad Sci U S A, 2011. **108**(44): p. 18032-18037.
26. Taylor, A., et al., *Consensus for genes to be included on cancer panel tests offered by UK genetics services: guidelines of the UK Cancer Genetics Group.* Journal of Medical Genetics, 2018. **55**(6): p. 372-377.
27. Song, H., et al., *Contribution of Germline Mutations in the RAD51B, RAD51C, and RAD51D Genes to Ovarian Cancer in the Population.* J Clin Oncol, 2015. **33**(26): p. 2901-2907.
28. Loveday, C., et al., *Germline RAD51C mutations confer susceptibility to ovarian cancer.* Nat Genet, 2012. **44**(5): p. 475-476.
29. Meindl, A., et al., *Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene.* Nature genetics, 2010. **42**(5): p. 410-414.
30. Rafnar, T., et al., *Mutations in BRIP1 confer high risk of ovarian cancer.* Nat Genet, 2011. **43**(11): p. 1104-1107.
31. Liu, Y.L., et al., *Risk-Reducing Bilateral Salpingo-Oophorectomy for Ovarian Cancer: A Review and Clinical Guide for Hereditary Predisposition Genes.* JCO Oncol Pract, 2022. **18**(3): p. 201-209.
32. Ford, D., et al., *Risks of cancer in BRCA1-mutation carriers.* The Lancet (British edition), 1994. **343**(8899): p. 692-695.
33. Miki, Y., et al., *A Strong Candidate for the Breast and Ovarian Cancer Susceptibility Gene BRCA1.* Science, 1994. **266**(5182): p. 66-71.
34. Hall, J.M., et al., *Linkage of Early-Onset Familial Breast Cancer to Chromosome 17q21.* Science, 1990. **250**(4988): p. 1684-1689.
35. Wooster, R., et al., *Identification of the breast cancer susceptibility gene BRCA2.* Nature, 1995. **378**(6559): p. 789-792.
36. Kuchenbaecker, K.B., et al., *Risks of Breast, Ovarian, and Contralateral Breast Cancer for BRCA1 and BRCA2 Mutation Carriers.* JAMA, 2017. **317**(23): p. 2402-2416.
37. Metcalfe, K., et al., *Predictors of contralateral breast cancer in BRCA1 and BRCA2 mutation carriers.* Br J Cancer, 2011. **104**(9): p. 1384-1392.
38. Mavaddat, N., et al., *Pathology of breast and ovarian cancers among BRCA1 and BRCA2 mutation carriers: results from the Consortium of Investigators of*

Modifiers of BRCA1/2 (CIMBA). Cancer Epidemiol Biomarkers Prev, 2012. **21**(1): p. 134-147.
39. Li, S., et al., *Cancer Risks Associated With BRCA1 and BRCA2 Pathogenic Variants.* J Clin Oncol, 2022. **40**(14): p. 1529-1541.
40. Lee, E., et al., *Characteristics of Triple-Negative Breast Cancer in Patients With a BRCA1 Mutation: Results From a Population-Based Study of Young Women.* J Clin Oncol, 2011. **29**(33): p. 4373-4380.
41. Couch, F.J., 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): p. 3419-3419.
42. Mavaddat, N., et al., *Pathology of Tumors Associated With Pathogenic Germline Variants in 9 Breast Cancer Susceptibility Genes.* JAMA Oncol, 2022. **8**(3): p. e216744-e216744.
43. Harbeck, N., et al., *Breast cancer.* Nature reviews. Disease primers, 2019. **5**(1).
44. *Nasjonalt handlingsprogram med retningslinjer for diagnostikk, behandling og oppfølging av pasienter med brystkreft (Helsedirektoratet)*. [cited 2022 April 27]; Available from: https://www.helsebiblioteket.no/retningslinjer/brystkreft/arvelig-brystkreft/utredning-for-arvelig-brystkreft
45. Kim, G., et al., *FDA Approval Summary: Olaparib Monotherapy in Patients with Deleterious Germline BRCA-Mutated Advanced Ovarian Cancer Treated with Three or More Lines of Chemotherapy.* Clin Cancer Res, 2015. **21**(19): p. 4257-4261.
46. Ledermann, J., et al., *Olaparib Maintenance Therapy in Platinum-Sensitive Relapsed Ovarian Cancer.* N Engl J Med, 2012. **366**(15): p. 1382-1392.
47. Robson, M., et al., *Olaparib for Metastatic Breast Cancer in Patients with a Germline BRCA Mutation.* N Engl J Med, 2017. **377**(6): p. 523-533.
48. Kaur, S.D., et al., *Recent advances in cancer therapy using PARP inhibitors.* Medical oncology (Northwood, London, England), 2022. **39**(12): p. 241-241.
49. Kaufman, B., et al., *Olaparib Monotherapy in Patients With Advanced Cancer and a Germline BRCA1/2 Mutation.* J Clin Oncol, 2015. **33**(3): p. 244-250.
50. Lee, J.m., J.A. Ledermann, and E.C. Kohn, *PARP Inhibitors for BRCA1/2 mutation-associated and BRCA-like malignancies.* Ann Oncol, 2014. **25**(1): p. 32-40.
51. *BioRender.com*. [cited 2022 November 05]; Available from: https://app.biorender.com/biorender-templates.
52. Savage, K. and D.P. Harkin, *BRCA1 and BRCA2: Role in the DNA Damage Response, Cancer Formation and Treatment*. 2009, Dordrecht: Springer Netherlands: Dordrecht. p. 415-443.
53. Baer, R. and T. Ludwig, *The BRCA1/BARD1 heterodimer, a tumor suppressor complex with ubiquitin E3 ligase activity.* Curr Opin Genet Dev, 2002. **12**(1): p. 86-91.
54. Mallery, D.L., C.J. Vandenberg, and K. Hiom, *Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains.* EMBO J, 2002. **21**(24): p. 6755-6762.

55. Xia, Y., et al., *Enhancement of BRCA1 E3 Ubiquitin Ligase Activity through Direct Interaction with the BARD1 Protein.* J Biol Chem, 2003. **278**(7): p. 5255-5263.
56. Clark, S.L., et al., *Structure-Function Of The Tumor Suppressor BRCA1.* Comput Struct Biotechnol J, 2012. **1**(1): p. e201204005.
57. Rodríguez, J.A. and B.R. Henderson, *Identification of a Functional Nuclear Export Sequence in BRCA1.* J Biol Chem, 2000. **275**(49): p. 38589-38596.
58. De Siervi, A., et al., *Transcriptional Autoregulation by BRCA1.* Cancer Res, 2010. **70**(2): p. 532-542.
59. Roy, R., J. Chun, and S.N. Powell, *BRCA1 and BRCA2: different roles in a common pathway of genome protection.* Nat Rev Cancer, 2012. **12**(1): p. 68-78.
60. Joo, W.S., et al., *Structure of the 53BP1 BRCT region bound to p53 and its comparison to the Brca1 BRCT structure.* Genes Dev, 2002. **16**(5): p. 583-593.
61. Leung, C.C.Y. and J.N.M. Glover, *BRCT domains: Easy as one, two, three.* Cell Cycle, 2011. **10**(15): p. 2461-2470.
62. Venkitaraman, A.R., *Cancer Suppression by the Chromosome Custodians, BRCA1 and BRCA2.* Science, 2014. **343**(6178): p. 1470-1475.
63. Dunker, A.K., et al., *Flexible nets. The roles of intrinsic disorder in protein interaction networks.* FEBS J, 2005. **272**(20): p. 5129-5148.
64. Savage, K.I. and D.P. Harkin, *BRCA1, a 'complex' protein involved in the maintenance of genomic stability.* FEBS J, 2015. **282**(4): p. 630-646.
65. Anantha, R.W., et al., *Functional and mutational landscapes of BRCA1 for homology-directed repair and therapy resistance.* Elife, 2017. **6**.
66. Savage, Kienan I., et al., *Identification of a BRCA1-mRNA Splicing Complex Required for Efficient DNA Repair and Maintenance of Genomic Stability.* Mol Cell, 2014. **54**(3): p. 445-459.
67. Weinberg, R.A., *The biology of cancer*. 2nd ed. ed. 2014, New York: Garland Science.
68. Monteiro, A.N.A., A. August, and H. Hanafusa, *Evidence for a Transcriptional Activation Function of BRCA1 C-Terminal Region.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13595-13599.
69. Bakkan, S.M.E. *Functional analysis of BRCA1 variants of uncertain significance (VUS)*. [cited 2022 September 08]; Available from: https://bora.uib.no/bora-xmlui/bitstream/handle/11250/3000140/FinalMasterThesis-01-06-Last.pdf?sequence=1&isAllowed=y.
70. Jasin, M. and R. Rothstein, *Repair of Strand Breaks by Homologous Recombination.* Cold Spring Harb Perspect Biol, 2013. **5**(11): p. a012740-a012740.
71. Panier, S. and D. Durocher, *Push back to respond better: regulatory inhibition of the DNA double-strand break response.* Nat Rev Mol Cell Biol, 2013. **14**(10): p. 661-672.
72. Orthwein, A., et al., *A mechanism for the suppression of homologous recombination in G1 cells.* Nature, 2015. **528**(7582): p. 422-426.

73. Vilma, P.-B., et al., *The Homologous Recombination Deficiency Scar in Advanced Cancer: Agnostic Targeting of Damaged DNA Repair.* Cancers, 2022. **14**(12): p. 2950.
74. Cortez, D., et al., *Requirement of ATM-Dependent Phosphorylation of Brca1 in the DNA Damage Response to Double-Strand Breaks.* Science, 1999. **286**(5442): p. 1162-1166.
75. Tibbetts, R.S., et al., *Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress.* Genes Dev, 2000. **14**(23): p. 2989-3002.
76. Chung, J.H., et al., *hCds1-mediated phosphorylation of BRCA1 regulates the DNA damage response.* Nature, 2000. **404**(6774): p. 201-204.
77. Chen, J., *Ataxia telangiectasia-related protein is involved in the phosphorylation of BRCA1 following deoxyribonucleic acid damage.* Cancer Res, 2000. **60**(18): p. 5037-5039.
78. Zhang, F., et al., *PALB2 Links BRCA1 and BRCA2 in the DNA-Damage Response.* Curr Biol, 2009. **19**(6): p. 524-529.
79. Sy, S.M.H., M.S.Y. Huen, and J. Chen, *PALB2 Is an Integral Component of the BRCA Complex Required for Homologous Recombination Repair.* Proc Natl Acad Sci U S A, 2009. **106**(17): p. 7155-7160.
80. Bhattacharyya, A., et al., *The Breast Cancer Susceptibility Gene BRCA1 Is Required for Subnuclear Assembly of Rad51 and Survival following Treatment with the DNA Cross-linking Agent Cisplatin.* J Biol Chem, 2000. **275**(31): p. 23899-23903.
81. Yamamoto, H. and A. Hirasawa, *Homologous Recombination Deficiencies and Hereditary Tumors.* Int J Mol Sci, 2021. **23**(1): p. 348.
82. Chapman, M.S. and I.M. Verma, *Transcriptional activation by BRCA1.* Nature, 1996. **382**(6593): p. 678-679.
83. Pascal, J.M., *The comings and goings of PARP-1 in response to DNA damage.* DNA Repair (Amst), 2018. **71**: p. 177-182.
84. Valencia, O.M., et al., *The Role of Genetic Testing in Patients With Breast Cancer: A Review.* JAMA Surg, 2017. **152**(6): p. 589-594.
85. Gadzicki, D., et al., *Genetic testing for familial/hereditary breast cancer—comparison of guidelines and recommendations from the UK, France, the Netherlands and Germany.* J Community Genet, 2011. **2**(2): p. 53-69.
86. Marmolejo, D.H., et al., *Overview of hereditary breast and ovarian cancer (HBOC) guidelines across Europe.* Eur J Med Genet, 2021. **64**(12): p. 104350.
87. *ClinVar database*. [cited 2022 March 17]; Available from: https://www.ncbi.nlm.nih.gov/clinvar.
88. Heramb, C., et al., *BRCA1 and BRCA2 mutation spectrum - an update on mutation distribution in a large cancer genetics clinic in Norway.* Hered Cancer Clin Pract, 2018. **16**(1): p. 3-3.
89. Møller, P., et al., *The BRCA1 syndrome and other inherited breast or breast–ovarian cancers in a Norwegian prospective series.* Eur J Cancer, 2001. **37**(8): p. 1027-1032.
90. Corso, G., et al., *BRCA1/2 germline missense mutations: a systematic review.* Eur J Cancer Prev, 2018. **27**(3): p. 279-286.

91. Wu, Q., et al., *Structure of BRCA1-BRCT/Abraxas Complex Reveals Phosphorylation-Dependent BRCT Dimerization at DNA Damage Sites.* Mol Cell, 2016. **61**(3): p. 434-448.
92. Ruffner, H., et al., *Cancer-Predisposing Mutations within the RING Domain of BRCA1: Loss of Ubiquitin Protein Ligase Activity and Protection from Radiation Hypersensitivity.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5134-5139.
93. Plon, S.E., et al., *Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results.* Hum. Mutat, 2008. **29**(11): p. 1282-1291.
94. Richards, S., et al., *Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.* Genet Med, 2015. **17**(5): p. 405-424.
95. *Evidence-based Network for the Interpretation of Germline Mutant Alleles*. [cited 2022 January 24]; Available from: https://enigmaconsortium.org/library/general-documents/enigma-classification-criteria/.
96. *CanVIG-UK Gene Specific Recommendations: BRCA1/BRCA2 (v 1.16)*. [cited 2022 June 01]; Available from: https://www.cangene-canvaruk.org/gene-specific-recommendations.
97. *CanVIG-UK Consensus Specification for Cancer Susceptibility Genes ACGS Best Practice Guidelines for Variant Classification (v2.16)*. [cited 2022 June 01]; Available from: https://www.cangene-canvaruk.org/canvig-uk-guidance.
98. *The Human Gene Mutation Database (HGMD). Institute of Medical Genetics, Cardiff.* [cited 2022 July 18]; Available from: https://www.hgmd.cf.ac.uk/ac/index.php.
99. Karczewski, K.J., et al., *The mutational constraint spectrum quantified from variation in 141,456 humans.* Nature, 2020. **581**(7809): p. 434-443.
100. *BRCA Exchange. Global Alliance for Genomics and Health*. [cited 2022 August 01]; Available from: https://brcaexchange.org/.
101. O'Leary, E., et al., *Expanded Gene Panel Use for Women With Breast Cancer: Identification and Intervention Beyond Breast Cancer Risk.* Ann Surg Oncol, 2017. **24**(10): p. 3060-3066.
102. Beitsch, P.D., et al., *Underdiagnosis of Hereditary Breast Cancer: Are Genetic Testing Guidelines a Tool or an Obstacle?* J Clin Oncol, 2019. **37**(6): p. 453-460.
103. Kurian, A.W., et al., *Uptake, Results, and Outcomes of Germline Multiple-Gene Sequencing After Diagnosis of Breast Cancer.* JAMA Oncol, 2018. **4**(8): p. 1066-1072.
104. Chang, J., et al., *Clinical Management of Patients at Risk for Hereditary Breast Cancer with Variants of Uncertain Significance in the Era of Multigene Panel Testing.* Ann Surg Oncol, 2019. **26**(10): p. 3389-3396.
105. Yue, P., Z. Li, and J. Moult, *Loss of Protein Structure Stability as a Major Causative Factor in Monogenic Disease.* J Mol Biol, 2005. **353**(2): p. 459-473.

106. Lindor, N.M., et al., *BRCA1/2 Sequence Variants of Uncertain Significance: A Primer for Providers to Assist in Discussions and in Medical Management.* Oncologist, 2013. **18**(5): p. 518-524.
107. Sian Ellard, E.L.B., et al. *ACGS Best Practice Guidelines for Variant Classification in*

Rare Disease 2020. Available from: https://www.acgs.uk.com/media/11631/uk-practice-guidelines-for-variant-classification-v4-01-2020.pdf.
108. Vears, D.F., et al., *Analysis of VUS reporting, variant reinterpretation and recontact policies in clinical genomic sequencing consent forms.* 2018.
109. Storvik, A.G. *21 kvinner fikk fjernet bryst og eggstokker – skulle ikke vært operert*. [cited 2022 January 30]; Available from: https://www.dagensmedisin.no/artikler/2017/02/17/21-kvinner-fikk-fjernet-bryst-og-eggstokker--skulle-ikke-vart-operert/.
110. Møller, P. and E. Hovig, *Retraction Note to: The BRCA2 variant c.68-7 T > A is associated with breast cancer.* Hered Cancer Clin Pract, 2018. **16**(1): p. 10-10.
111. Althari, S., et al., *Unsupervised Clustering of Missense Variants in HNF1A Using Multidimensional Functional Data Aids Clinical Interpretation.* Am J Hum Genet, 2020. **107**(4): p. 670-682.
112. Malikova, J., et al., *Functional analyses of HNF1A-MODY variants refine the interpretation of identified sequence variants.* J Clin Endocrinol Metab, 2020. **105**(4): p. e1377-e1386.
113. Ransburgh, D.J.R.D.J.R., et al., *The effect of BRCA1 missense mutations on homology directed recombination.* Cancer research (Chicago, Ill.), 2010. **70**(3): p. 988-995.
114. Monteiro, A.N., A. August, and H. Hanafusa, *Evidence for a Transcriptional Activation Function of BRCA1 C-Terminal Region.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13595-13599.
115. Monteiro, A.N.A., A. August, and H. Hanafusa, *Common BRCA1 Variants and Transcriptional Activation.* Am J Hum Genet, 1997. **61**(3): p. 761-762.
116. Lyra, J.P.C.M., et al., *Integration of functional assay data results provides strong evidence for classification of hundreds of BRCA1 variants of uncertain significance.* Genet Med, 2021. **23**(2): p. 306-315.
117. Langerud, J., et al., *Trans-activation-based risk assessment of BRCA1 BRCT variants with unknown clinical significance.* Hum Genomics, 2018. **12**(1): p. 51-51.
118. Jarhelle, E., et al., *Characterization of BRCA1 and BRCA2 variants found in a Norwegian breast or ovarian cancer cohort.* Fam Cancer, 2016. **16**(1): p. 1-16.
119. Møller, P., et al., *Causes for Frequent Pathogenic BRCA1 Variants Include Low Penetrance in Fertile Ages, Recurrent De-Novo Mutations and Genetic Drift.* Cancers (Basel), 2019. **11**(2): p. 132.
120. Møller, P., et al., *Genetic epidemiology of BRCA1 mutations in Norway.* Eur J Cancer, 2001. **37**(18): p. 2428-2434.
121. *Høringsforslag behandlingsrettet helseregister med tolkede genetiske varianter*. [cited 2022 August 01]; Available from:

https://www.regjeringen.no/no/dokumenter/prop.-112-l-20202021/id2839981/?ch=8.
122. Lebo, M.S., et al., *Data sharing as a national quality improvement program: reporting on BRCA1 and BRCA2 variant-interpretation comparisons through the Canadian Open Genetics Repository (COGR).* Genet Med, 2018. **20**(3): p. 294-302.
123. Balmaña, J., et al., *Conflicting Interpretation of Genetic Variants and Cancer Risk by Commercial Laboratories as Assessed by the Prospective Registry of Multiplex Testing.* J Clin Oncol, 2016. **34**(34): p. 4071-4078.
124. Amendola, L.M., et al., *Performance of ACMG-AMP Variant-Interpretation Guidelines among Nine Laboratories in the Clinical Sequencing Exploratory Research Consortium.* Am J Hum Genet, 2016. **99**(1): p. 247-247.
125. Mighton, C., et al., *Data sharing to improve concordance in variant interpretation across laboratories: results from the Canadian Open Genetics Repository.* J Med Genet, 2021.
126. Rehm, H.L., et al., *ClinGen — The Clinical Genome Resource.* N Engl J Med, 2015. **372**(23): p. 2235-2242.
127. Easton, D.F., 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): p. 873-883.
128. Tavtigian, S.V., et al., *Classification of rare missense substitutions, using risk surfaces, with genetic- and molecular-epidemiology applications.* Hum Mutat, 2008. **29**(11): p. 1342-1354.
129. Vallée, M.P., et al., *Adding In Silico Assessment of Potential Splice Aberration to the Integrated Evaluation of BRCA Gene Unclassified Variants.* Human Mutation, 2016. **37**(7): p. 627-639.
130. Ghosh, R., N. Oak, and S.E. Plon, *Evaluation of in silico algorithms for use with ACMG/AMP clinical variant interpretation guidelines.* Genome biology, 2017. **18**(1): p. 225-225.
131. Ernst, C., et al., *Performance of in silico prediction tools for the classification of rare BRCA1/2 missense variants in clinical diagnostics.* BMC Med Genomics, 2018. **11**(1): p. 35-35.
132. Moghadasi, S., 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): p. 74-79.
133. Grimm, D.G., et al., *The Evaluation of Tools Used to Predict the Impact of Missense Variants Is Hindered by Two Types of Circularity.* Human Mutation, 2015. **36**(5): p. 513-523.
134. Hicks, S., et al., *Prediction of missense mutation functionality depends on both the algorithm and sequence alignment employed.* Hum. Mutat, 2011. **32**(6): p. 661-668.
135. Leong, I.U.S., et al., *Assessment of the predictive accuracy of five in silico prediction tools, alone or in combination, and two metaservers to classify long QT syndrome gene mutations.* BMC Med Genet, 2015. **16**(1): p. 34-34.
136. Rodrigues, C., et al., *Performance of In Silico Tools for the Evaluation of UGT1A1 Missense Variants.* Human Mutation, 2015. **36**(12): p. 1215-1225.

