BRCA2 related cancer, haploinsufficiency and telomere dysfunction

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Thesis for the degree of Philosophiae Doctor

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Stakstæð áhrif og telomere-gallar í BRCA2-tengdum krabbameinum

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Ritgerð til doktorsgráðu

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Ágrip

Kímlínustökkbreytingar í *BRCA1* og *BRCA2* genum auka verulega líkurnar á brjósta- og eggjastokkakrabbameini. Landnemastökkbreytingin *BRCA2*^{999del5} (*BRCA2*$^{c.767\_771delCAAAT}$) er algengust á Íslandi og skýrir nánast öll tilfelli brjósta- og eggjastokkakrabbameina sem tengjast *BRCA2* stökkbreytingum. Stökkbreytingin veldur óvirkri próteinafurð. BRCA2 próteinið gegnir lykilhlutverki við endurröðunarviðgerð á tvíþátta brotum í DNA og ver einnig eftirmyndunarkvíslar sem hafa stöðvast fyrir niðurbroti af núkleösum. Tap á BRCA2 leiðir til mikilla litningagalla sem einkennir krabbamein tengd *BRCA* stökkbreytingum. BRCA2 er því mikilvægt fyrir viðhald á stöðugleika erfðamengisins.

BRCA genin eru flokkuð sem æxlisbæligen og almennt er gert ráð fyrir því að tap á villigerðarsamsætunni eigi sér stað í æxlum. Hins vegar hefur verið sýnt fram á að í hluta krabbameina í *BRCA* arfberum verður ekki tap á villigerðarsamsætunni. Þetta er sérstaklega áberandi í brjóstakrabbameini í konum þar sem villigerðarsamsætan er varðveitt í um 50% tilfella í *BRCA2* arfberum, sem gefur til kynna að um stakstæð áhrif BRCA2 sé að ræða.

Gallar á telomerum finnast í æxlum og frumulínum úr *BRCA2* arfberum. Telomerar, sem vernda litningaenda, gætu verið viðkvæmari fyrir BRCA2 vöntun en aðrir hlutar erfðamengisins vegna erfiðleika við eftirmyndun á endurteknum röðum sem einkenna þá. Markmið fyrsta hluta verkefnisins var að mæla telomera-lengd og meta telomera-galla hjá arfberum *BRCA2*^{999del5} stökkbreytingarinnar til að kanna hvort hægt væri að nota mælingar á telomerum til að spá fyrir um brjóstakrabbameinsáhættu.

Telomera-lengd var mæld með multiplex monochrome qPCR aðferð í blóðsýnum úr vel skilgreindum rannsóknarhópi sem samanstóð af *BRCA2* arfberum, brjóstakrabbameinstilfellum og viðmiðunarhópi. Enginn munur var á telomera-lengd milli *BRCA2* arfbera og þeirra sem ekki eru arfberar en styttri telomera-lengd var tengd við aukna brjóstakrabbameinsáhættu innan arfberahópsins. Þessi niðurstaða var fengin með því að skoða aðeins þær konur sem gefið höfðu blóðsýni fyrir brjóstakrabbameinsgreiningu og undirstrikar mikilvægi forspárrannsókna í þessu samhengi.

Telomera-lengd var einnig mæld í paraffin-steyptum sýnum úr eðlilegum brjóstavef með Q-FISH aðferð. Merki um telomere-galla voru fengin með því

að samlita fyrir telomerum og próteininu 53BP1 sem merkir tvíþátta DNA brot. Kirtilþekjufrumur hafa stystu telomere-lengd og mestu DNA skemmdir af frumugerðum í eðlilegum brjóstavef. Líkt og í blóðsýnum mældist enginn marktækur munur á lengd telomera í eðlilegum brjóstavef milli *BRCA2* arfbera og þeirra sem ekki eru arfberar. Hins vegar var fylgni á milli styttri telomera-lengdar og lægri greiningaraldurs brjóstakrabbameins innan arfberahópsins. Niðurstöður úr blóði og brjóstavef benda því til þess að styttri telomera-lengd auki brjóstakrabbameinsáhættu hjá *BRCA2* arfberum en stuttir telomerar geta bent til galla í viðhaldi telomera.

Til að rannsaka frekar stakstæð áhrif BRCA2 hefur