137. Radhakrishnan, I., et al., *Solution Structure of the KIX Domain of CBP Bound to the Transactivation Domain of CREB: A Model for Activator:Coactivator Interactions.* Cell, 1997. **91**(6): p. 741-752.
138. Rosen, M.K., et al., *Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein.* Nature, 2000. **404**(6774): p. 151-158.
139. Kriwacki, R.W., et al., *p27 binds cyclin-CDK complexes through a sequential mechanism involving binding-induced protein folding.* Nat Struct Mol Biol, 2004. **11**(4): p. 358-364.
140. Kriwacki, R.W., et al., *Structural studies of p21Waf1/Cip1/Sdi1 in the free and Cdk2-bound state: conformational disorder mediates binding diversity.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11504-11509.
141. Mark, W.-Y., et al., *Characterization of Segments from the Central Region of BRCA1: An Intrinsically Disordered Scaffold for Multiple Protein–Protein and Protein–DNA Interactions?* J Mol Biol, 2005. **345**(2): p. 275-287.
142. Loong, L., et al., *Reclassification of clinically-detected sequence variants: Framework for genetic clinicians and clinical scientists by CanVIG-UK (Cancer Variant Interpretation Group UK).* Genet Med, 2022. **24**(9): p. 1867-1877.
143. Machackova, E., et al., *Twenty years of BRCA1 and BRCA2 molecular analysis at MMCI : current developments for the classification of variants.* 2019.
144. Lee, J.-S., et al., *Reclassification of BRCA1 and BRCA2 variants of uncertain significance: a multifactorial analysis of multicentre prospective cohort.* J Med Genet, 2018. **55**(12): p. 794-802.
145. Mighton, C., et al., *Variant classification changes over time in BRCA1 and BRCA2.* Genet Med, 2019. **21**(10): p. 2248-2254.
146. Murray, M.L., et al., *Follow-up of carriers of BRCA1 and BRCA2 variants of unknown significance: variant reclassification and surgical decisions.* Genet Med, 2011. **13**(12): p. 998-1005.
147. Kast, K., P. Wimberger, and N. Arnold, *Changes in classification of genetic variants in BRCA1 and BRCA2.* Arch Gynecol Obstet, 2018. **297**(2): p. 279-280.
148. Macklin, S., et al., *Observed frequency and challenges of variant reclassification in a hereditary cancer clinic.* Genet Med, 2018. **20**(3): p. 346-350.
149. Mersch, J., et al., *Prevalence of Variant Reclassification Following Hereditary Cancer Genetic Testing.* JAMA, 2018. **320**(12): p. 1266-1274.
150. Aljarf, R., et al., *Understanding and predicting the functional consequences of missense mutations in BRCA1 and BRCA2.* Scientific reports, 2022. **12**(1): p. 10458-10458.
151. Cubuk, C., et al., *Clinical likelihood ratios and balanced accuracy for 44 in silico tools against multiple large-scale functional assays of cancer susceptibility genes.* Genet Med, 2021. **23**(11): p. 2096-2104.
152. Carvalho, M.A., et al., *Determination of cancer risk associated with germ line BRCA1 missense variants by functional analysis.* Cancer Res, 2007. **67**(4): p. 1494-1501.

153. Phelan, C.M., et al., *Classification of BRCA1 missense variants of unknown clinical significance.* J Med Genet, 2005. **42**(2): p. 138-146.
154. Vallon-Christersson, J., et al., *Functional analysis of BRCA1 C-terminal missense mutations identified in breast and ovarian cancer families.* Hum Mol Genet, 2001. **10**(4): p. 353-360.
155. Williams, R.S., et al., *Detection of Protein Folding Defects Caused by BRCA1-BRCT Truncation and Missense Mutations.* J Biol Chem, 2003. **278**(52): p. 53007-53016.
156. Williams, R.S. and J.N.M. Glover, *Structural Consequences of a Cancer-causing BRCA1-BRCT Missense Mutation.* J Biol Chem, 2003. **278**(4): p. 2630-2635.
157. Starita, L.M., et al., *A Multiplex Homology-Directed DNA Repair Assay Reveals the Impact of More Than 1,000 BRCA1 Missense Substitution Variants on Protein Function.* Am J Hum Genet, 2018. **103**(4): p. 498-508.
158. Kais, Z., et al., *Functional differences among BRCA1 missense mutations in the control of centrosome duplication.* Oncogene, 2012. **31**(6): p. 799-804.
159. Kais, Z. and J.D. Parvin, *Regulation of centrosomes by the BRCA1-dependent ubiquitin ligase.* Cancer Biol Ther, 2008. **7**(10): p. 1540-1543.
160. Starita, L.M., et al., *BRCA1-Dependent Ubiquitination of γ-Tubulin Regulates Centrosome Number.* Mol Cell Biol, 2004. **24**(19): p. 8457-8466.
161. Sankaran, S., et al., *Identification of domains of BRCA1 critical for the ubiquitin-dependent inhibition of centrosome function.* Cancer Res, 2006. **66**(8): p. 4100-4107.
162. Morris, J.R., et al., *Genetic analysis of BRCA1 ubiquitin ligase activity and its relationship to breast cancer susceptibility.* Hum. Mol. Genet, 2006. **15**(4): p. 599-606.
163. Parvin, J.D., et al., *BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A.* Nat Genet, 1998. **19**(3): p. 254-256.
164. Findlay, G.M., et al., *Accurate classification of BRCA1 variants with saturation genome editing.* Nature, 2018. **562**(7726): p. 217-222.
165. Fernandes, V.C., et al., *Impact of amino acid substitutions at secondary structures in the BRCT domains of the tumor suppressor BRCA1: Implications for clinical annotation.* J Biol Chem, 2019. **294**(15): p. 5980-5992.
166. Petitalot, A., et al., *Combining Homologous Recombination and Phosphopeptide-binding Data to Predict the Impact of BRCA1 BRCT Variants on Cancer Risk.* Mol Cancer Res, 2019. **17**(1): p. 54-69.
167. Bouwman, P., et al., *Functional categorization of BRCA1 variants of uncertain clinical significance in homologous recombination repair complementation assays.* Clin Cancer Res, 2020. **26**(17): p. 4559-4568.
168. Jarhelle, E., et al., *Characterization of BRCA1 and BRCA2 variants found in a Norwegian breast or ovarian cancer cohort.* Fam Cancer, 2017. **16**(1): p. 1-16.
169. Lee, M.S., et al., *Comprehensive Analysis of Missense Variations in the BRCT Domain of BRCA1 by Structural and Functional Assays.* Cancer Res, 2010. **70**(12): p. 4880-4890.
170. Glover, J.N.M., et al., *Structural basis of phosphopeptide recognition by the BRCT domain of BRCA1.* Nat Struct Mol Biol, 2004. **11**(6): p. 519-525.

171. Adamovich, A.I., et al., *The functional impact of BRCA1 BRCT domain variants using multiplexed DNA double-strand break repair assays.* Am J Hum Genet, 2022. **109**(4): p. 618-630.
172. Christou, C.M., et al., *The BRCA1 Variant p.Ser36Tyr Abrogates BRCA1 Protein Function and Potentially Confers a Moderate Risk of Breast Cancer.* PLoS One, 2014. **9**(4): p. e93400-e93400.
173. Nepomuceno, T.C., et al., *Assessment of small in-frame indels and C-terminal nonsense variants of BRCA1 using a validated functional assay.* Scientific reports, 2022. **12**(1): p. 16203-16203.
174. Gaboriau, D.C.A., et al., *Protein stability versus function: effects of destabilizing missense mutations on BRCA1 DNA repair activity.* Biochem J, 2015. **466**(3): p. 613-624.
175. Bershtein, S., et al., *Protein Quality Control Acts on Folding Intermediates to Shape the Effects of Mutations on Organismal Fitness.* Mol Cell, 2013. **49**(1): p. 133-144.
176. Bershtein, S., et al., *Soluble oligomerization provides a beneficial fitness effect on destabilizing mutations.* Proc Natl Acad Sci U S A, 2012. **109**(13): p. 4857-4862.
177. Velasco, K., et al., *Functional evaluation of 16 SCHAD missense variants: Only amino acid substitutions causing congenital hyperinsulinism of infancy lead to loss-of-function phenotypes in vitro.* J Inherit Metab Dis, 2021. **44**(1): p. 240-252.
178. Brnich, S.E.A.T., Ahmad N ; Couch, Fergus J ; Cutting, Garry R ; Greenblatt, Marc S ; Heinen, Christopher D ; Kanavy, Dona M ; Luo, Xi ; McNulty, Shannon M ; Starita, Lea M ; Tavtigian, Sean V ; Wright, Matt W ; Harrison, Steven M ; Biesecker, Leslie G ; Berg, Jonathan S, *Recommendations for application of the functional evidence PS3/BS3 criterion using the ACMG/AMP sequence variant interpretation framework.* Genome medicine, 2019. **12**(1): p. 3-3.
179. Rodriguez, J.A., W.W.Y. Au, and B.R. Henderson, *Cytoplasmic mislocalization of BRCA1 caused by cancer-associated mutations in the BRCT domain.* Exp Cell Res, 2004. **293**(1): p. 14-21.
180. Heeke, A.L., et al., *Prevalence of Homologous Recombination-Related Gene Mutations Across Multiple Cancer Types.* JCO Precis Oncol, 2018. **2018**(2): p. 1-13.
181. Stewart, M.D., et al., *Homologous Recombination Deficiency: Concepts, Definitions, and Assays.* Oncologist, 2022. **27**(3): p. 167-174.
182. Lovelock, P.K., et al., *Identification of BRCA1 missense substitutions that confer partial functional activity: potential moderate risk variants?* Breast Cancer Res, 2007. **9**(6): p. R82-R82.
183. Lu, C., et al., *Patterns and functional implications of rare germline variants across 12 cancer types.* Nat Commun, 2015. **6**(1): p. 10086.
184. Yu, X. and J. Chen, *DNA Damage-Induced Cell Cycle Checkpoint Control Requires CtIP, a Phosphorylation-Dependent Binding Partner of BRCA1 C-Terminal Domains.* Mol Cell Biol, 2004. **24**(21): p. 9478-9486.

185. Chen, L., et al., *Cell Cycle-dependent Complex Formation of BRCA1·CtIP·MRN Is Important for DNA Double-strand Break Repair.* The Journal of biological chemistry, 2008. **283**(12): p. 7713-7720.
186. Nakanishi, M., et al., *NFBD1/MDC1 Associates with p53 and Regulates Its Function at the Crossroad between Cell Survival and Death in Response to DNA Damage.* J Biol Chem, 2007. **282**(31): p. 22993-23004.
187. Drikos I, B.E., Kastritis PL, Vorgias CE, *BRCA1-BRCT mutations alter the subcellular localization of BRCA1 in vitro.* Anticancer Res2021;41:2953-62, 2020.
188. Brnich, S.E., et al., *Recommendations for application of the functional evidence PS3/BS3 criterion using the ACMG/AMP sequence variant interpretation framework.* Genome medicine, 2019. **12**(1): p. 1-12.
189. Garrett, A., et al., *Combining evidence for and against pathogenicity for variants in cancer susceptibility genes: CanVIG-UK consensus recommendations.* J Med Genet, 2021. **58**(5): p. 297-304.
190. Michailidou, K., et al., *Polygenic Risk Scores for Prediction of Breast Cancer and Breast Cancer Subtypes.* Am J Hum Genet, 2019. **104**(1): p. 21-34.
191. Yadav, L.R., et al., *Functional assessment of intrinsic disorder central domains of BRCA1.* J Biomol Struct Dyn, 2015. **33**(11): p. 2469-2478.
192. van der Lee, R., et al., *Classification of Intrinsically Disordered Regions and Proteins.* Chem. Rev, 2014. **114**(13): p. 6589-6631.
193. Babu, M.M., et al., *Intrinsically disordered proteins: regulation and disease.* Curr Opin Struct Biol, 2011. **21**(3): p. 432-440.
194. Uversky, V.N., C.J. Oldfield, and A.K. Dunker, *Intrinsically disordered proteins in human diseases: introducing the D2 concept.* Annu Rev Biophys, 2008. **37**: p. 215-246.
195. Julien, M., et al., *Intrinsic Disorder and Phosphorylation in BRCA2 Facilitate Tight Regulation of Multiple Conserved Binding Events.* Biomolecules (Basel, Switzerland), 2021. **11**(7): p. 1060.

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ORIGINAL ARTICLE

BRCA1 Norway: comparison of classification for *BRCA1* germline variants detected in families with suspected hereditary breast and ovarian cancer between different laboratories

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Abstract
Pathogenic germline variants in *Breast cancer susceptibility gene 1* (*BRCA1*) predispose carriers to hereditary breast and ovarian cancer (HBOC). Through genetic testing of patients with suspected HBOC an increasing number of novel *BRCA1* variants are discovered. This creates a growing need to determine the clinical significance of these variants through correct classification (class 1–5) according to established guidelines. Here we present a joint collection of all *BRCA1* variants of class 2–5 detected in the four diagnostic genetic laboratories in Norway. The overall objective of the study was to generate an overview of all *BRCA1* variants in Norway and unveil potential discrepancies in variant interpretation between the hospitals, serving as a quality control at the national level. For a subset of variants, we also assessed the change in classification over a ten-year period with increasing information available. In total, 463 unique *BRCA1* variants were detected. Of the 126 variants found in more than one hospital, 70% were interpreted identically, while 30% were not. The differences in interpretation were mainly by one class (class 2/3 or 4/5), except for one larger discrepancy (class 3/5) which could affect the clinical management of patients. After a series of digital meetings between the participating laboratories to disclose the cause of disagreement for all conflicting variants, the discrepancy rate was reduced to 10%. This illustrates that variant interpretation needs to be updated regularly, and that data sharing and improved national inter-laboratory collaboration greatly improves the variant classification and hence increases the accuracy of cancer risk assessment.

Keywords Breast and ovarian cancer · *BRCA1* · Variant classification · Variants of uncertain significance

Introduction

While most cancer cases are sporadic, 5–10% are hereditary and caused by disease-causing germline variants in cancer susceptibility genes. Hereditary breast and ovarian cancer (HBOC) can be caused by alterations in several genes, among which the tumour suppressors *Breast cancer susceptibility gene 1* and 2 (*BRCA1* and *BRCA2*) are the most prevalent and studied. Carriers of (likely) pathogenic germline variants of *BRCA1*, which is the focus of this study, have a lifetime risk of 56–75% for breast cancer and 36–51% for ovarian cancer [1].

In recent years, technological development and reduced costs have led to rapid growth in the use of genetic testing of patients with suspected HBOC. An increasing number of novel *BRCA1* variants are thus being discovered, and to date more than 11 000 *BRCA1* variants are registered in ClinVar

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[2]. Accurate assessment of the clinical relevance of a given *BRCA1* variant is crucial for risk assessment, genetic counselling, and clinical management including cancer prevention in both the patient and healthy relatives with the same hereditary predisposition. A joint consensus of standards and guidelines for the interpretation of genetic variants in general has been made by ACMG-AMP (The American College of Medical Genetics and Genomics and the Association for Molecular Pathology) [3]. The pathogenicity of a variant is interpreted according to a five-tier score system with the following designations: benign (class 1), likely benign (class 2), uncertain significance (class 3), likely pathogenic (class 4), and pathogenic (class 5) [4]. In addition, the expert consortium ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) has developed classification criteria specific for *BRCA1* and *BRCA2* [5].

While *BRCA1* variants classified as either likely benign or benign are not associated with increased risk of cancer, variants classified as likely pathogenic or pathogenic increase the risk of cancer by impairing protein structure or function. Carriers of such variants are offered regular surveillance and prophylactic surgery according to national guidelines [6–8]. However, for a large number of *BRCA1* variants the knowledge is either very limited or conflicting, and accordingly these are classified as variants of uncertain significance (VUS). The expanding use of genetic testing increases the number of new and rare VUSs identified. Hence, even though *BRCA1* is a well-characterized gene, interpretation of variants in this gene is still a challenge for the individual clinical laboratories.

Discrepancies in the interpretation of the same gene variants at different laboratories have previously been observed in several countries, including Canada and USA [9–12]. The consequences can be tragic. Recently, an example of misinterpretation was unveiled in a hospital in Norway, where twenty-one female carriers had their breast and/or ovaries removed by prophylactic surgery without sufficient evidence that their variant, *BRCA2* (NM_000059.3) c.68–7 T > A, was pathogenic [13, 14]. The other Norwegian hospitals did not classify this *BRCA2* variant as pathogenic, but this was unknown at the time, as there is no general practice for data sharing or a common national variant database. There are several serious consequences of a misclassified variant including unnecessary interventions in patients and misallocation of resources for the society. Furthermore, family members harbouring the same genetic variant may receive different medical advice if they live in different parts of the country. This may lead to increasing uncertainty and anxiety among carriers of such variants.

In this study, based on inter-laboratory collaboration, we aim to give an overview of all class 2–5 *BRCA1* variants identified at the four diagnostic genetic laboratories in Norway. Furthermore, we compare the corresponding classifications at the different hospitals to explore the national consistency of *BRCA1* variant interpretation. In addition, for a subset of variants, we aim to assess the change in classification over time with increasing information available. Ideally, the collaboration will give an increased consensus regarding *BRCA1* variant classification and create a forum for future discussions.

Materials and methods

BRCA1 variants were collected from the four diagnostic genetic laboratories in Norway; Haukeland University Hospital in Bergen (HUH, 177 variants), Oslo University Hospital (OUH, 303 variants), the University Hospital of North Norway in Tromsø (UNN, 88 variants) and St. Olav's University Hospital in Trondheim (TUH, 84 variants). All *BRCA1* variants had been detected by genetic testing of patients or healthy family members of patients with suspected HBOC from late 1990s to July 2019. Samples were mainly analysed by Sanger sequencing and/or NGS (Illumina custom made gene panel). Each variant was classified at the hospitals according to local protocols based on the ACMG-AMP guidelines or equivalent procedures. Nomenclature was assigned according to the Human Genome Variation Society (HGVS), and the reference sequence NM_007294.3 was used [15]. Variants reported as benign (class 1) and copy number variants identified by multiplex ligation-dependent probe amplification (MLPA) were not included in the dataset.

For VUSs observed in only one hospital and with a classification report older than three years in 2019 ($n = 45$), a reassessment of the variants was performed by HUH. For variants detected in more than one hospital, classifications were compared. For all variants with conflicting classifications, the corresponding laboratories were asked to reassess the variant. Following this reclassification, a series of digital meetings between all laboratories were arranged to disclose the cause of disagreement and to try to reach consensus. At least one laboratory geneticist from each hospital participated in these discussions.

Finally, for a subset of variants (variants found at HUH in the period from 2007 to 2017 ($n = 115$)) the classifications at HUH and ClinVar over time were compared. A heat map of these classifications was generated using R (v. 4.0.2) and the package ggplot2 [16]. Data was cleaned and managed with tidy data principles using the tidyverse collection of packages (v 1.3.1) [17]. The colour scale used for variant classification was generated using the RColorBrewer package (v. 1.1–2). Multiple heat maps and a bar plot were combined using the package cowplot (v. 1.1.1).

The program Alamut Visual (Version 2.13) was used as a tool during reassessment of variants [18]. The variant allele frequencies were retrieved from GnomAD v2.1.1 [19].

Results

In total, 652 *BRCA1* variants were submitted from the four hospitals. The number of variants from each hospital, in addition to the distribution of variants within each class, is shown in Fig. 1. After removal of overlapping variants, 463 *BRCA1* variants were shown to be unique (Supplementary table 1). Of the 463 unique variants, 126 variants (27%) were detected in more than one hospital; 76 (16%), 37 (8%) and 13 (3%) variants were detected at two, three and four hospitals, respectively (Table 1). The remaining 337 (73%) *BRCA1* variants were observed in one hospital only.

For the 126 variants detected in more than one hospital, the corresponding classifications were compared. For 30% (38/126) of these variants, there were discrepancies in interpretations between the hospitals (Table 2). The differences in interpretation were mainly by one pathogenicity class (class 2/3 or 4/5) as shown in Figs. 2 and 3A. Alarmingly, one class 3/5 discrepancy was detected for the variant *BRCA1* c.457_458ins21. This variant was observed in three hospitals; one of the hospitals reported the variant as class 3 (variant of uncertain significance), while the two other hospitals reported it as class 5 (pathogenic). In the class 5 reports, the variant was described as an insertion of 21 nucleotides leading to a premature stop codon (ATTAGCA*G*GAAACCAGTCTCA). This did not correspond with the class 3 report, where the inserted nucleotide sequence was different, and did not contain a stop codon (ATTA*C*CA*A*GAAACCAGTCTCA). Thorough investigations of the raw data revealed that the discrepancy was caused by a misread of the inserted sequence due to software weakness (Sequence Pilot, JSI medical systems), and that a stop codon was indeed present in the insertion. The mistake was corrected and all hospitals now classify *BRCA1* c.457_458ins21 as pathogenic (class 5).

Table 1 Number of *BRCA1* variants detected in more than one hospital

Number of hospitals	Number of variants	Percentage
1	337	73
2	76	16
3	37	8
4	13	3
Total	463	100

After removal of overlapping variants, 463 variants were shown to be unique. 126 variants were detected in more than one hospital, while the remaining 337 variants were observed in one hospital only

Comparison of the *BRCA1* variants in Supplementary table 1 with previously published *BRCA1* variants found in certain regions of Norway [20–22] revealed that the two variants *BRCA1* c.5123C > T (VUS) and c.4883 T > C (likely benign) have previously been incorrectly classified as pathogenic in a recent publication [20]. The authors were informed on the discovery, and the mistakes were later corrected [23]. According to the authors, the mistakes were caused by problems related to formatting of a table in the article, and the incorrect classifications had not been utilized in the clinic.

In order to ensure updated classifications, VUSs with only one interpretation report older than three years ($n = 45$) were reassessed. In total, eleven variants were reclassified to likely benign variants, while one variant

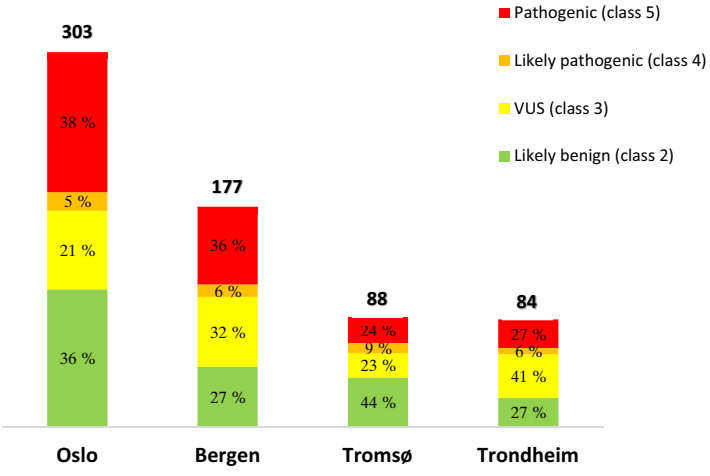

Fig. 1 Number of *BRCA1* variants submitted from the participating hospitals and the local distribution of variants within each class. In total 652 variants were submitted from the four laboratories

Table 2 *BRCA1* variants with conflicting classification between different Norwegian hospitals and resulting reclassification after collaboration

Variant	Oslo (OUH)		Bergen (HUH)		Tromsø (UNN)		Trondheim (TUH)		Reclassified	
	Class	Date	Class	Date	Class	Date	Class	Date	Class	Date
c.19C > T	3	2015	2	2015	3	2018			3	2021
c.140G > T	4	2014	5	2008					4	2021
c.301 + 7G > A					3	2013	2	2015	$2^{\Delta\bigcirc}$, $1^{\square}$	2021
c.441G > C	3	2017	3	2013	2	2010			3	2021
c.441 + 21C > T			2	2018			3	2018	3	2021
c.457_458ins21	5	2014	3	2016			5	2016	5	2019
c.547 + 14del			3	2011	2				2^{Δ},$1^{\square}$	2021
c.557C > A			3	2014			2	2015	2	2021
c.670 + 16G > A			3	2018	2	2010	2	2016	2	2021
c.736 T > G			3	2014	2	2010	2	2018	2	2021
c.889A > G	2	2019	3	2016					2^{*},3^{Δ}	2021
c.1287del	5	2008	5	2015	4	2014			5	2021
c.1508A > G	2	2017					3	2017	$2^{*\bigcirc}$,3^{Δ}	2021
c.1534C > T	2	2018	2	2018	3	2015	3	2018	2	2021
c.1568 T > G	2	2018			3	2017	3	2017	$2^{*\bigcirc}$,$3^{\Delta\square}$	2021
c.1687C > T	5	2018	4	2013	5	2018			5	2021
c.1772 T > C	2	2019	3	2011					2	2021
c.1879G > A	2	2018	3	2019	3	2016			2^{*},$3^{\Delta\square}$	2021
c.2131A > C	2	2019	3	2019					2^{*},3^{Δ}	2021
c.2183G > A	2	2018			3	2016			2^{*},$3^{\Delta\square}$	2021
c.2315 T > C	3	2015					2	2019	1^{*},$2^{\Delta\bigcirc}$	2021
c.2773A > G	2	2017	3	2014					2^{*},3^{Δ}	2021
c.2798G > A	2	2018	3	2016					2^{*},3^{Δ}	2021
c.3041 T > A	3	2012	2	2016					3	2021
c.3228_3229del	5	2018	5	2019	4	2012	5	2018	5	2021
c.3319G > T	5	2018			4	2010			5	2021
c.3454G > A	2	2018	3	2018					2	2021
c.3640G > A	3	2010	2	2019					2	2021
c.3659A > T	2	2018			3	2012	3	2018	2^{*},$3^{\Delta\square\bigcirc}$	2021
c.4096 + 3A > G	3	2019	2	2017			3	2017	3	2021
c.4300del	5		5	2015	4	2016			5	2021
c.4315C > T	2	2019	3	2019	3	2018	3	2018	2^{*},$3^{\Delta\square\bigcirc}$	2021
c.4484G > A	5	2015			5	2015	4	2015	5	2021
c.5047G > T	5	2018	5	2008	4	2014			5	2021
c.5096G > A	4	2018	4	2013	5	2019	5	2017	5	2021
c.5213G > A	5		4	2010					5	2021
c.5348 T > C	2	2017	3	2018					2	2021
c.5477A > T	2	2017			3	2016	3	2017	2	2021

For *BRCA1* variants with conflicting classifications after reassessment, the following symbols indicate the corresponding laboratories: * = OUH (Oslo University Hospital), $^{\Delta}$ = HUH (Haukeland University Hospital in Bergen), $^{\square}$ = UNN (University Hospital of North Norway in Tromsø), $^{\bigcirc}$ = TUH (St. Olav's University Hospital in Trondheim)

was reclassified as a benign variant. The remaining 33 variants were still assessed as VUSs.

For the 38 variants with conflicting classifications between hospitals, each laboratory was asked to reassess the variants, resulting in a reduction of the rate of discrepancies from 30% (38/126) to 14% (18/126). All laboratories then participated in a series of digital meetings discussing the causes of disagreement, further reducing the discrepancy rate to 10% (13/126) (Fig. 3B).Thus, after reassessment of the variants, 66% (25/38) of the original conflicting interpretations eventually reached consensus.

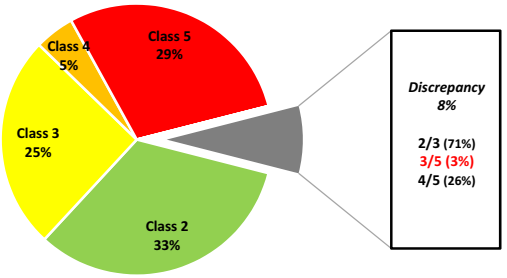

Fig. 2 Distribution of *BRCA1* variants within each pathogenicity class

For a sub-cohort of the variants (detected at HUH in the period from 2007 to 2017) a schematic presentation of their classification over time at the hospital as well as in ClinVar was made. The heat map shows that among the variants that changed classification over time, the majority were VUSs reclassified to likely benign both at HUH and ClinVar (Fig. 4). The following were observed; (1) nine variants from HUH and 22 variants from ClinVar were downgraded, (2) three variants from HUH and eight variants from ClinVar were upgraded, and (3) no variants from HUH and 16 variants from ClinVar were both upgraded and downgraded. Fifteen of the variants from HUH were not reported in ClinVar. The concordance in classifications between HUH and ClinVar was relatively high.

Discussion

Even though *BRCA1* sequencing of HBOC patients has been performed in Norway since late 1990s, previous studies characterizing *BRCA1* variants in Norway have included only specific regions of the country [20–22, 24]. This study is the first to include *BRCA1* variant data from all four medical genetic departments in Norway, and gives a complete overview of the Norwegian *BRCA1* variant spectrum. Comparison of variant classification between the different hospitals revealed several discrepancies and clearly illustrates the pivotal role of sharing variant interpretation data. Furthermore, the change in variant classification over time for a subset of the data demonstrates the importance of updating variant classifications regularly.

Due to the complexity of variant interpretation, some discrepancies among hospitals are expected [9], and the discrepancy rate of 30% for Norwegian *BRCA1* variants found in this study is within the range of previous findings in similar studies [25]. A study from Canada that investigated variants uploaded to a national database by eleven participating diagnostic laboratories found that 38.9% (350/900) of the *BRCA1* and *BRCA2* variants classified by two or more laboratories had conflicting interpretations when using a five-tier classification model. After reassessment of the variants, 21.4% (75/350) of the conflicting interpretations reached consensus. The laboratories reported that the main reasons for reclassifying a variant was availability of new evidence (52.7%), and the use of revised classification criteria (28.4%) [11]. Several other studies performed on gene variants in general have found similar discrepancy rates [9, 10]. In addition, analysis of all gene variants reported to ClinVar has shown that 17% (2229/12895) of the variants

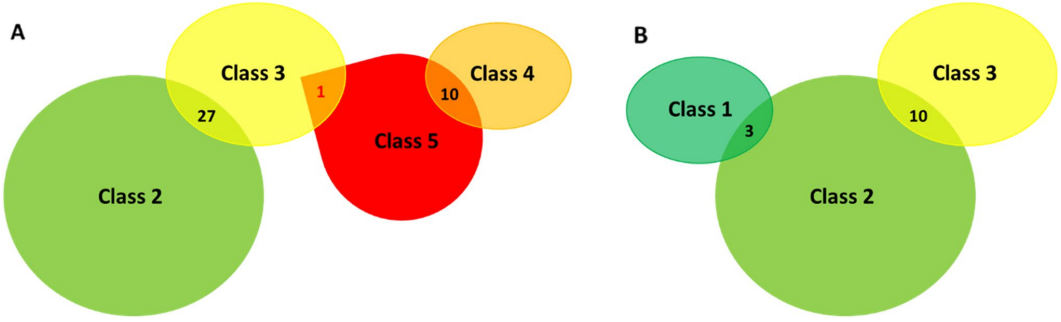

Fig. 3 Distribution of *BRCA1* variants with conflicting interpretations. **A** For 38 of the variants detected in more than one hospital there were discrepancies in interpretations between the hospitals. The majority of discrepancies (37/38) were one class apart. Only one variant was found to have a discrepancy extending two classes. **B** After a series of collaborative meetings between the different hospitals to discuss the causes of disagreement, the number of conflicting classifications were reduced to 14. All discrepancies were one class apart, mainly between class 2 and 3

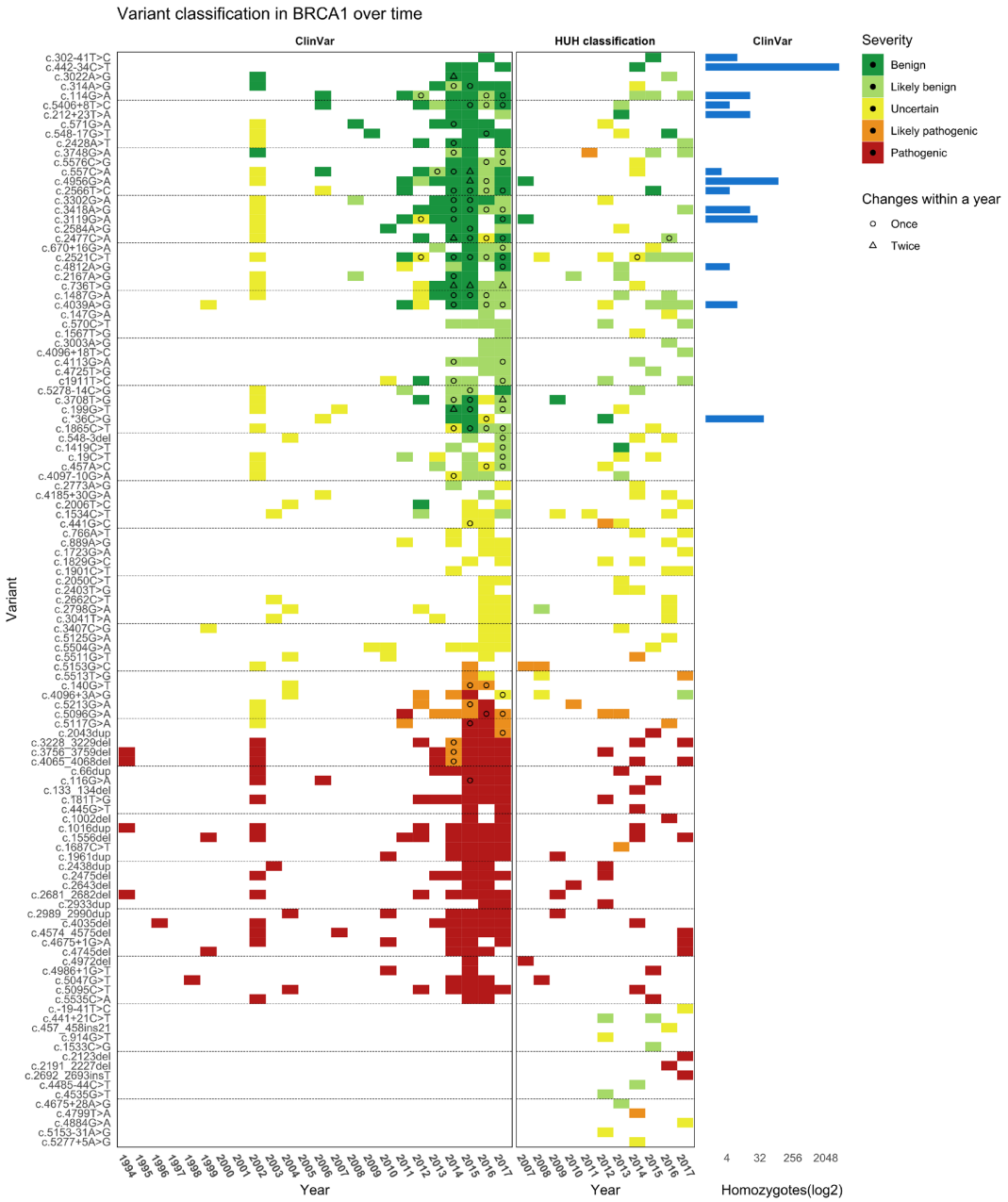

Fig. 4 Heat map: Schematic heat map presentation of changes in classification over time for variants detected at Haukeland University hospital in the period from 2007 to 2017. The figure presents classifications performed locally at the hospital compared to classifications reported to the open access database ClinVar in the same timeframe

submitted by more than one laboratory were interpreted differently [26]. Currently, 4% (374/8829) of *BRCA1* variants reported in ClinVar are registered with conflicting interpretations [2]. Some studies report discrepancy rates much lower than the examples described above [27, 28]. However, in contrast to our study, in these studies a five-tier classification system was not used, but rather a two-tier system reporting discrepancies only between variants described as non-actionable (class 1–3) and clinically actionable (class 4–5). These results are therefore still in concordance with the findings in our study, as we only found one discrepancy that would affect the management of patients. Overall, our results indicate that the *BRCA1* variant classification in Norwegian hospitals is relatively consistent.

Unveiling potential conflicting interpretations that may impact the management of patients is of high value. Of particular interest, this study revealed one classification deviation (class 3/5) for the variant *BRCA1* c.457_458ins21. There is a major difference in the clinical management of patients harbouring a VUS and a pathogenic *BRCA1* variant, and misclassification of this pathogenic variant as a VUS has serious consequences by depriving the affected family of appropriate treatment. Healthy carriers of pathogenic variants are offered surveillance and risk reducing surgery to prevent cancer [29–35], and accurate assessment of a genetic variant is crucial to ensure that carriers receive satisfactory genetic counselling regarding these options. Patients with *BRCA1* deficient cancers are also candidates for treatment with Poly (ADP-ribose) polymerase (PARP)-inhibitors, thus *BRCA1* variant interpretation status is extremely important for treatment decisions [36–41]. Accordingly, identification of a pathogenic *BRCA1* variant in the family affects both the patient and healthy family members who might have inherited the same variant. After discovering the misclassification, all family members were re-advised for further genetic testing and correct clinical management was offered. Luckily, no new cancer cases had occurred in the family during the period of misclassification (2016–2019).

The majority of the identified classification discrepancies did not affect the clinical management of patients. There were 27 variants with conflicting interpretation between class 2 (likely benign) and class 3 (VUS), and ten variants with conflicting interpretations between class 4 (likely pathogenic) and class 5 (pathogenic). As variants of both class 4 and 5 are clinically actionable, such discrepancies are of lower clinical relevance. If a likely benign variant is detected in a patient, it is assumed that this is not the explanation for the cancer in the family (but it does not rule out other hereditary causes). If a VUS is detected, it is not possible to determine if this is the cause of the cancer and the classification report will be inconclusive. A VUS is thus not clinically actionable [4], but it might still produce significant anxiety among the carriers if reported back to the patient. Such findings will often need further analysis of the variant like functional analysis, and reassessment of the pathogenicity when new knowledge is unveiled.

All 38 variants with conflicting classifications were discussed between the participating hospitals, aiming to disclose the cause of disagreements and increase the national consensus regarding *BRCA1* variant classification in Norway. The main reasons for conflicting classifications were found to be differences in how strictly the different laboratories followed the ACMG-AMP classification guidelines, in addition to different understandings of some of the guidelines. The BP1 evidence (missense variant in a gene for which primarily truncating variants are known to cause disease) was one of the most debated criteria. As only two supporting benign evidence are enough to classify a variant as likely benign according to ACMG-AMP, use of this evidence would more easily lead to classification of *BRCA1* missense variants outside the RING and BRCT domains as likely benign. Since there is only limited knowledge about the regions located outside these protein domains, it was debated whether or not this criteria should be used as supportive benign evidence. Several publications have suggested that most *BRCA1* missense substitutions located outside of critical domains could be classified as likely benign, arguing that pathogenic missense variants are infrequent in these regions. This is supported by the fact that the ClinVar dataset contains hardly any (likely) pathogenic *BRCA1* missense variants located outside the critical domains. It was however debated that this does not necessarily mean that such variants do not exist. During the folding of proteins, amino acid residues originally located outside well established domains in the primary structure can come in contact with important structural and functional elements in the three dimensional structure of the native folded protein. Thus, it is reasonable to believe that the consequence of introducing a missense variant involving an amino acid with major differences in the size, polarity and physiochemical properties compared to the original residue could be fatal, also for residues located outside well established domains. Since the structural knowledge of BRCA1 is sparse and there is only limited knowledge about the regions located outside these protein domains, functional studies similar to the saturation editing data for the BRCT and RING domains are needed to further address this issue. After the discussions, an agreement was made to use BP1 with caution, and always to compile with data on amino acid conservation as well as comparison of the physiochemical properties of the original and new amino acid residue. There were also differences in the use of the ACMG-AMP BS1 evidence (allele frequency is greater than expected for disorder) as some of the laboratories use different cut-off values regarding allele frequencies to decide the strength of the BS1 evidence. Some laboratories use new and updated guidelines like CanVig-UK

[42] in addition to the ACMG-AMP guidelines. The degree of emphasis on classifications performed by the expert consortium ENIGMA were also the reason for some of the discrepancies. If ENIGMA had classified a variant as benign (class 1), some of the laboratories weighted this stronger than any of the ACMG-AMP guidelines. Other reasons for inter-lab discrepancies were in-house information regarding family history and findings of additional pathogenic variants in combination with the variant of interest.

Both the criteria for eligibility of having a genetic test and the criteria used to classify a variant have changed during the years included in the study. The ACMG-AMP guidelines for the interpretation of sequence variants were published in 2015. The ENIGMA criteria for classifying *BRCA1* variants were first published in 2009, and lastly updated in 2017. In addition, even though laboratories use standardized methods when interpreting variants, the available information is often sparse or sometimes even conflicting. Noteworthy, the resulting classification of a variant is dependent on the information available in the local laboratory at the time of interpretation. Thus, a given classification is most correct at the specific time of interpretation based on available information, but is outdated and should ideally be reassessed when new information is available. Such discrepancies can be solved by data sharing between the hospitals and regular reassessment of variants, but in Norway a variant is often only reassessed if it is identified in a new individual.

After observing that 12 of the 45 VUSs with old classification reports could be reclassified to likely benign after a new assessment, we wanted to further investigate how the *BRCA1* classification had potentially changed over time in general. Thus, the classification history for a subset of *BRCA1* variants reported between 2007 and 2017 at HUH and in ClinVar when available was generated. Both at HUH and in ClinVar the classification of several variants had changed over time following the rapid increase of available information. The majority of reclassified *BRCA1* variants were downgraded from VUS to (likely) benign variants in concordance with other studies reporting reclassification of *BRCA1* variants [43–49]. Most likely this is due to open-access databases like ClinVar (made available in 2012), and gradually increasing knowledge about variant frequencies in the general population made available in GnomAD in 2017 [19] and its precursor ExAC in 2014. Data on allele frequencies shows that many variants are relatively abundant among assumed healthy adults, and can therefore be excluded as pathogenic. A few variants were upgraded from VUSs to likely pathogenic variants. This is probably based on functional studies, discovery of the variants in more individuals with HBOC (or absence of the variants in healthy controls), and / or extensive segregation in families. For 15 of the variants from HUH there were no registered classifications in ClinVar. Most likely these variants are very rare and only occur in individuals/families in Western Norway. Since Norway has a relatively small population, there is often limited clinical information on a variant, while classification reports from the same variant in ClinVar can be based on a larger amount of information from several institutions and countries.

The *BRCA1* Norway collaboration has shown that data sharing increases the amount of evidence and contributes to national standardization and harmonization of variant classification and patient management. Data sharing is especially important for rare variants for which there is often limited evidence available. In small families, there are frequently insufficient family members to perform an informative segregation analysis. In addition, reduced penetrance for certain variants might add to the complexity. Consequently, such variants are often classified as VUSs. Hence, gathering of multiple observations of the same *BRCA1* variant and comparison of independent interpretations will increase the credibility of a given classification and increase the accuracy of cancer risk assessments. Data sharing can also help constrain laboratory resources.

To date, sharing information among the medical genetic departments in Norway has been limited, mostly due to the strict laws about the privacy of patients. When this project was initiated, the Norwegian law defined rare variants as information that may be used for the identification of individuals, and sharing of databases containing such information between different hospitals was therefore not allowed. Prior to initiating this study, the scientific community had though expressed a desire for increased sharing of data regarding variant interpretation between the laboratories, and currently there is a proposal to a change in the law that will make it possible to share such information. The major challenge is to find a common platform where important information about the variants can be exchanged in accordance with the guidelines for patient privacy policies in an efficient manner. The collaboration between all the diagnostic genetic laboratories in Norway will be extended to include variant interpretation of several other cancer genes like *BRCA2* and the *MMR* genes. At the clinical and diagnostic level, a national working group with participants from all departments of medical genetics in the field of hereditary cancer has already been established. In addition, a national network for hereditary cancer organised by the Norwegian Directorate of Health works on the national guidelines for different cancers to ensure that recommendations concerning genetics are up-to-date and communicated to non-genetic clinicians.

To summarize, the *BRCA1* Norway study shows that collaboration and data sharing can; (1) provide a more detailed overview of the *BRCA1* variant spectrum in the Norwegian population, (2) reveal discrepancies in variant interpretation among different laboratories, (3) reveal outdated

classification reports and ensure up to date interpretations, (4) reduce the number of VUSs, (5) reduce time spent on variant interpretation, (6) ensure more trustworthy classifications in accordance with increasing information, and (7) guide patients and clinicians to make well-informed clinical decisions.

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Data availability The dataset generated and analysed during the current study is available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the Regional Committee for Medical and Health Research Ethics of Haukeland University Hospital (2018/2467).

References

1. Kuchenbaecker KB, et al (2017) Evaluation of polygenic risk scores for breast and ovarian cancer risk prediction in *BRCA1* and *BRCA2* mutation carriers
2. The ClinVar Database. 11.09.20. https://www.ncbi.nlm.nih.gov/clinvar/?term=BRCA1%5Bgene%5D.
3. Richards S et al (2015) Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 17(5):405–423
4. Plon SE et al (2008) Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. Hum Mutat 29(11):1282–1291
5. ENIGMA Classification Criteria. 11.09.20. https://enigmaconsortium.org/library/general-documents/enigma-classification-criteria/.
6. The Norwegian Breast Cancer Group. Nasjonalt handlingsprogram med retningslinjer diagnostikk, behandling og oppfølging av pasienter med brystkreft, 13th edition. 11.09.2020. https://www.helsedirektoratet.no/retningslinjer/brystkreft-handlingsprogram.
7. Eccles DM et al (2016) Selecting patients with ovarian cancer for germline BRCA mutation testing: findings from guidelines and a systematic literature review. Adv Ther 33(2):129–150
8. National Comprehensive Cancer Network. NCCN Guideliner Genetics/familial High-Risk Assessment: Breast, Ovarian and Pancreatic, version 1.2020. 11.09.20. https://www.nccn.org/professionals/physician_gls/pdf/genetics_bop.pdf.
9. Amendola, Laura M, et al (2016) Performance of ACMG-AMP variant-interpretation guidelines among nine laboratories in the clinical sequencing exploratory research consortium. Am J Hum Genet 99(1):247–247.
10. Balmaña J et al (2016) Conflicting interpretation of genetic variants and cancer risk by commercial laboratories as assessed by the prospective registry of multiplex testing. J Clin Oncol 34(34):4071–4078
11. Lebo MS et al (2017) Data sharing as a national quality improvement program: reporting on BRCA1 and BRCA2 variant-interpretation comparisons through the Canadian Open Genetics Repository (COGR). Genet Med 20(3):294–302
12. Vail PJ et al (2015) Comparison of locus-specific databases for BRCA1 and BRCA2 variants reveals disparity in variant classification within and among databases. J Community Genet 6(4):351–359
13. *Dagens medisin* 11.09.20. https://www.dagensmedisin.no/artikler/2017/02/17/21-kvinner-fikk-fjernet-bryst-og-eggstokker--skulle-ikke-vart-operert/.
14. Møller P, Hovig E (2018) Retraction note to: the *BRCA2* variant c.68–7 T > A is associated with breast cancer. Hered Cancer Clin Pract 16(1):10–10
15. *HGVS*. 14.09.20. https://varnomen.hgvs.org/.
16. Wickham H (2016) Programming with ggplot2. Springer, Cham, 241–253
17. Wickham H et al (2019) Welcome to the Tidyverse. J Open Source Softw 4(43):1686
18. Alamut. 14.09.20. http://www.interactive-biosoftware.com/alamut-visual/.
19. *GnomAD*. 14.09.20. http://gnomad.broad institute.org/.
20. Møller P et al (2019) Causes for frequent pathogenic BRCA1 variants include low penetrance in fertile ages, recurrent de-novo mutations and genetic drift. Cancers (Basel) 11(2):132
21. Heramb C et al (2018) BRCA1 and BRCA2 mutation spectrum—an update on mutation distribution in a large cancer genetics clinic in Norway. Hered Cancer Clin Pract 16(1):3–15
22. Jarhelle E et al (2017) Characterization of BRCA1 and BRCA2 variants found in a Norwegian breast or ovarian cancer cohort. Fam Cancer 16(1):1–16
23. Møller P, et al (2020) Correction: Møller, P.; et al. Causes for frequent pathogenic BRCA1 variants include low penetrance in fertile ages, recurrent de-novo mutations and genetic drift. Cancers 2019, 11, 132. Cancers (Basel) 12(2):410.
24. Møller P et al (2001) Genetic epidemiology of BRCA1 mutations in Norway. Eur J Cancer 37(18):2428–2434
25. Mighton C, et al (2021) Data sharing to improve concordance in variant interpretation across laboratories: results from the Canadian Open Genetics Repository. J Med Genet
26. Rehm HL et al (2015) ClinGen—the clinical genome resource. N Engl J Med 372(23):2235–2242
27. Yang S et al (2017) Sources of discordance among germ-line variant classifications in ClinVar. Genet Med 19(10):1118–1126

28. Maxwell, Kara N, et al (2016) Evaluation of ACMG-guideline-based variant classification of cancer susceptibility and non-cancer-associated genes in families affected by breast cancer. Am J Hum Genet 98(5):801–817.
29. Rebbeck TR, Kauff ND, Domchek SM (2009) Meta-analysis of risk reduction estimates associated with risk-reducing Salpingo-oophorectomy in BRCA1 or BRCA2 Mutation Carriers. J Natl Cancer Inst 101(2):80–87
30. Mavaddat N et al (2013) Cancer risks for BRCA1 and BRCA2 mutation carriers: results from prospective analysis of EMBRACE. J Natl Cancer Inst 105(11):812–822
31. Brohet RM et al (2014) Breast and ovarian cancer risks in a large series of clinically ascertained families with a high proportion of BRCA1 and BRCA2 Dutch founder mutations. J Med Genet 51(2):98–107
32. Heijnsdijk E et al (2012) Differences in natural history between breast cancers in BRCA1 and BRCA2 mutation carriers and effects of MRI Screening-MRISC, MARIBS, and Canadian studies combined. Cancer Epidemiol Biomarkers Prev 21(9):1458–1468
33. Gareth ED et al (2014) MRI breast screening in high-risk women: cancer detection and survival analysis. Breast Cancer Res Treat 145(3):663–672
34. Rebbeck TR et al (2004) Bilateral prophylactic mastectomy reduces breast cancer risk in BRCA1 and BRCA2 mutation carriers: the PROSE Study Group. J Clin Oncol 22(6):1055–1062
35. Ingham SL et al (2013) Risk-reducing surgery increases survival in BRCA1/2 mutation carriers unaffected at time of family referral. Breast Cancer Res Treat 142(3):611–618
36. Lee JM, Ledermann JA, Kohn EC (2014) PARP Inhibitors for *BRCA1/2* mutation-associated and BRCA-like malignancies. Ann Oncol 25(1):32–40.
37. Robson M et al (2017) Olaparib for metastatic breast cancer in patients with a germline BRCA mutation. N Engl J Med 377(6):523–533
38. Ledermann J et al (2012) Olaparib maintenance therapy in platinum-sensitive relapsed ovarian cancer. N Engl J Med 366(15):1382–1392
39. Golan T et al (2019) Maintenance olaparib for germline BRCA -mutated metastatic pancreatic cancer. N Engl J Med 381(4):317–327
40. Kaufman B et al (2015) Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. J Clin Oncol 33(3):244–250
41. Mateo J et al (2019) A decade of clinical development of PARP inhibitors in perspective. Ann Oncol 30(9):1437–1447
42. Garrett A et al (2020) Cancer variant interpretation group UK (CanVIG-UK): an exemplar national subspecialty multidisciplinary network. J Med Genet 57(12):829–834
43. Machackova E, et al (2019) Twenty years of BRCA1 and BRCA2 molecular analysis at MMCI: current developments for the classification of variants.
44. Lee J-S et al (2018) Reclassification of BRCA1 and BRCA2 variants of uncertain significance: a multifactorial analysis of multicentre prospective cohort. J Med Genet 55(12):794–802
45. Mighton C et al (2019) Variant classification changes over time in BRCA1 and BRCA2. Genet Med 21(10):2248–2254
46. Murray ML et al (2011) Follow-up of carriers of BRCA1 and BRCA2 variants of unknown significance: variant reclassification and surgical decisions. Genet Med 13(12):998–1005
47. Kast K, Wimberger P, Arnold N (2018) Changes in classification of genetic variants in BRCA1 and BRCA2. Arch Gynecol Obstet 297(2):279–280
48. Turner SA et al (2018) The impact of variant classification on the clinical management of hereditary cancer syndromes. Genet Med 21(2):426–430
49. Macklin S et al (2017) Observed frequency and challenges of variant reclassification in a hereditary cancer clinic. Genet Med 20(3):346–350

Supplementary table 1. Overview of all *BRCA1* variants found at the four genetic laboratories in Norway

	Oslo (OUH)		Bergen (HUH)		Tromsø (UNN)		Trondheim (TUH)		Reclassified	
Variant	Class	Date	Class	Date	Class	Date	Class	Date	Class	Date
c.-125C>T							3	2016		
c.-107_-103del					2	2012				
c.-86C>T							3	2018		
c.-66C>G							3	2018		
c.-20+11C>T							3	2018		
c.-20+46G>C							3			
c.-19-85_-19-81del			2	2019						
c.-19-41T>C			2	2017						
c.1A>G	5	2018	5	2015	5	2009				
c.19C>T	3	2015	2	2015	3	2018			3	2021
c.65T>C	5	2018								
c.66dup	5		5	2018			5	2015		
c.68_69del	5	2017								
c.69G>T	3	2015							3	2019
c.75C>T			2	2018	2	2013	2	2017		
c.80+7C>A	2	2017								
c.81-14C>T			2	2018	2	2016				
c.81-13C>A	2	2018								
c.81-12C>G	2	2018								
c.81-11del	2	2018								
c.81-2del			5	2019						
c.114G>A			2	2017						
c.115T>G	4	2016								
c.116G>A	5	2018	5	2015						
c.130T>A	5	2018					5	2015		
c.133_134del			5	2014						
c.140G>T	4	2014	5	2008					4	2021
c.147G>A			3	2016						
c.154C>T							3	2018		
c.169G>C					3	2014			3	2019
c.178C>T	5	2017								
c.181T>G	5	2019	5	2019						
c.199G>T			3	2013					2	2019
c.212+1G>T					5	2018				
c.212+21G>A							3	2018		
c.213-5T>A	3	2019								
c.241C>T	5	2012								
c.255G>A	2	2019								
c.301+7G>A					3	2013	2	2015	$2^{\Delta\circ}$, $1^{\square}$	2021
c.302-124del					2	2013				
c.302-2A>C	4	2018								
c.305C>G	3	2015							2	2019
c.314A>G			3	2014					2	2019
c.334A>G	2	2018								
c.386del			5	2019						
c.397C>T			3	2019						
c.441G>C	3	2017	3	2013	2	2010			3	2021

c.441+18C>T					2	2013					
c.441+21C>T			2	2018			3	2018	3	2021	
c.441+41dup			5								
c.445G>T	5	2015	5	2014							
c.448del	5	2017									
c.457_458ins21	5	2014	3	2016			5	2016	5	2019	
c.457A>C			3	2015					3	2019	
c.486G>T	2	2017									
c.509G>A	2	2017									
c.510del	5	2016					5	2016			
c.514C>T	5						5	2015			
c.538A>G	3	2016									
c.547+2dup	3	2017									
c.547+14del			3	2011	2				2^{Δ},$1^{\square}$	2021	
c.548-17G>T			2	2016	2	2010	2	2017			
c.548-3del			3	2016							
c.557C>A			3	2014			2	2015	2	2021	
c.5586C>T	2	2019	2	2019							
c.564A>G	2	2015									
c.570C>T	2	2014	2	2017	2	2011					
c.571G>A			3	2012					2	2019	
c.591C>T					2	2010					
c.594-34T>C			2	2011							
c.594-20A>G	2	2017									
c.670+7G>A	2	2014									
c.670+16G>A			3	2018	2	2010	2	2016	2	2021	
c.671-12del	3	2015									
c.671-10A>G	3	2019									
c.692C>T	3	2015							3	2019	
c.697_698del	5	2018	5	2018	5	2009					
c.712C>T	3	2015							3	2019	
c.734A>T	3	2014							3	2019	
c.736T>G			3	2014	2	2010	2	2018	2	2021	
c.765G>A	2	2018									
c.766A>T	3	2018	3	2017							
c.794_795del	5	2017									
c.814G>T			5	2018							
c.825C>T	2	2018									
c.834T>G	2	2017									
c.843_846del	5	2015									
c.848T>A	5										
c.889A>G	2	2019	3	2016					2*,3^{Δ}	2021	
c.914G>C			3	2012					3	2019	
c.914G>T			3	2012					3	2019	
c.929del			5	2019							
c.995G>A	2	2018									
c.1002del			5	2016							
c.1016dup	5	2018	5	2019	5	2018	5	2018			
c.1021G>A	2	2018									
c.1040T>A	3	2015							3	2019	
c.1040T>C							3	2018			

c.1058G>A	5	2018									
c.1059G>A	5	2018									
c.1066C>T	5	2017									
c.1072del	5	2017	5	2015							
c.1076_1080del					4	2014					
c.1081T>C	2	2017									
c.1082_1092del	5	2018									
c.1107_1111del					4	2011					
c.1125A>G	2	2015									
c.1149T>C	2	2014									
c.1169A>C	2	2018									
c.1175_1214del	5	2015									
c.1196A>G	3	2015								3	2019
c.1242C>T	2	2014									
c.1287del	5	2008	5	2015	4	2014				5	2021
c.1292dup	5	2014	5	2019							
c.1333G>C							3	2015		3	2019
c.1360_1361del	5										
c.1392C>T	2	2016									
c.1405G>A	3	2014								3	2019
c.1419C>T	2	2018			2	2015	2	2018			
c.1427A>G	2	2018									
c.1434_1435del	5	2015									
c.1441C>G	2	2017									
c.1450G>T	5	2017	5	2015							
c.1486C>T			2	2019							
c.1487G>A			2	2018	2	2009	2	2016			
c.1500T>A	3	2013								3	2019
c.1508A>G	2	2017					3	2017		2*°,3$^{\Delta}$	2021
c.1510C>T	3	2016									
c.1511G>A	2	2018									
c.1521_1531del	5	2017									
c.1533C>G			2	2015							
c.1534C>T	2	2018	2	2018	3	2015	3	2018		2	2021
c.1556del	5	2019	5	2018	5	2016	5	2018			
c.1567T>G	3	2016	3	2014							
c.1568T>G	2	2018			3	2017	3	2017		2*°,3$^{\Delta\square}$	2021
c.1580A>G							3	2015		3	2019
c.1600C>T	5	2016									
c.1616C>T	2	2018									
c.1640A>C					3	2019					
c.1674dup	5	2012									
c.1687C>T	5	2018	4	2013	5	2018				5	2021
c.1695dup	5										
c.1714G>A	3	2016									
c.1722C>T							3	2015		2	2019
c.1723G>A			3	2017							
c.1724A>G	3	2017									
c.1745C>T	3	2015								3	2019
c.1746G>A	2	2015									
c.1772T>C	2	2019	3	2011						2	2021

c.1793T>G	5	2014									
c.1823_1826del	5	2015									
c.1824_1826del	3	2018									
c.1829G>C			3	2014						3	2019
c.1834A>G	3	2016									
c.1840A>T									5	2015	
c.1846_1848del	2	2018									
c.1865C>T			3	2015						3	2019
c.1866G>A	2	2018			2	2016					
c.1879G>A	2	2018	3	2019	3	2016				2*,3[Δ□]	2021
c.188T>A	5	2016									
c.1893A>C	2	2016									
c.1901C>T			3	2017							
c.1911T>C	2	2018	2	2018							
c.1927A>G	3	2015								3	2019
c.1961del					5	2011					
c.1961dup	5		5	2009							
c.1978G>A	3	2014								3	2019
c.2006T>C			3	2017							
c.2019del	5	2018									
c.2024C>G	3	2015								3	2019
c.2027C>T	3	2014								3	2019
c.2043dup			5	2015							
c.2050C>T	3	2014	3	2013	3	2017					
c.2063C>A									3	2018	
c.2083G>T	2	2017									
c.2123C>A	3	2015							3	2015	3 2019
c.2123del			5	2017							
c.2131A>C	2	2019	3	2019						2*,3[Δ]	2021
c.2135G>A	3	2015			3	2018					
c.2138C>G	5	2016									
c.2140A>G					3	2017					
c.2146A>G			3	2008						3	2019
c.2167A>G			2	2012					2	2016	
c.2183G>A	2	2018			3	2016				2*,3[Δ□]	2021
c.2185G>T	5	2010									
c.2191_2227del			5	2016							
c.2196del	5	2018									
c.2245G>A	3	2015								3	2019
c.2252T>C	2	2016									
c.2257dup	5	2015									
c.2293G>T	5	2015									
c.2312T>C	2	2019									
c.2315T>C	3	2015							2	2019	1*,2[Δ○] 2021
c.2336C>T									3	2018	
c.2347A>G	2	2016			2	2018					
c.2351_2357del	5	2017	5	2019							
c.2352G>A	2	2017									
c.2368A>G	2	2016									
c.2389G>T	5										
c.2403T>G	3	2013	3	2014					3	2017	

c.2412G>C	2	2016									
c.2428A>T			2	2017							
c.2438dup	5		5	2012	5	2014	5	2017			
c.2475del	5	2017	5	2012	5	2016					
c.2477C>A			2	2016							
c.2495C>T	3	2010							3	2019	
c.2503C>T	3	2014							3	2019	
c.2518A>G	2	2016									
c.2521C>T			2	2018	2	2009	2	2018			
c.2522G>A	3	2015							2	2019	
c.2544A>C	3	2011							3	2019	
c.2558ins356	5	2017									
c.2584A>G			3	2013					2	2019	
c.2591C>G	5	2018									
c.2606T>C	3	2018									
c.2643del			5	2010							
c.2662C>T			3	2016							
c.2666C>T			3	2018							
c.2668G>A			3	2019	3	2017					
c.2681_2682del	5	2015	5	2019							
c.2685_2686del	5	2018									
c.2692_2693ins			5	2017							
c.2692A>G							3	2015	3	2019	
c.2727_2730del	5	2017									
c.2765C>G			3	2019							
c.2773A>G	2	2017	3	2014					2*,3$^{\Delta}$	2021	
c.2783G>A	2	2017									
c.2798G>A	2	2018	3	2016					2*,3$^{\Delta}$	2021	
c.2814A>G	2	2015									
c.2836A>G	2	2016									
c.2849C>T	2	2018									
c.2864C>A	5	2015									
c.2869C>T	5	2016									
c.2933dup			5	2012	5	2018					
c.2981_2982del	5	2015									
c.2989_2990dup	5	2015	5	2009			5	2016			
c.3003A>G	2	2014	2	2016							
c.3005del			5	2015							
c.3022A>G			2	2016			2	2017			
c.3041T>A	3	2012	2	2016					3	2021	
c.3048_3052dup	5	2018	5	2019							
c.3083G>A							2	2016			
c.3084_3094del	5	2018	5	2015	5	2009	5	2016			
c.3085A>G	3	2016									
c.3119G>A					2	2009					
c.3126C>G							3	2018			
c.3178G>T	5	2018	5	2015			5	2015			
c.3181del	5	2018									
c.3185G>T	3	2015							3	2019	
c.3210A>C			2	2019							
c.3228_3229del	5	2018	5	2019	4	2012	5	2018	5	2021	

c.3233C>T			3	2012					3	2019	
c.3257T>A	5	2019									
c.3270A>G	2	2016									
c.3296C>T					2	2013					
c.3302G>A			2	2013							
c.3319G>T	5	2018			4	2010			5	2021	
c.3327_3329del					3	2013					
c.3327A>C	2	2014									
c.3328_3330del	2	2018									
c.3329dup	5	2018									
c.3331_3334del	5	2018	5	2015	5	2018					
c.3344_3346del	3	2019	3	2018							
c.3377C>T	3	2016									
c.3378A>G	2	2016									
c.3392A>G							3	2018			
c.3400G>T	5	2015									
c.3407C>G			3	2013					3	2019	
c.3418A>G			2	2018	2	2009	2	2016			
c.3448C>T	2	2018									
c.3454G>A	2	2018	3	2018					2	2021	
c.3477_3479delAA AinsC	5	2017									
c.3477A>C	2	2016									
c.3541G>A	3	2015			3	2019					
c.3544C>T	5	2014									
c.3554A>G			3	2019							
c.3555G>T	2	2019									
c.3600G>C	2	2018									
c.3607C>T	5	2017	5	2018	5	2018	5	2016			
c.3608G>A	3	2012							1	2019	
c.3629_3630del	5	2015									
c.3640G>A	3	2010	2	2019					2	2021	
c.3644_3648del	5	2016									
c.3657G>C	3	2012							2	2019	
c.3659A>T	2	2018			3	2012	3	2018	2*,3[Δ□○]	2021	
c.3689T>G	5										
c.3700_3704del	5	2017					5	2016			
c.3708T>G	2	2018			2	2016	2	2015			
c.3710delT	5	2016									
c.3713C>T			3	2019							
c.3722C>A	2	2019									
c.3740T>C	2	2019									
c.3748G>A			2	2017	2	2012	2	2019			
c.3756_3759del	5	2015	5	2012							
c.3767C>T							3	2019			
c.3770_3771del	5										
c.3779del							5	2015			
c.3813dup	5	2015									
c.3817C>T	5										
c.3824T>C							3	2017			
c.3835del	5	2017									

c.3874del	5										
c.3889T>C	3	2014								3	2019
c.3937C>T			5	2015							
c.3965A>G	2	2018									
c.3966del	5	2017	5	2018							
c.4035del	5	2017	5	2014	5	2015	5	2017			
c.4036_4038del	3	2017									
c.4036G>A	3	2014								2	2019
c.4039A>G			2	2018	2	2009					
c.4045A>C	3	2015								2	2019
c.4065_4068del	5	2019	5	2017	5	2017	5	2018			
c.4073A>G	3	2018									
c.4096+3A>G	3	2019	2	2017			3	2017		3	2021
c.4096+18T>C			2	2018							
c.4096+30C>T							3	2018			
c.4097-20C>T	2	2015									
c.4097-10G>A			3	2014							
c.4097-2A>G					5	2019					
c.4113G>A			2	2014			2	2017			
c.4132G>A					2	2013					
c.4146_4155dup	5	2016									
c.4185+16G>A			3	2019							
c.4185+21_4185+22del							2	2018			
c.4185+30G>A			3	2018			3	2018			
c.4186-11C>T					2	2015					
c.4186C>T	5	2014									
c.4288C>G	2	2018									
c.4300del	5		5	2015	4	2016				5	2021
c.4308T>C					2	2010					
c.4315C>T	2	2019	3	2019	3	2018	3	2018		2*,3[Δ□○]	2021
c.4327C>G	2	2014									
c.4327C>T	5	2017									
c.4347A>G	2	2014									
c.4357+17A>G			2	2018	2	2015	2	2017			
c.4358-10C>T	2	2017									
c.4364T>C	2	2016									
c.4441G>A	2	2016									
c.4484G>A	5	2015			5	2015	4	2015		5	2021
c.4484+3A>C	4	2016									
c.4484+14A>G			2	2018							
c.4484+61G>C			3	2019							
c.4484+181_4484+182del					2	2010					
c.4485-44C>T			2	2014							
c.4485-10A>G	3	2015									
c.4501T>A	2	2018									
c.4508C>A	5	2017									
c.4515T>C	2	2016									
c.4532A>C	2	2017									
c.4574_4575del			5	2017							
c.4579G>T							4	2015			

c.4603G>A	3	2016									
c.4605G>A	2	2018									
c.4612C>T			5	2015							
c.4636G>A	2	2016									
c.4636G>T	2	2016							2	2018	
c.4644G>A	2	2018									
c.4675+1G>A			5	2017							
c.4675+28A>G			2	2013							
c.4675G>A	5	2018									
c.4676-19C>T	2	2014									
c.4676-8C>G	2	2016									
c.4676-7C>T	2	2015									
c.4683C>A									2	2016	
c.4689C>G	5		5	2015							
c.4718A>G	2	2018									
c.4725T>G			2	2015							
c.4745del	5	2019	5	2019	5	2010					
c.4750G>T	3	2011									2 2019
c.4765C>T	2	2018									
c.4766G>A	2	2017									
c.4775del			5	2019							
c.4798T>C	2	2016									
c.4799T>A			4	2014					4	2015	
c.4812A>G			2	2013	2	2010					
c.4837A>G					2	2010					
c.4860T>C	2	2016									
c.4882A>G	2	2016									
c.4883T>C			2	2018	2	2013					
c.4884G>A			3	2017							
c.4930G>T									5	2017	
c.4932_4933dup	5	2013									
c.4941C>A					2	2013					
c.4956G>A					2	2009					
c.4964_4982del			5	2019							
c.4964C>T	4	2016									
c.4972del	5	2019	5	2007							
c.4986+1G>T	5	2014	5	2015							
c.4987-20A>G			2	2018							
c.4987-4T>G	2	2018									
c.4992C>T					2	2016					
c.5002T>C					3	2017					
c.5005G>T	2	2018									
c.5017_5019del	4	2018									
c.5030_5033del	5	2017									
c.5030_5033dup	5	2017									
c.5037A>G									2	2017	
c.5047G>T	5	2018	5	2008	4	2014					5 2021
c.5049G>A	2	2017									
c.5074+2T>C	4	2014									
c.5075-53C>T			2	2019							
c.5075-2A>C	5	2018	5	2015	5	2014	5	2017			

c.5075A>C	4	2017									
c.5095C>T	5	2017	5	2014							
c.5096G>A	4	2018	4	2013	5	2019	5	2017	5	2021	
c.5100A>G	2	2019									
c.5117G>A			4	2016							
c.5117G>C	2	2017			2	2015					
c.5123C>A			4	2010							
c.5123C>T	3	2018									
c.5124G>A	2	2015									
c.5125G>A	3	2017	3	2016	3	2015					
c.5131A>C	3	2017									
c.5153-31A>G			3	2012			3	2017			
c.5153-26A>G			3	2018							
c.5153-1G>C			5	2019							
c.5153G>C	4	2015	4	2008							
c.5158A>G					2	2018					
c.5175A>G	2	2016	2	2018							
c.5193+2del	5	2016									
c.5193+43_5193+46del			3	2019							
c.5194-30T>C							3	2016			
c.5212G>A	4	2017									
c.5213G>A	5		4	2010					5	2021	
c.5248A>T					4	2018					
c.5251C>T	5	2018									
c.5258G>C	4	2018					4	2017			
c.5266dup	5	2018	5	2019	5	2018	5	2018			
c.5277+5A>G			2	2014							
c.5277+48_5277+59dup					2	2013					
c.5277+48_5277+60dup			2	2012							
c.5278-14C>G	2	2018	2	2014			2	2018			
c.5306A>G	2	2017									
c.5309G>T	4	2018									
c.5326C>T	2	2018									
c.5332+4A>G	3	2017					3	2019			
c.5333-8C>T	2	2015									
c.5333-3del							3	2018			
c.5346G>A	5	2018									
c.5347A>C	2	2017									
c.5348T>C	2	2017	3	2018					2	2021	
c.5377A>T	5	2017									
c.5406+8T>C			2	2014							
c.5406+33A>T			2	2018							
c.5407-36G>T			2	2018							
c.5407-25T>A	4	2018	4	2019							
c.5407-10G>A	5	2017									
c.5407-2A>G	5	2015					5	2016			
c.5411T>A					2	2018					
c.5412C>T	2	2017	2	2019							
c.5429T>C	3	2018									

c.5434C>G	5	2018						
c.5467+148del					2	2012		
c.5477A>T	2	2017			3	2016	3	2017	2	2021
c.5503C>T	5	2014	5	2019						
c.5504G>A	3	2018	3	2018	3	2017				
c.5511G>A	5	2019	5	2019						
c.5511G>T			4	2014						
c.5513T>G	4	2017	4	2017			4	2017		
c.5534del	5	2018	5	2019						
c.5535C>A			5	2015						
c.5576C>G			3	2019			3	2018		

Note: For *BRCA1* variants with conflicting classifications after reassessment, the following symbols indicate the corresponding laboratories: * = OUH, $^{\Delta}$ = HUH, $^{\square}$ = UNN, $^{\circ}$ = TUH.

Functional analyses of rare germline missense *BRCA1* variants located within and outside protein domains with known functions

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Abstract

The BRCA1 protein is implicated in numerous important cellular processes to prevent genomic instability and tumorigenesis, and pathogenic germline variants predispose carriers to hereditary breast and ovarian cancer (HBOC). Most functional studies of missense variants in *BRCA1* focus on variants located within the RING, coiled-coil and BRCT domains, and several missense variants in these regions have been shown to be pathogenic. However, the majority of these studies focus on domain specific assays, and have been performed using isolated protein domains and not the full-length BRCA1 protein. Furthermore, it has been suggested that *BRCA1* missense variants located outside domains with known function are of no functional importance, and could be classified as (likely) benign. However, very little is known about the role of the regions outside the well-established domains of BRCA1, and only a few functional studies of missense variants located within these regions have been published. In this study, we have therefore functionally evaluated the effect of 14 rare *BRCA1* missense variants considered to be of uncertain clinical significance and located outside the well-established domains, as well as one variant within the RING domain. In order to investigate the hypothesis stating that most BRCA1 variants located outside the known protein domains are benign and of no functional importance, multiple protein assays including protein expression and stability, subcellular localisation and protein interactions have been performed, utilising the full-length protein to better mimic the native state of the protein. Two variants located outside the known domains (p.Met297Val and p.Asp1152Asn) were found to make the BRCA1 protein more prone to proteasome-mediated degradation. In addition, two variants (p.Leu1439Phe and p.Gly890Arg) also located outside known domains, were found to have reduced protein stability compared to the wild type protein. These findings indicate that also variants located outside the RING, BRCT and coiled-coiled domains could affect the BRCA1 protein function. For the nine remaining variants, no significant effect on BRCA1 protein functions were observed. Based on this, reclassification of seven variants from VUS to likely benign could be suggested.

Introduction

Through interaction with a myriad of protein partners, the multifunctional BRCA1 protein is involved in numerous important cellular processes to prevent genomic instability and tumorigenesis. While pathogenic germline alterations including missense variants in *BRCA1* predispose carriers to hereditary breast and ovarian cancer (HBOC), the role of variants of uncertain significance (VUSs) is unclear [1]. Rare missense variants constitute a major part of all *BRCA1* VUSs, and are particularly challenging to classify due to limited or conflicting evidence.

The *BRCA1* gene encodes a large protein of 220 kDa, primarily located in the nucleus, which consists of several functional domains (Figure 1). The N-terminal RING domain (aa 22-64) binds to BRCA1-Associated RING Domain protein 1 (BARD1), where heterodimerisation of the complex provides E3 ubiquitin ligase activity [2-4]. Two nuclear localisation sequences (NLS) (aa 503-508 and 607-614) allocate the BRCA1 protein to the nucleus where it exerts its functions. The coiled-coil domain (aa 1364-1437) located towards the C-terminal is involved in binding to Partner And Localiser of BRCA2 (PALB2). Through the BRCA1 C-terminal (BRCT) domain (aa 1646-1736 and 1760-1855), BRCA1 interacts with multiple proteins involved in transcription and DNA damage response [5, 6]. In addition to the established protein domains, BRCA1 contains an approximately 1500 residue unstructured central non-conserved region, of which very little is known [7].

Functional assays are considered as evidence of supportive to very strong strength for variant classification in the ACMG-AMP guidelines (BS3 or PS3 evidence) [8, 9]. According to the *BRCA1* specific guideline for variant interpretation from CanVIG-UK, five functional protein studies are suggested with specific recommendations regarding the strength of their respective functional evidence [10-15]. However, only two of these studies use the full-length BRCA1 protein [10, 11]. This is also the case for several other BRCA1 functional studies published to date, which focus primarily on variants located in the RING and BRCT domains using plasmid constructs expressing only parts of the full-length protein [16-21]. In addition, several of the previously published studies perform assays to study only one of the multiple functional characteristics of the BRCA1 protein separately, like ubiquitination, transcriptional activation or homologous recombination repair (HRR). However, as *BRCA1* VUSs are distributed throughout the entire protein including regions outside well-established domains, examining only a single assay may be misleading [22]. Hence, to clarify how variants in the more non-

conserved parts of the protein potentially can affect its functions, there is a need for several functional assays utilising the full-length protein to mimic the more native state of the BRCA1 protein. Some of the protein functions of BRCA1 also involve several domains of the protein, and consequently, there is a need for multiple functional assays covering different activities.

Several publications have suggested that most *BRCA1* missense substitutions located outside the well-established and conserved RING, coiled-coil and BRCT domains could be classified as (likely) benign, arguing that pathogenic missense variants are infrequent in these regions, which are thought to tolerate variations and be without essential functions [23-25]. In a recent publication, classification of *BRCA1* missense variants available in the public database ClinVar was used to illustrate this, and the authors suggest incorporation of a criteria regarding coldspots to improve the ACMG-AMP guidelines for *BRCA1* variant interpretation, as a counterweight to hotspots [26]. Noteworthy, a coldspot criteria is included in the BP1 evidence in the *BRCA1/BRCA2* gene-specific guidelines for variant interpretation from CanVIG-UK, which states that the location of a missense variant outside the RING, coiled-coil and BRCT domains is a supporting evidence towards benign effect [27]. On the other hand, the approximately 1500 residue central region of BRCA1 has been suggested to act as a long flexible scaffold for intermolecular interactions which obtain a more ordered structure upon binding to protein partners, and may thus still be functionally important in the DNA damage response [7, 28-31]. Furthermore, amino acid residues located outside well-established domains in the primary structure of the polypeptide chain can potentially interact with or become part of important structural and functional elements in the native folded three-dimensional structure of the BRCA1 protein. This indicates that replacing amino acid residues located outside an important protein domain could still possibly affect both the structure and function of the protein.

The purpose of this study was therefore to functionally characterise a set of 14 *BRCA1* VUSs, of which 13 variants are located outside the known domains, by multiple different protein assays utilising the full-length BRCA1 protein. The *BRCA1* VUSs were selected from our recently published study of *BRCA1* variants detected in families with suspected HBOC in Norway, "*BRCA1* Norway" [32]. Since the majority of the VUSs investigated in this study are located outside the known protein domains of BRCA1, we aimed to use not only BRCA1 specific assays, but also more general protein assays to assess their impact on protein expression, protein stability and subcellular localisation. Based on this, we wanted to investigate the hypothesis stating that *BRCA1* variants located outside the known protein

domains are benign and of no functional importance. Furthermore, we aimed to use the data gathered from the different functional assays, in combination with other available information, as a tool to clarify the pathogenicity of these variants.

Materials and methods

Plasmids and construction of *BRCA1* variants

The plasmid pDEST-mCherry-LacR-BRCA1 encoding mCherry-tagged wild type (WT) full-length human BRCA1 protein was a gift from Daniel Durocher (Addgene plasmid #71115; http://n2t.net/addgene:71115; RRID:Addgene_71115) [33]. This plasmid will hereafter be assigned *BRCA1* WT, or WT only. The *BRCA1* missense variants (listed in Table 1) were introduced in the WT plasmid using the QuikChange II XL Site Directed Mutagenesis Kit (Agilent Technologies, California, USA). Primers used to produce variants of interest and control variants are available upon request. The empty vector (EV) plasmid pDEST-mCherry-LacR, hereafter assigned EV, was also kindly provided by Daniel Durocher [33]. All plasmids were prepared by QIAfilter Plasmid Maxi Kit (QIAGEN, Hilden, Germany), and the presence of the altered variants, in addition to the whole *BRCA1* insert, were verified by Sanger sequencing. The variants were all selected from our previous study "*BRCA1* Norway" and were reported as VUSs in ClinVar or classified as VUS by one or more of the Norwegian medical genetic departments at the time of selection [32]. Some of the variants were classified as both VUS and likely benign by different departments, and these were specifically included aiming to harmonise the variant classification between the different departments. Intentionally, variants throughout the whole *BRCA1* gene were selected, and all variants except one (located within the RING domain) are located outside the well-established RING, coiled-coil and BRCT domains (Figure 1). For each assay, benign and pathogenic control variants were chosen. If possible, variants tested previously by the same type of assay were preferred as controls. A recurring issue and a limitation for all assays performed in this study was the lack of well-established relevant pathogenic missense control variants located in the regions outside of the known domains. No pathogenic variants outside of these regions were found in ClinVar or the literature. This made it difficult to fulfil the requirement of a sufficient number of control variants as suggested by Brnich *et al.* [9]. For investigations of co-immunoprecipitation assays with BARD1 and PALB2, controls were chosen from the relevant regions (RING and coiled-coiled domain, respectively).

In the co-immunoprecipitation assay, the plasmids pcDNA6.2-BARD1-V5, hereafter called *BARD1-V5* WT, and pDEST-FRT/T0-Flag-PALB2, hereafter called *Flag-PALB2* WT, were used. *BARD1-V5* WT was a gift from Masanori Kurihara and Atsushi Iwata [34], and *Flag-PALB2* WT was a gift from Daniel Durocher (Addgene plasmid #71114; http://n2t.net/addgene:71114 ; RRID:Addgene_71114) [33]. The corresponding empty vectors (pcDNA6.2 -V5 and pDEST FRT/TO-FLAG) were used as controls.

Cell culture and transfection

HEK293FT and MDA-MB-231 cells were cultured in DMEM high glucose GlutaMAX™ medium or DMEM medium (Thermo Fisher Scientific, Massachusetts, USA), respectively, supplemented with 10% FBS (Thermo Fisher Scientific) and 1% PenStrep (Sigma-Aldrich, Missouri, USA). Both cell lines were maintained in 5% CO_2 at 37˚C. JetPrime® (Polyplus-Transfection, Illkirch-Graffenstaden, France) was used for transient transfection of the cells according to the manufacturer's protocol.

Assessment of BRCA1 protein expression by immunoblotting

For western blot analyses, cells were lysed in RIPA buffer (supplemented with cOmplete Mini EDTA-free Protease inhibitor cocktail tablets, Roche, Basel, Switzerland) 48 hours post transfection, and centrifuged at 13 000 g for 10 minutes at 4°C. Following measurements of the protein concentration by Pierce BCA protein assay kit (Thermo Fisher Scientific), 5 µg total protein were analysed by SDS-PAGE using 3-5% Tris-Acetate gels (150 V, 75 minutes) and transferred to a nitrocellulose membrane (30 V, 60 minutes). One BRCA1 WT sample was always included in each gel to ensure comparable results with the variants investigated. To detect BRCA1 protein, the following antibodies were used: primary anti-BRCA1 (sc-6954, Santa Cruz Biotechnology, Texas, USA) and secondary m-IgGκ BP-HRP (sc-516102, Santa Cruz). Anti-β-Actin antibody (sc-47778, Santa Cruz) was used as loading control and for quantification of relative BRCA1 protein expression levels. Proteins were visualised using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and the ChemiDOC™ MP imaging system. The signals were quantified using the Image Lab™ Software from BioRad (version 6.0). As benign controls for protein expression, the variants p.Lys45Gln, p.Arg504His, and p.Val1378Ile were included (all classified as benign by the ENIGMA expert panel) [35]. As negative controls for protein expression, empty vector and the pathogenic variants p.Ala1708Glu and p.Val1838Gly were included [17, 36].

RNA purification and qPCR

HEK293FT cells were seeded in 12-well plates (0.35 x 10^6 cells/well), and transfected with *BRCA1* WT and variant plasmids. Forty-eight hours after transfection, RNA was purified using RNeasy Mini Kit (QIAGEN) as described by the manufacturer. The quality of the RNA samples was analysed by the Agilent RNA 2200 ScreenTape System. Purified RNA (1 µg) was used to synthesise single-stranded cDNA applying the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen). The synthesised cDNA was then used as a template for analysis of expression of *BRCA1* variants and the house keeping gene β-actin by qPCR using TaqMan® Gene Expression Assays (Applied Biosystems, Life technologies).

MG132 assay for assessment of proteasomal degradation

HEK293FT cells were seeded in 12-well plates (0.35 x 10^6 cells/well) and transfected with *BRCA1* WT and variants. Twenty-four hours post transfection, cells were incubated with 20 µM MG132 (Sigma-Aldrich) dissolved in DMSO or DMSO only for 24 hours. Cells were then lysed in 100 µl RIPA buffer (with protease inhibitor). Samples containing 10 µg of protein were analysed by western blotting, and compared to WT and p.Val1838Gly used as benign and pathogenic controls, respectively.

Cycloheximide chase assay for measurement of BRCA1 protein stability

HEK293FT cells were seeded in 12-well plates (0.35 x 10^6 cells/well) and transfected with *BRCA1* WT and variants. Twenty-four hours post transfection, the medium was removed and replaced with fresh medium containing 50 µg/ml cycloheximide (Sigma-Aldrich) dissolved in DMSO or DMSO only. Cells were harvested after 0, 2 and 8 hours treatment. For each time point, the cells were lysed in RIPA buffer (supplemented with protease inhibitor) and frozen at -20°C immediately after harvest. Centrifugation (13 000 g, 10 minutes, 4°C) was performed for all samples in parallel >24 hours post freezing. Samples containing 5 µg of protein were analysed by western blotting. As benign controls for protein stability, WT and three benign variants (p.Lys45Gln, p.Arg504His, p.Vall378Ile) were included. As pathogenic controls for protein stability, two variants known to harbour reduced protein stability were used (p.Cys49Tyr and p.Ala1708Glu) [36-38]. The resulting % protein expression presented are relative to the protein levels for the corresponding variant at the starting point (0 hours, corresponding to 100%).

Fractionation assay for assessment of subcellular localisation

Subcellular localisation was tested by a fractionation assay separating the cytosolic and nuclear cell fractions [39, 40]. HEK293FT cells seeded in 10 cm dishes (4.8 x 10^6 cells/dish) were transfected with 10 µg plasmid encoding *BRCA1* WT or variants. Forty-eight hours post transfection, the cells were washed in PBS and pelleted at 1200 rpm for 5 minutes, before resuspending the cells in 250 µl buffer A (10 µM HEPES pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.10% IGEPAL, 0.5 mM DTT, EDTA free protease inhibitor) and incubating for 30 minutes. The suspension was then pelleted at 13 000 rpm for 5 minutes at 4°C. The resulting supernatant, which is the cytosolic fraction, was then frozen at -80°C for later analyses. The pellet was washed once with 100 µl buffer A and resuspended in 100 µl buffer B (20 mM HEPES pH 7.8, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, EDTA free protease inhibitor) by pipetting up/down 30 times. After 30 minutes incubation on ice with vortexing every minute, the resuspension was centrifuged at 13 000 rpm for 15 minutes at 4°C. The resulting supernatant, which is the nuclear fraction, was then frozen at -80°C for later analyses. Cytosolic and nuclear samples containing 5 µg of total protein were analysed by western blotting. Anti-Topoisomerase IIα (D10G9, Cell Signaling Technology, Massachusetts, USA) and Anti-HSP 90α/β (sc-13119, Santa Cruz) were used to confirm the purity of the nuclear and cytosol fractions, respectively, and were used for normalisation. The % of BRCA1 protein in each fraction was then calculated, and the level of protein in the nucleus was presented.

Co-immunoprecipitation assay

HEK293FT cells were seeded in 10 cm petri dishes (4.8 x 10^6), and co-transfected with 5 µg plasmid encoding *BRCA1* WT or the selected *BRCA1* variants in combination with either 5 µg plasmid encoding *BARD1-V5* WT or *Flag-PALB2* WT. After 48 hours, cells were lysed in 500 µl ice-cold IP Lysis/Wash Buffer (supplemented with protease inhibitor) per dish. The cell lysate was centrifuged at 13 000 g for 10 minutes at 4°C, before measuring protein concentration by Pierce BCA protein assay kit. Co-immunoprecipitation (Co-IP) was performed using the Dynabeads™ Protein G Immunoprecipitation Kit (Invitrogen) according to the manufacturer's protocol, with the following specifications: 5 µg of V5 antibody (for WT *BARD1-V5*) or 5 µg of Flag antibody (for WT *Flag-PALB2*) was coupled to 50 µl magnetic beads. Equal amounts of cell lysate proteins (2 mg, input) were incubated with the antibody-coupled beads for 90 minutes at 4˚C. After non-denaturing elution of the protein complexes, the proteins bound to the beads (IP) were separated by SDS-PAGE, and BRCA1 WT or variants in combination with BARD1-V5 or Flag-PALB2 were visualised by western blotting using

anti-V5 (46-0705, Invitrogen) or anti-Flag (F1804, Sigma-Aldrich), respectively. BRCA1 protein levels in the IP samples were quantified and normalised to the anti-V5 signal or anti-Flag signal in the IP samples. The data for each of the variants were presented as % compared to WT (set to 100%). As controls for the Co-IP assay with BRCA1-PALB2, the benign variant p.Val1378Ile and the pathogenic variant p.Met1411Thr (both located in the coiled-coil domain of BRCA1), were included [41, 42]. As controls for the Co-IP assay with BRCA1-BARD1, the benign variant p.Lys45Gln and the pathogenic variant p.Cys39Tyr (both located in the RING domain of BRCA1), were included [37, 38].

Statistics

All experiments were carried out on at least three independent occasions unless otherwise specified in the figure legends, with the exception of the empty vector, which was performed in one replicate only. The standard deviations were calculated for WT and each variant in all assays. The statistical significance was evaluated with the Student's t-test with p values < 0.05.

Assessment of variant classifications

The Alamut Software (Version 2.15, SOPHiA GENETICS) and the Human Gene Mutation Database (HGMD) professional 2022.1 (QIAGEN) was used for gathering information on the *BRCA1* variants. Reinterpretation of the variants was performed based on new knowledge using the ACMG-AMP criteria supplemented with the *BRCA1/BRCA2* gene-specific criteria by CanVIG-UK [8, 15].

Results

Effects of *BRCA1* variants on protein expression

To test the effect of the selected *BRCA1* missense variants (Figure 1) on the protein expression level, the corresponding plasmids were transfected into HEK293FT cells, and the cell lysates were analysed by western blot analysis (Supplementary Figure 1). As expected, a band located just above 220 kDa corresponding to the theoretical molecular weight of mCherry-BRCA1 (248 kDa) was detected for both the WT and variants. Nine variants had similar relative expression levels as the WT (100%) and/or benign controls (44-70%). Four of the variants (p.Leu52Phe, p.Met297Val, p.Asp1152Asn and p.Leu1439Phe) displayed severely reduced protein levels, i.e. <20% protein compared to WT, similar to the included pathogenic controls (9-14%) (Figure 2). In addition, the variant p.Leu523Val was found to have reduced protein expression (27%) compared to the WT, at an intermediate expression level between pathogenic (9-14%) and

benign controls (44-70%). For comparison, the assay was repeated in MDA-MB-231 cells, where a similar trend for protein expression was seen (results not shown).

qPCR for assessment of mRNA levels

The four protein variants (p.Leu52Phe, p.Met297Val, p.Asp1152Asn and p.Leu1439Phe) found to be expressed at levels lower or similar to the included pathogenic controls in HEK293FT cells, were subsequently analysed by qPCR to investigate if the low protein expression was caused by a reduction of the mRNA levels. After normalisation of the data by actin, the relative mRNA levels for each variant compared to the *BRCA1* WT were calculated (Figure 3). The results suggest that the plasmids encoding p.Leu52Phe, p.Met297Val, p.Asp1152Asn and p.Leu1439Phe produce similar amounts of mRNA as the WT plasmid. Thus, for these variants, reduced protein levels are unlikely caused by reduced transcription or transfection efficiency, but are more likely caused by increased protein degradation or reduced stability.

MG132 assay for assessment of proteasomal degradation

To check if the low protein levels in HEK293FT cells observed for p.Leu52Phe, p.Met297Val, p.Asp1152Asn and p.Leu1439Phe could be due to degradation by the ubiquitin-proteasome system, transfected HEK293FT cells were treated with proteasome inhibitor MG132 for 24 hours. As shown in Figure 4, protein expression clearly increased for the pathogenic control (p.Val1838Gly) and three of the variants (p.Leu52Phe, p.Met297Val and p.Asp1152Asn) after treatment with MG132 compared to the control samples treated with DMSO only. For the variant p.Leu1439Phe, comparable amounts of protein were observed in the MG132 treated sample and the DMSO control sample.

Cycloheximide chase assay for assessment of protein stability

For the BRCA1 variants which showed protein expression levels above 20% compared to the BRCA1 WT protein in the western blot analysis (Figure 2), including the variant p.Leu1439Phe which showed equal amounts of protein in the MG132 assay (Figure 4), the protein stability was analysed by cycloheximide chase assay to follow protein degradation over time in transfected HEK293FT cells. The results from one representative replicate after 0, 2 and 8 hours treatment with the protein synthesis inhibitor cycloheximide compared to DMSO only for a minor selection of variants (p.Gly890Arg and p.Leu1439Phe) and controls (p.Arg504His and p.Ala1708Glu) are shown in Supplementary Figure 2. Figure 5 shows the mean % BRCA1 protein level remaining for each variant in transfected HEK293FT cells treated with

cycloheximide for 8 hours. For the BRCA1 WT, the protein expression level decreased to 83% after 8 hours treatment with cycloheximide. For all the variants, including the benign controls, a more prominent degradation of BRCA1 protein was observed during cycloheximide treatment. The protein levels for all the benign controls were reduced to 28-34%, while there was respectively 0% and 9% protein detected for the pathogenic controls p.Ala1708Glu and p.Cys39Tyr. Protein levels of the two BRCA1 VUSs p.Gly890Arg and p.Leu1439Phe were reduced to 11% and 10%, respectively, after 8 hours cycloheximide treatment, similar to the pathogenic controls. The nine remaining variants showed reduction in protein levels comparable to the benign controls after 8 hours treatment.

Assessment of subcellular localisation by fractionation assay

According to literature, the BRCA1 protein is known to be mainly located to the nucleus, and the two NLS of BRCA1 are located at aa 503-508 and 607-614 [43]. To investigate if some of the variants of interest could alter the nuclear localisation of the protein, subcellular localisation was assessed by a nuclear/cytosol fractionation assay. This was especially interesting to evaluate for the variants p.Lys503Arg, p.Arg504Cys, p.Arg610Thr and p.Arg612Gly, which are all located within the nuclear localisation sequences. Similar to the BRCA1 WT protein (84% located in the nucleus), all analysed variants were found to be mainly located in the nucleus fraction (Supplementary Figure 3).

Assessment of protein-protein interactions with BARD1 and PALB2 by co-immunoprecipitation

Co-IP assays were performed to test the potential effect of the VUSs on the binding of BRCA1 protein to two of its binding partners: BARD1 and PALB2. The resulting western blots for the WT, EV, control variants and a selection of the analysed VUSs are illustrated in Figure 6A and B. As seen in the blots, the BRCA1 WT protein captured both PALB2 (Figure 6A) and BARD1 (Figure 6B). It was observed a strong binding to the respective benign controls p.Val1378Ile and p.Lys45Gln, and a weak binding to the respective pathogenic controls p.Met1411Thr and p.Cys39Tyr. Mean values for all variants (% binding capacity compared to WT) are shown in Figure 6C and D. In the initial analysis, the variant p.Lys503Arg appeared to have a reduced binding to PALB2 (Figure 6C), but this interaction was shown to be similar to the WT/benign controls when quantifying against the amount of the variant input sample (not shown). Thus, none of the variants of interest showed to have significantly reduced binding to either BARD1 or PALB2.

Summary of functional assays

When summarising the data from each of the different functional assays (Table 1), five *BRCA1* variants were found to have one or more features strongly deviating from the WT protein, while the remaining nine variants showed no or only minor deviations. The data gathered throughout the study were combined with other relevant information and used to suggest an updated classification of the pathogenicity of these rare *BRCA1* variants (Table 2). The molecular properties and previous known information on each of these variants will be discussed below.

Discussion

In this study, we have examined the effects of 14 rare *BRCA1* missense VUSs to investigate the hypothesis stating that no pathogenic *BRCA1* missense variants are present outside of known protein domains in BRCA1 [23-25]. We have investigated the effect of each variant by multiple functional protein assays using the full-length BRCA1 protein to better mimic the native state of the protein.

BRCA1 protein domains and amino acid conservation

Use of the BP1 criteria in the gene-specific *BRCA1/BRCA2* guidelines from CanVIG-UK indicating that no pathogenic *BRCA*1 missense variants are present outside of known protein domains, was debated in our "*BRCA1* Norway" publication [15, 32]. Counterarguments stated that amino acid residues located outside well-established domains in the primary structure of the polypeptide chain can potentially interact with or become part of important structural and functional elements in the native folded three-dimensional structure of the BRCA1 protein. Thus, replacement of amino acid residues located outside an important domain in the primary structure could possibly affect both structure and function of the protein. In fact, it has been suggested that the majority of loss of function missense mutation are indirectly caused by destabilisation of the protein's three-dimensional-structure, rather than directly disrupting important functional characteristics like binding sites [36, 44-46]. In contrast to the highly conserved RING and BRCT domains for which the structure is known, it has been suggested that the central 1500 residue region of BRCA1 acts as a long flexible scaffold for intermolecular interactions even though the central region lacks substantial conserved motifs [7]. Such intrinsically non-conserved disordered regions are known to obtain a more folded structure upon interaction with its protein partners [7, 28-31]. Thus, although being a disordered region in the absence of its binding partners, this central region might still be functionally important in the DNA damage response [7].

Protein expression and protein stability of the BRCA1 variants

For many genes, the protein expression level of a variant is known to correlate with the pathogenicity of the variant [39, 40]. In contrast, it has been demonstrated that BRCA1 protein variants displaying low protein levels may still sustain structure/function similar to the WT protein, and that variants with protein levels similar to the WT protein may fail to sustain function [12, 47]. These studies were however performed by expression of isolated protein domains and not the full-length BRCA1 protein. The effect of missense changes on the expression of full-length BRCA1 protein, particularly those located outside of the known domains, has to our knowledge not previously been thoroughly investigated. We therefore aimed to investigate the effect of our selected *BRCA1* variants on protein expression levels in HEK293FT cells by western blotting. The benign control variants showed reduced protein level (44-70%) compared to the WT protein, but considerably higher levels than the pathogenic control variants (9-14%). In concordance with previous studies [47], this indicates that even significantly reduced BRCA1 expression levels are sufficient to maintain BRCA1 protein functions, and that protein expression levels do not necessarily correlate with the level of protein activity. In addition, the lower threshold for BRCA1 protein expression associated with pathogenicity is currently unknown. Nine of the investigated variants showed protein expression levels comparable to the benign controls/WT protein (27-60%), while the four variants p.Leu52Phe, p.Met297Val, p.Asp1152Asn and p.Leu1439Phe showed reduced protein levels in the range of the pathogenic controls (7-18%). Low protein expression can be caused by among others, low transcription levels, protein instability or increased protein degradation. Although protein expression analyses alone are not adequate to distinguish between benign and pathogenic variants, protein expression analysis can, in combination with additional protein assays, still provide important insights regarding the underlying mechanism for the loss of protein function. To investigate the cause of the reduced protein levels, we therefore analysed the four variants p.Leu52Phe, p.Met297Val, p.Asp1152Asn and p.Leu1439Phe using qPCR. The mRNA levels for the four variants were found to be in the same range as the WT, indicating that the underlying mechanism for the low protein expression levels is at the protein level.

It has previously been shown that several missense variants in the BRCT domain lead to increased susceptibility to degradation of BRCA1 and destabilisation of the protein structure, among others by the ubiquitin-proteasome system [48-53]. When investigating the aforementioned four variants by inhibiting the ubiquitin-proteasome degradation pathway by MG132, an increased protein level was observed for three of the variants (p.Leu52Phe,

p.Met297Val and p.Asp1152Asn) and the pathogenic control p.Val1838Gly, which has been previously shown to have reduced protein levels in HEK293 cells [17]. This indicates that these variants, of which two are located outside of known protein domains, make the BRCA1 protein more prone to ubiquitin-mediated degradation. In contrast, the variant p.Leu1439Phe seems not to be removed by the proteasomal system.

To evaluate the protein stability of the BRCA1 variants over time, a cycloheximide chase assay was performed for the BRCA1 variants showing protein expression levels comparable to the benign controls/WT protein. In addition, the p.Leu1439Phe variant which was found not to be removed by the proteasomal system, was included. The BRCA1 WT protein showed a stability of 83% after treatment with cycloheximide. All benign control variants surprisingly illustrated 28-34% protein levels compared to the WT protein after eight hours, indicating that a protein variant could have pronounced reduction in stability without affecting the pathogenicity. In comparison, the pathogenic control variants p.Ala1708Glu and p.Cys39Tyr showed respectively 0% and 9% protein expression after treatment with cycloheximide. Similar to the two pathogenic controls, the two VUSs p.Gly890Arg and p.Leu1439Phe showed severely reduced protein stability comparable to the WT protein (11% and 10%, respectively). After cycloheximide treatment, four of the VUSs (p.Lys503Arg, p.Ile925Val, p.Gly933Asp and p.Thr1256Ile) demonstrated protein levels in the range 19-23% of the WT, at an intermediate level between the pathogenic and benign controls. The remaining five VUSs showed protein levels comparable to the benign controls (29-48%). In order to improve the capacity of the assay to better discriminate the benign/pathogenic thresholds, more pathogenic and benign controls should be included in this assay [9].

The effect of BRCA1 variants on BARD1 and PALB2 interaction

The BRCA1 protein is known to interact with a myriad of other proteins. Among others, BRCA1 interacts with BARD1 though the RING domain, and with PALB2 through the coiled-coil domain. Although only one of the 14 VUSs analysed in this study is located in the RING domain, and none in the coiled-coil domain, we wanted to investigate if any of our variants of interest could alter these interactions. The effect of an abolished BRCA1-BARD1 interaction was illustrated by the pathogenic BRCA1 control variant p.Cys39Tyr located in the RING domain. Even though initial protein levels of the variant appeared to be within the normal range (data not shown), this variant demonstrated reduced binding to BARD1 during Co-IP (Figure 6D) and severely reduced protein stability in the cycloheximide chase assay (Figure 5B). The

reduced stability can potentially be explained by the fact that variants impairing the interaction between BRCA1 and BARD1 can result in proteolytic degradation of both proteins, thus our results are in agreement with previously published data [37, 38]. In contrast, the benign control p.Lys45Gln which is also located within the RING domain, showed both normal protein expression levels, stability and BRCA1-BARD1 binding. In the BRCA1-PALB2 assay, the variant p.Met1411Thr located in the coiled-coil domain was used as a pathogenic control. This missense variant has in agreement with our results, previously been shown to abolish BRCA1 interaction with PALB2 [41, 42]. The benign control p.Val1378Ile, equally located in the coiled-coil domain, showed normal BRCA1-PALB2 binding. However, none of the variants of interest showed significantly reduced binding to either BARD1 or PALB2.

Variant interpretation of the investigated VUSs

Even though the general protein based analyses performed in this study are not among the functional assays suggested by CanVIG-UK, our data indicate that the new knowledge could provide useful information regarding the pathogenicity of variants located outside of the known protein domains of BRCA1. We therefore wanted to investigate if our newly achieved functional data could contribute to re-classification of the 14 investigated VUSs (Table 2). In our study, the three variants p.Leu52Phe, p.Met297Val and p.Asp1152Asn were shown to have reduced protein expression levels (<20% protein compared to WT), probably due to removal by proteasomal degradation. The p.Leu52Phe variant has previously been functionally assessed by others, with conflicting results. This variant has been shown to have normal binding to BARD1 [54, 55], normal HRR activity [54, 56], and normal saturation genome editing assay [10]. However, defective ubiquitination [18], impact on centrosome duplication [57], and changes in E3 ligase activity [55] have also been reported. Furthermore, the variant allele frequency in the East Asian population is 0.09% according to the gnomAD database, which is above the expected frequency of a pathogenic variant (BS1 criteria) [15]. In all cases where there were conflicts between our newly achieved functional data and the data in any of the five functional BRCA1 protein studies suggested by the CanVIG-UK, we chose not to include the functional evidence criteria (BS3 or PS3) when classifying the variants [10-15]. Due to the conflicting evidence from functional studies, we therefore still classify p.Leu52Phe as a VUS. For the p.Met297Val variant, no previous experimental evidence demonstrating its impact on protein function has been reported. In cases where the variants of interest were not investigated in any of the functional studies recommended by CanVIG-UK, we chose to apply the functional criteria (PS3 or BS3) as supportive strength. Thus, due to lack of evidence, also p.Met297Val

is still assessed as a VUS. The variant p.Asp1152Asn is by in silico tools predicted as benign, and according to CanVIG-UK, this variant could therefore theoretically be classified as likely benign (BP1 and BP4 criteria). This variant has been shown to harbour normal HRR activity [11, 58], and to be neutral in cisplatin and olaparib assays [11], which would qualify for the BS3 criteria. However, in our study, the variant showed low protein expression when analysed in HEK293FT cells, and even lower in MDA-MB-231 cells (data not shown). Due to conflicting functional evidence, we therefore still chose to classify p.Asp1152Asn as a VUS due to the remarkably low protein expression levels and increased proteasomal degradation, and suggest that the variant should be analysed by further studies. In the initial western blot analysis, the variant p.Gly890Arg showed similar protein expression levels as the benign controls, but a severely reduced protein stability over time compared to the WT in the cycloheximide chase assay. The variant p.Leu1439Phe was shown to have both reduced initial protein expression levels and reduced protein stability over time. Both p.Gly890 and p.Leu1439 are weakly conserved amino acids. For p.Gly890Arg, no experimental evidence demonstrating its impact on protein function have previously been reported. The variant p.Leu1439Phe has been found to be neutral in HRR and a cisplatin sensitivity assay, but showed inconclusive results in a olaparib sensitivity assay. Due to our findings of reduced protein stability, we still assess these variants as VUSs.

For the nine remaining variants, no significant effect on the BRCA1 protein expression, protein stability, subcellular localisation or BARD1/PALB2 interaction were observed. When including information on allele frequency, conservation, literature, and in silico predictions, seven of these variants were suggested reclassified as likely benign. The original and new classifications for each variant are summarised in Table 2.

Conclusion

In this study, we have assessed the effect of 14 *BRCA1* missense VUSs using the full-length protein and multiple functional assays, aiming to investigate the hypothesis stating that no pathogenic *BRCA1* missense variants are present outside of protein domains with known function. Although our findings should be confirmed using additional pathogenic and benign control variants to improve the discrimination, the findings indicate that also variants located outside the RING, BRCT and coiled-coiled domains could affect the BRCA1 protein, and that the BP1 criteria should be used with care. This study also illustrates the importance of not

relying on one functional assay only, but rather to include several assays when investigating variants in the multifunctional BRCA1 protein.

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References

1. Antoniou, A., 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): p. 1117-1130.
2. Baer, R. and T. Ludwig, *The BRCA1/BARD1 heterodimer, a tumor suppressor complex with ubiquitin E3 ligase activity.* Curr Opin Genet Dev, 2002. **12**(1): p. 86-91.
3. Mallery, D.L., C.J. Vandenberg, and K. Hiom, *Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains.* EMBO J, 2002. **21**(24): p. 6755-6762.
4. Xia, Y., et al., *Enhancement of BRCA1 E3 Ubiquitin Ligase Activity through Direct Interaction with the BARD1 Protein.* J Biol Chem, 2003. **278**(7): p. 5255-5263.
5. De Siervi, A., et al., *Transcriptional Autoregulation by BRCA1.* Cancer Res, 2010. **70**(2): p. 532-542.
6. Roy, R., J. Chun, and S.N. Powell, *BRCA1 and BRCA2: different roles in a common pathway of genome protection.* Nat Rev Cancer, 2011. **12**(1): p. 68-78.
7. Mark, W.-Y., et al., *Characterization of Segments from the Central Region of BRCA1: An Intrinsically Disordered Scaffold for Multiple Protein–Protein and Protein–DNA Interactions?* J Mol Biol, 2005. **345**(2): p. 275-287.
8. Richards, S., et al., *Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.* Genet Med, 2015. **17**(5): p. 405-424.
9. Brnich, S.E.A.T., Ahmad N ; Couch, Fergus J ; Cutting, Garry R ; Greenblatt, Marc S ; Heinen, Christopher D ; Kanavy, Dona M ; Luo, Xi ; McNulty, Shannon M ; Starita, Lea M ; Tavtigian, Sean V ; Wright, Matt W ; Harrison, Steven M ; Biesecker, Leslie G ; Berg, Jonathan S, *Recommendations for application of the functional evidence PS3/BS3 criterion using the ACMG/AMP sequence variant interpretation framework.* Genome medicine, 2019. **12**(1): p. 3-3.
10. Findlay, G.M., et al., *Accurate classification of BRCA1 variants with saturation genome editing.* Nature, 2018. **562**(7726): p. 217-222.
11. Bouwman, P., et al., *Functional categorization of BRCA1 variants of uncertain clinical significance in homologous recombination repair complementation assays.* Clin Cancer Res, 2020. **26**(17): p. 4559-4568.
12. Fernandes, V.C., et al., *Impact of amino acid substitutions at secondary structures in the BRCT domains of the tumor suppressor BRCA1: Implications for clinical annotation.* J Biol Chem, 2019. **294**(15): p. 5980-5992.
13. Petitalot, A., et al., *Combining Homologous Recombination and Phosphopeptide-binding Data to Predict the Impact of BRCA1 BRCT Variants on Cancer Risk.* Mol Cancer Res, 2019. **17**(1): p. 54-69.
14. Starita, L.M., et al., *A Multiplex Homology-Directed DNA Repair Assay Reveals the Impact of More Than 1,000 BRCA1 Missense Substitution Variants on Protein Function.* Am J Hum Genet, 2018. **103**(4): p. 498-508.

15. *CanVIG-UK Gene Specific Recommendations: BRCA1/BRCA2* 01.06.2022]; Available from: https://www.cangene-canvaruk.org/gene-specific-recommendations.
16. Langerud, J., et al., *Trans-activation-based risk assessment of BRCA1 BRCT variants with unknown clinical significance.* Hum Genomics, 2018. **12**(1): p. 51-51.
17. Jarhelle, E., et al., *Characterization of BRCA1 and BRCA2 variants found in a Norwegian breast or ovarian cancer cohort.* Fam Cancer, 2016. **16**(1): p. 1-16.
18. Morris, J.R., et al., *Genetic analysis of BRCA1 ubiquitin ligase activity and its relationship to breast cancer susceptibility.* Hum. Mol. Genet, 2006. **15**(4): p. 599-606.
19. Lee, M.S., et al., *Comprehensive Analysis of Missense Variations in the BRCT Domain of BRCA1 by Structural and Functional Assays.* Cancer Res, 2010. **70**(12): p. 4880-4890.
20. Glover, J.N.M., et al., *Structural basis of phosphopeptide recognition by the BRCT domain of BRCA1.* Nat Struct Mol Biol, 2004. **11**(6): p. 519-525.
21. Adamovich, A.I., et al., *The functional impact of BRCA1 BRCT domain variants using multiplexed DNA double-strand break repair assays.* Am J Hum Genet, 2022. **109**(4): p. 618-630.
22. Christou, C.M., et al., *The BRCA1 Variant p.Ser36Tyr Abrogates BRCA1 Protein Function and Potentially Confers a Moderate Risk of Breast Cancer.* PLoS One, 2014. **9**(4): p. e93400-e93400.
23. Easton, D.F., 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): p. 873-883.
24. Tavtigian, S.V., et al., *Classification of rare missense substitutions, using risk surfaces, with genetic- and molecular-epidemiology applications.* Hum Mutat, 2008. **29**(11): p. 1342-1354.
25. Vallée, M.P., et al., *Adding In Silico Assessment of Potential Splice Aberration to the Integrated Evaluation of BRCA Gene Unclassified Variants.* Human Mutation, 2016. **37**(7): p. 627-639.
26. Dines, J.N., et al., *Systematic misclassification of missense variants in BRCA1 and BRCA2 "coldspots".* Genet Med, 2020. **22**(5): p. 825-830.
27. Garrett, A., et al., *Cancer Variant Interpretation Group UK (CanVIG-UK): an exemplar national subspecialty multidisciplinary network.* J Med Genet, 2020. **57**(12): p. 829-834.
28. Radhakrishnan, I., et al., *Solution Structure of the KIX Domain of CBP Bound to the Transactivation Domain of CREB: A Model for Activator:Coactivator Interactions.* Cell, 1997. **91**(6): p. 741-752.
29. Rosen, M.K., et al., *Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein.* Nature, 2000. **404**(6774): p. 151-158.
30. Kriwacki, R.W., et al., *p27 binds cyclin-CDK complexes through a sequential mechanism involving binding-induced protein folding.* Nat Struct Mol Biol, 2004. **11**(4): p. 358-364.
31. Kriwacki, R.W., et al., *Structural studies of p21Waf1/Cip1/Sdi1 in the free and Cdk2-bound state: conformational disorder mediates binding diversity.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11504-11509.
32. Hovland, H.N., et al., *BRCA1 Norway: comparison of classification for BRCA1 germline variants detected in families with suspected hereditary breast and ovarian cancer between different laboratories.* Fam Cancer, 2022.
33. Orthwein, A., et al., *A mechanism for the suppression of homologous recombination in G1 cells.* Nature, 2015. **528**(7582): p. 422-426.
34. Kurihara, M., et al., *Colocalization of BRCA1 with Tau Aggregates in Human Tauopathies.* Brain Sci, 2019. **10**(1): p. 7.
35. *Evidence-based Network for the Interpretation of Germline Mutant Alleles*. 24.01.22]; Available from: https://enigmaconsortium.org/library/general-documents/enigma-classification-criteria/.
36. Gaboriau, D.C.A., et al., *Protein stability versus function: effects of destabilizing missense mutations on BRCA1 DNA repair activity.* Biochem J, 2015. **466**(3): p. 613-624.
37. Tarsounas, M. and P. Sung, *The antitumorigenic roles of BRCA1-BARD1 in DNA repair and replication.* Nat Rev Mol Cell Biol, 2020. **21**(5): p. 284-299.

38. Ruffner, H., et al., *Cancer-Predisposing Mutations within the RING Domain of BRCA1: Loss of Ubiquitin Protein Ligase Activity and Protection from Radiation Hypersensitivity.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5134-5139.
39. Malikova, J., et al., *Functional analyses of HNF1A-MODY variants refine the interpretation of identified sequence variants.* J Clin Endocrinol Metab, 2020. **105**(4): p. e1377-e1386.
40. Althari, S., et al., *Unsupervised Clustering of Missense Variants in HNF1A Using Multidimensional Functional Data Aids Clinical Interpretation.* Am J Hum Genet, 2020. **107**(4): p. 670-682.
41. Sy, S.M.H., M.S.Y. Huen, and J. Chen, *PALB2 Is an Integral Component of the BRCA Complex Required for Homologous Recombination Repair.* Proc Natl Acad Sci U S A, 2009. **106**(17): p. 7155-7160.
42. Woods, N.T., et al., *Functional assays provide a robust tool for the clinical annotation of genetic variants of uncertain significance.* NPJ Genom Med, 2016. **1**(1): p. 16001.
43. Clark, S.L., et al., *Structure-Function Of The Tumor Suppressor BRCA1.* Comput Struct Biotechnol J, 2012. **1**(1): p. e201204005.
44. Yue, P., Z. Li, and J. Moult, *Loss of Protein Structure Stability as a Major Causative Factor in Monogenic Disease.* J Mol Biol, 2005. **353**(2): p. 459-473.
45. Shi, Z., J. Sellers, and J. Moult, *Protein stability and in vivo concentration of missense mutations in phenylalanine hydroxylase.* Proteins, 2012. **80**(1): p. 61-70.
46. Yates, C.M. and M.J.E. Sternberg, *The Effects of Non-Synonymous Single Nucleotide Polymorphisms (nsSNPs) on Protein–Protein Interactions.* J Mol Biol, 2013. **425**(21): p. 3949-3963.
47. Nepomuceno, T.C., et al., *Assessment of small in-frame indels and C-terminal nonsense variants of BRCA1 using a validated functional assay.* Scientific reports, 2022. **12**(1): p. 16203-16203.
48. Kim, S., et al., *Regulating BRCA1 protein stability by cathepsin S-mediated ubiquitin degradation.* Cell Death Differ, 2019. **26**(5): p. 812-825.
49. Rowling, P.J.E., R. Cook, and L.S. Itzhaki, *Toward Classification of BRCA1 Missense Variants Using a Biophysical Approach.* J Biol Chem, 2010. **285**(26): p. 20080-20087.
50. Glover, J.N.M., R.S. Williams, and R. Green, *Crystal structure of the BRCT repeat region from the breast cancer-associated protein BRCA1.* Nat Struct Biol, 2001. **8**(10): p. 838-842.
51. Williams, R.S., et al., *Detection of Protein Folding Defects Caused by BRCA1-BRCT Truncation and Missense Mutations.* J Biol Chem, 2003. **278**(52): p. 53007-53016.
52. Wang, X., et al., *HUWE1 interacts with BRCA1 and promotes its degradation in the ubiquitin–proteasome pathway.* Biochem Biophys Res Commun, 2014. **444**(4): p. 549-554.
53. Miyahara, K., et al., *BRCA1 degradation in response to mitochondrial damage in breast cancer cells.* Sci Rep, 2021. **11**(1): p. 8735-8735.
54. Ransburgh, D.J.R., et al., *Identification of Breast Tumor Mutations in BRCA1 That Abolish Its Function in Homologous DNA Recombination.* Cancer Res, 2010. **70**(3): p. 988-995.
55. Starita, L.M., et al., *Massively Parallel Functional Analysis of BRCA1 RING Domain Variants.* Genetics, 2015. **200**(2): p. 413-422.
56. Towler, W.I., et al., *Analysis of BRCA1 Variants in Double-Strand Break Repair by Homologous Recombination and Single-Strand Annealing.* Human Mutation, 2013. **34**(3): p. 439-445.
57. Kais, Z., et al., *Functional differences among BRCA1 missense mutations in the control of centrosome duplication.* Oncogene, 2012. **31**(6): p. 799-804.
58. Lu, C., et al., *Patterns and functional implications of rare germline variants across 12 cancer types.* Nat Commun, 2015. **6**(1): p. 10086.
59. Karczewski, K.J., et al., *The mutational constraint spectrum quantified from variation in 141,456 humans.* Nature, 2020. **581**(7809): p. 434-443.
60. Garrett, A., et al., *Combining evidence for and against pathogenicity for variants in cancer susceptibility genes: CanVIG-UK consensus recommendations.* J Med Genet, 2021. **58**(5): p. 297-304.

61. *CanVIG-UK Consensus Specification for Cancer Susceptibility Genes ACGS Best Practice Guidelines for Variant Classification (v2.16)*. 01.06.22]; Available from: https://www.cangene-canvaruk.org/canvig-uk-guidance.
62. Ioannidis, N.M., et al., *REVEL: An Ensemble Method for Predicting the Pathogenicity of Rare Missense Variants.* Am J Hum Genet, 2016. **99**(4): p. 877-885.
63. Anantha, R.W., et al., *Functional and mutational landscapes of BRCA1 for homology-directed repair and therapy resistance.* Elife, 2017. **6**.

Tables

Table 1 – Summary of results from functional assays.

Variant cDNA	Protein	Protein expression (% of WT)	qPCR (% of WT)	% reduction in protein levels (after 8 hours CHX treatment)	Inhibition of proteolytic degradation (MG132)	Co-IP BARD1 (% of WT)	Co-IP PALB2 (% of WT)	Nuclear localization (% of total)	Summary functional assays
	WT protein	100±31	100±23	83±23	Equal amounts	100±23	100±25	84±10	
c.154C>T	**p.Leu52Phe**	18±7	141±51		Increased protein levels			77±5	Reduced protein expression
c.889A>G	**p.Met297Val**	16±8	89±30		Increased protein levels			82±7	Reduced protein expression
c.1508A>G	**p.Lys503Arg**	34±12		22±11		81±21	18±9	81±12	No deviations detected
c.1510C>T	**p.Arg504Cys**	42±15		48±23		63±33	65±18	71±16	No deviations detected

c.1567T>G	**p.Leu523Val**	27±11		42±20		71±30	73±16	88±1	No deviations detected
c.1723G>A	**p.Glu575Lys**	55±22		43±24		77±27	66±13	87±10	No deviations detected
c.1829G>C	**p.Arg610Thr**	40±20		39±34		81±12	56±23	87±2	No deviations detected
c.1834A>G	**p.Arg612Gly**	60±28		29±13		71±9	59±9	88±9	No deviations detected
c.2668G>A	**p.Gly890Arg**	55±23		11±6		77±16	89±30	90±10	Reduced protein stability
c.2773A>G	**p.Ile925Val**	58±6		19±7		97±19	88±23	95±6	No deviations detected
c.2798G>A	**p.Gly933Asp**	40±28		19±6		73±37	115±39	88±12	No deviations detected
c.3454G>A	**p.Asp1152Asn**	13±9	99±10		Increased protein levels			88±10	Reduced protein expression
c.3767C>T	**p.Thr1256Ile**	52±29		23±7		55±26	79±8	90±2	No deviations detected

c.4315C>T	**p.Leu1439Phe**	7±4	69±19	10±13	Equal amounts	60±20	86±37	82±13	Reduced protein expression and stability
Benign control variants									
c.133A>C	**p.Lys45Gln**	70±15		28±14		76±14			
c.1511G>A	**p.Arg504His**	56±11		33±5					
c.4132G>A	**p.Val1378Ile**	44±5		34±17			72±11		
Pathogenic control variants									
c.116G>A	**p.Cys39Tyr**			9±7		8±3			
c.4232T>C	**p.Met1411Thr**						11±18		
c.5513T>G	**p.Val1838Gly**	9±3			Increased protein levels				
c.5123C>A	**p.Ala1708Glu**	14±2		0±0					

Abbreviations: CHX, cycloheximide; WT, wild type.

Table 2 – Characteristics and resulting reclassification for the studied *BRCA1* variants.

Variant	Region/ domain	Functional studies recommended by CanVIG-UK *	Results in functional assays in this study	GnomAD MAF % (allele count) **	REVEL ***	Splicing ****	ClinVar classifications	CanVIG-UK criteria	Original class *****	Revised class
c.154C>T p.(Leu52Phe)	RING	Functional HRR [14], functional saturating genome assay [10]	Reduced protein expression and increased proteasomal degradation	0.09354 (24)	0.68	Possible effect	VUSx7, LBx4, Bx2	BS1_strong	VUS	VUS
c.889A>G p.(Met297Val)			Reduced protein expression and increased proteasomal degradation	- (1)	0.58		VUSx6	BP1, PS3_sup, PM2_sup	LB, VUS	VUS

c.1508A>G p.(Lys503Arg)	NLS		No deviations detected	0.0003240 (2)	0.76		VUSx4, LBx3	BP1, BS3_sup, PP3, PM2_sup	LB, VUS	VUS
c.1510C>T p.(Arg504Cys)	NLS		No deviations detected	0.0003240 (4)	0.65		VUSx10	BP1, BS3_sup, PM2_sup	VUS	LB
c.1567T>G p.(Leu523Val)			No deviations detected	- (1)	0.61		VUSx2	BP1, BS3_sup, PM2_sup	VUS	LB
c.1723G>A p.(Glu575Lys)			No deviations detected	0.0009700 (5)	0.62		VUSx6	BP1, BS3_sup, PM2_sup	VUS	LB
c.1829G>C p.(Arg610Thr)	NLS		No deviations detected	- (0)	0.74		VUSx1	BP1, BS3_sup, PP3, PM2_mod	VUS	VUS
c.1834A>G p.(Arg612Gly)	NLS		No deviations detected	- (0)	0.60		VUSx6, LBx1, Bx1 (ENIGMA)	BS3_sup, BP6, PM2_mod	VUS	LB

c.2668G>A p.(Gly890Arg)			Reduced protein stability	- (0)	0.39	Possible effect	VUSx3, LBx5	PS3_sup, PM2_mod	VUS	VUS
c.2773A>G p.(Ile925Val)			No deviations detected	0.0003240 (2)	0.19		VUSx4, LBx3	BP1, BP4, BS3_sup, PM2_sup	LB, VUS	LB
c.2798G>A p.(Gly933Asp)		Neutral in cisplatin, olaparib and DR-GFP HRR assays [11]	No deviations detected	- (0)	0.29	Possible effect	VUSx5, LBx1	BS3_strong, BS4_sup, PM2_mod	LB, VUS	LB
c.3454G>A p.(Asp1152Asn)		Neutral in cisplatin, olaparib and DR-GFP HRR assays [11]	Reduced protein expression and increased proteasomal degradation	0.004516 (10)	0.37		VUSx10, LBx6, Bx1	BP1, BP4	LB	VUS •
c.3767C>T p.(Thr1256Ile)			No deviations detected	- (0)	0.42		VUSx1	BP1, BS3_sup, PM2_mod	VUS	LB

c.4315C>T p.(Leu1439Phe)		Neutral in cisplatin and DR-GFP HRR assays, not clear in olaparib assay [11]	Reduced protein expression and stability	0.0007760(3)	0.25		VUSx3, LBx1	BP1, BP4, PM2_sup	LB, VUS	VUS •

*According to the CanVIG-UK *BRCA1* specific guideline for variant interpretation, five functional protein studies are suggested with specific recommendations regarding the strength of their respective functional evidence [10-15]. **Minor allele frequencies were retrieved from GnomAD (v2.1.1., non-cancer) Popmax Filtering AF (95% confidence) [59]. As recommended by Garrett et al., the PM2 evidence was ignored when determining the final variant classifications in the presence of evidence towards benignity [60]. ***REVEL was used to assess in-silico predictions with a benign cut off at or below 0.4 and a pathogenic cut off at or above 0.7 as recommended in the Best Practice Guidelines for Variant Classification by CanVIG-UK [61, 62]. ****When investigating the variants' effect on splicing, SpliceSiteFinder-like and MaxEntScan in Alamut was used as recommended by CanVig-UK [15]. *****Original classification in the "*BRCA1* Norway" study [32]. •These variant could theoretically be classified as likely benign (BP1 and BP4 criteria) according to CanVIG-UK [15, 61], but due to conflicting functional evidence, they were still classified as VUSs. Abbreviations: B, Benign; LB, Likely Benign; MAF, Minor Allele Frequency; VUS, Variant of Uncertain Significance.

Figures

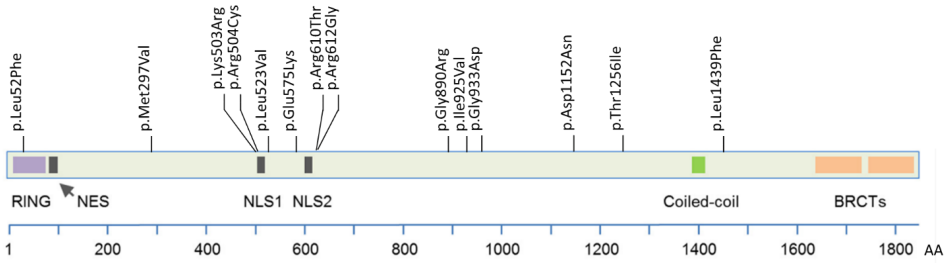


Figure 1 - Schematic presentation of *BRCA1* and location of the investigated missense variants. Figure adapted from *[63]*.

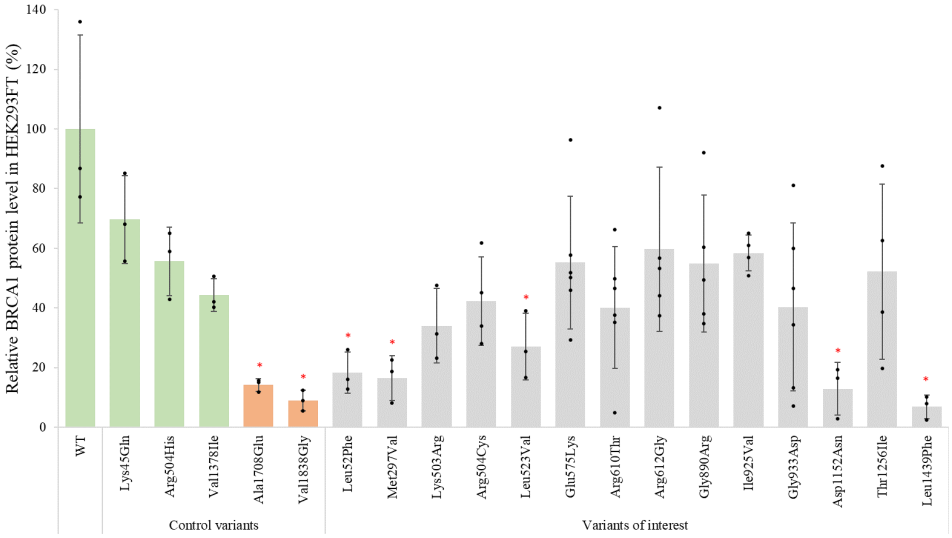


Figure 2 – Protein expression levels of BRCA1 variants determined by western blot analysis: HEK293FT cells were transiently transfected with *BRCA1* WT, known benign and pathogenic control variants and 14 missense *BRCA1* VUSs. Cells were harvested 48 h post transfection, and 5 µg cell lysate was analysed per lane by western blotting. BRCA1 was detected with anti-BRCA1 antibody. Actin was used as loading control to normalise the corresponding BRCA1 bands, and the relative amount of the protein variants compared to the WT was quantified using Image Lab software. The black dots represent individual normalised band intensities. Each column represents the mean of three to six independent replicates (n = 3-6). The benign (green) and pathogenic (orange) control variants are grouped to the left. Variants marked with a red * indicate $p < 0.05$. Error bars represent standard deviation.

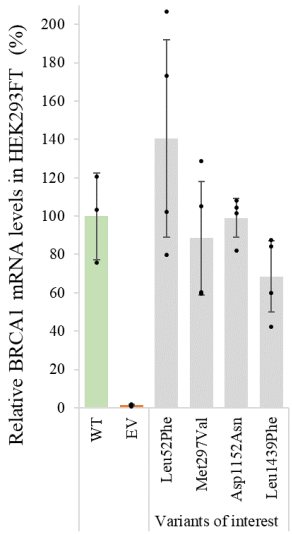


Figure 3 – mRNA levels of *BRCA1* variants in HEK293FT cells determined by qPCR: HEK293FT cells were transfected with plasmids encoding *BRCA1* WT and the four variants found to be expressed at protein levels lower or similar to the included pathogenic controls as shown in Figure 2. Cells were harvested 48 hours post transfection, RNA was purified, and cDNA was synthesised. The synthesised cDNA was used as a template for analysis of mRNA expression by qPCR. Each column represents the mean of three or four independent replicates (n = 3-4), and the black dots represent individual values after normalisation using actin. Error bars represent standard deviation.

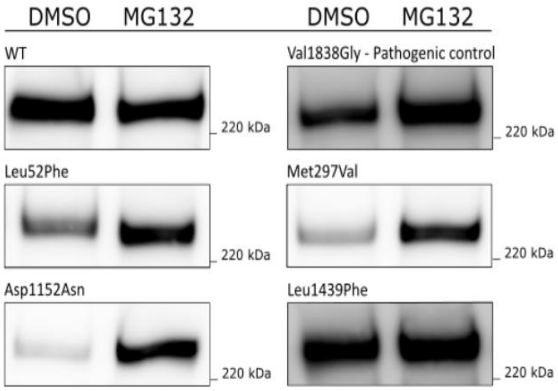


Figure 4 – Assessment of proteasomal degradation of BRCA1 variants by treatment with MG132: HEK293FT cells were transiently transfected with *BRCA1* WT, the pathogenic control p.Val1838Gly, and four missense *BRCA1* VUSs. Twenty-four hours after transfection, cells were treated with 20 µM MG132 or DMSO for 8 hours. After harvesting of cells, 10 µg total cell lysate per lane was analysed by western blot. BRCA1 (220 kDa) was detected with anti-BRCA1 antibody.

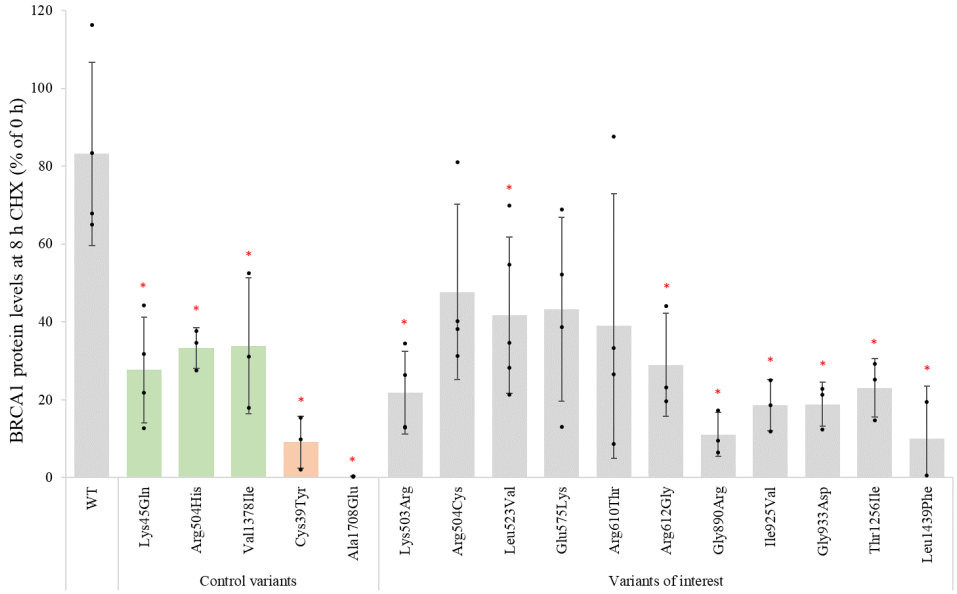
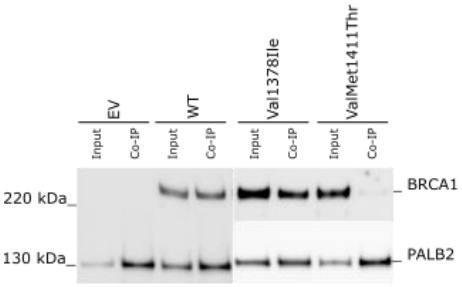
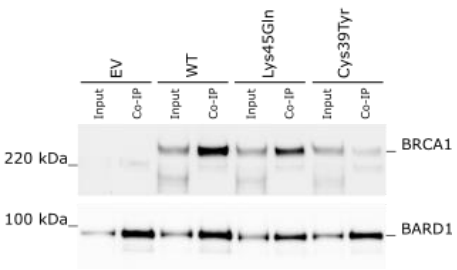


Figure 5 – Assessment of BRCA1 protein variant stability after 8 hours by cycloheximide chase assay: HEK293FT cells were transiently transfected with *BRCA1* WT, known benign and pathogenic control variants and 11 missense *BRCA1* VUSs. Cells were treated with cycloheximide (5 µg/ml) dissolved in DMSO or DMSO only 24 hours post-transfection. Cells were harvested after 8 hours of treatment, and 5 µg cell lysate was analysed per lane by western blotting. BRCA1 was detected with anti-BRCA1 antibody. Actin was used as loading control to normalise the corresponding BRCA1 bands, and the relative amount of the variants after 8 hours treatment compared to the starting point (0 hours) was quantified using Image Lab software. The columns show normalised mean protein levels of three to five independent replicates (n = 3-5) after 8 hours treatment with cycloheximide relative to the levels at 0 hours treatment (100%) for each individual variant. The black dots represent individual normalised band intensities. Error bars represent standard deviation. The benign and pathogenic control variants are coloured green and orange respectively. Variants marked with a red * indicate a significant reduction in protein stability compared with WT protein ($p < 0.05$).

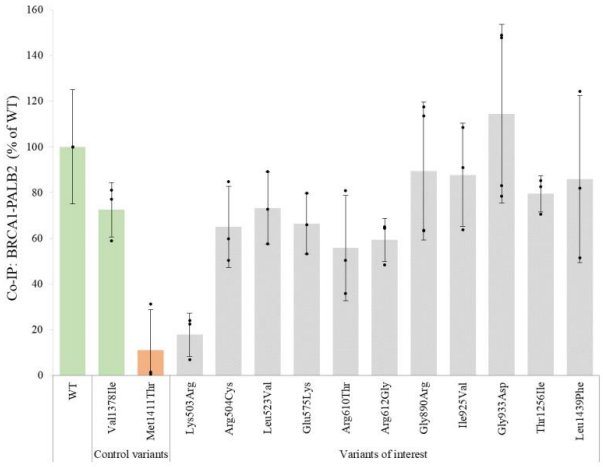
A


EV
WT
Val1378Ile
ValMet1411Thr
Input
Co-IP
220 kDa
130 kDa
BRCA1
PALB2

B


EV
WT
Lys45Gln
Cys39Tyr
Input
Co-IP
220 kDa
100 kDa
BRCA1
BARD1

C


Co-IP: BRCA1-PALB2 (% of WT)
0
20
40
60
80
100
120
140
160
WT
Val1378Ile
Met1411Tlr
Lys503Arg
Arg504Cys
Leu523Val
Glu575Lys
Arg610Thr
Arg612Gly
Gly890Arg
Ile925Val
Gly933Asp
Thr1256Ile
Leu1439Phe
Control variants
Variants of interest

D

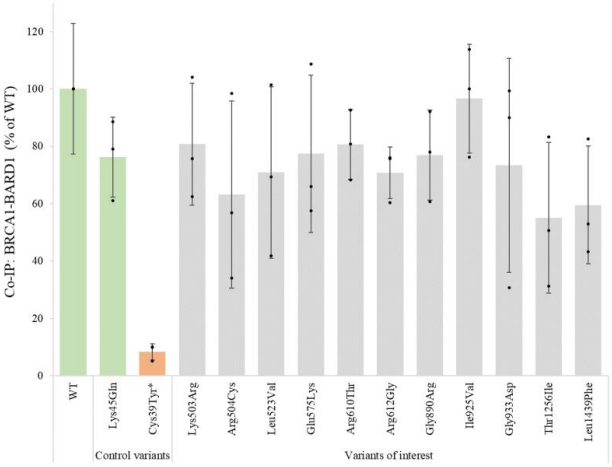
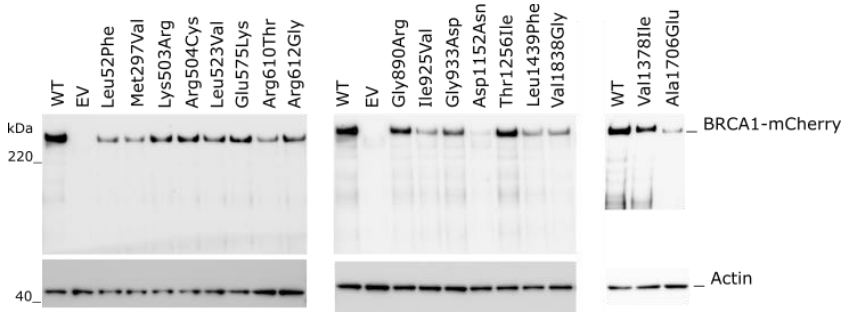

Co-IP: BRCA1-BARD1 (% of WT)
0
20
40
60
80
100
120
WT
Lys45Gln
Cys39Tyr*
Lys503Arg
Arg504Cys
Leu523Val
Glu575Lys
Arg610Thr
Arg612Gly
Gly890Arg
Ile925Val
Gly933Asp
Thr1256Ile
Leu1439Phe
Control variants
Variants of interest

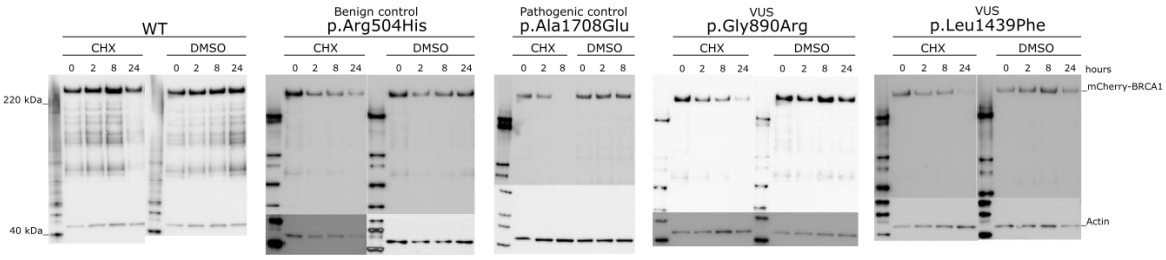
Figure 6 – Assessment of protein interactions between BRCA1 and BARD1 or PALB2 by Co-IP assay: A) HEK293FT cells were transiently co-transfected with EV or *BRCA1* construct together with *Flag-PALB2*. Cells were harvested 48 hours post transfection and co-immunoprecipitation (Co-IP) was performed using the DynabeadsTM Protein G Immunoprecipitation Kit and Flag antibody coupled to the magnetic beads. After elution of the protein complexes, 5 µg of input and Co-IP sample was analysed per lane by western blotting. Input = input cell lysates, Co-IP = eluates from the Flag-column. BRCA1 (220 kDa) was detected with anti-BRCA1. PALB2-Flag (130 kDa) was detected with anti-Flag. Representative results from one of in total three experiments are shown. **B)** Identical experiment as A, with *BARD1*-V5 and V5 antibody coupled to the magnetic beads. BARD1-V5 (100 kDa) was detected with anti-V5. **C)** Quantified results from BRCA1-PALB2 Co-IP. Western blot bands from three biological replicates were quantified by Image Lab software (n = 3). Black dots represent individual normalised band intensities. Graphs represent mean % compared to the WT. Error bars represent standard deviation. The benign (green) and pathogenic (orange) control variants are grouped to the left. In the initial analysis, the variant p.Lys503Arg appeared to have a reduced binding to PALB2, but this interaction was shown to be similar to the WT/benign controls when quantifying against the amount of the variant input sample (not shown). **D)** Identical experiment as C, but with BRCA1-BARD1 Co-IP.

Supplementary figures

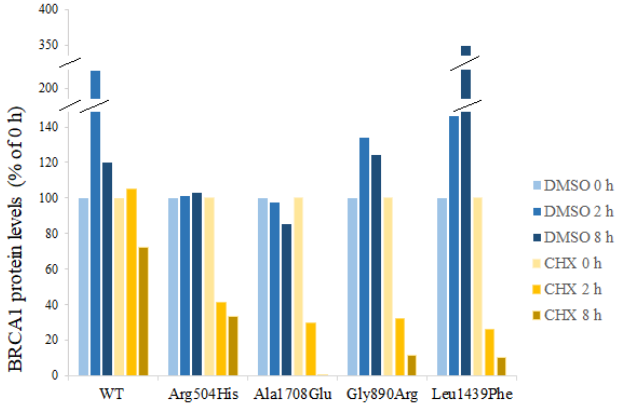


Supplementary Figure 1 - Protein expression levels of BRCA1 variants determined by western blot analysis: HEK293FT cells were transiently transfected with *BRCA1* WT, known benign and pathogenic control variants and 14 missense *BRCA1* VUSs. Cells were harvested 48 h post transfection, and 5 µg total protein retrieved from cell lysate was analysed per lane by western blotting. BRCA1 was detected with anti-BRCA1 antibody. Actin was used as loading control to normalise the corresponding BRCA1 bands. The figure shows images from one representative replicate.

A

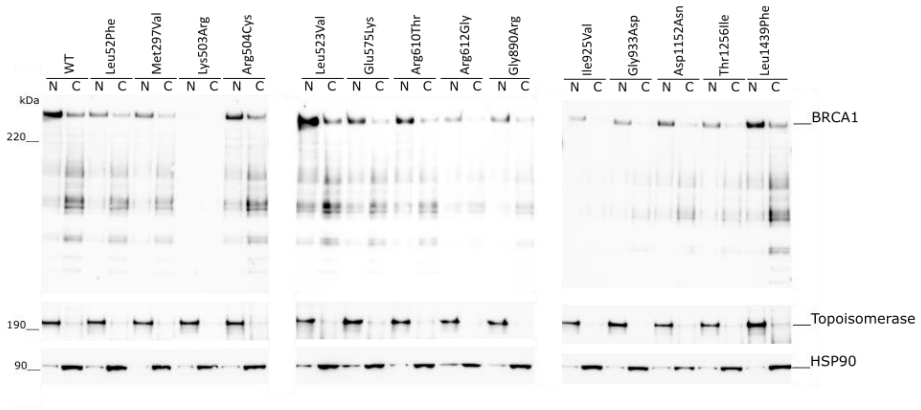


B

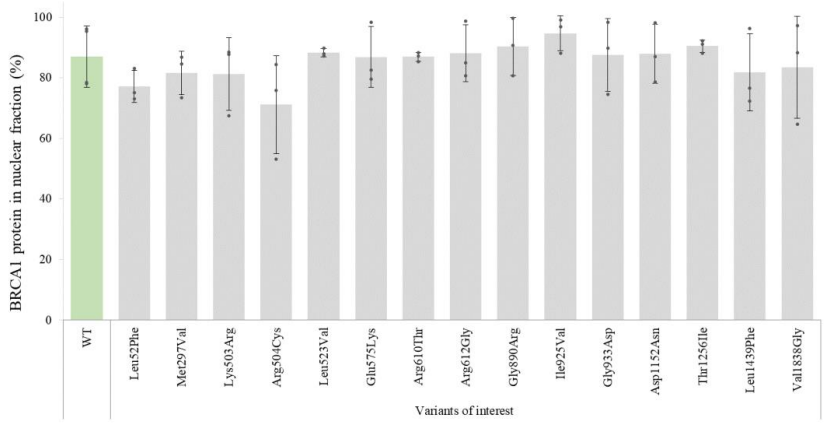


Supplementary Figure 2 – Assessment of protein stability after 0, 2 and 8 hours treatment with cycloheximide or DMSO only. A) The figure shows western blot images for HEK293FT cells transiently transfected with *BRCA1* WT, the two variants showing the highest levels of protein degradation (p.Gly890Arg and p.Leu1439Phe) after 0-8 hours treatment with cycloheximide (CHX), a benign control (p.Arg504His) and a pathogenic control (p.Ala1708Glu) (results for several controls and seven BRCA1 variants are not shown). After 24 hours, cells were treated with cycloheximide dissolved in DMSO or DMSO only for comparison. Samples were harvested after 0, 2 and 8 hours of treatment. Five µg total protein retrieved from cell lysates was analysed per lane by western blotting. BRCA1 was detected with anti-BRCA1 antibody. Actin was used as loading control to normalise the corresponding BRCA1 bands. **B)** Western blot bands from three to five biological replicates were quantified in Image Lab software. The normalised protein levels at each time point are shown relative to the amount of each variant at time 0 hours (100%). The graphs illustrate the mean % of all replicates after 0-8 hours treatment with cycloheximide or DMSO only.

A



B



Supplementary Figure 3 – Assessment of nuclear localisation of BRCA1 variants by cellular fractionation assay. A) HEK293FT cells were transiently transfected with *BRCA1* WT and VUSs. After 48 hours, cells were harvested and separated into nuclear and cytosolic fractions. Cytosolic and nuclear samples containing 5 µg of total protein were analysed by western blotting. BRCA1 was detected with anti-BRCA1 antibody. Topoisomerase IIα (190 kDa) and HSP 90α/β (90 kDa) were used as nucleus and cytosol markers, respectively. **B)** The signals from each band in the western blot images were used for normalisation using Image Lab software. The columns show the percentage of the total BRCA1 protein variant which is located in the nucleus (mean of three independent replicates). The black dots represent individual normalised band intensities. Each error bar represents the standard deviation.

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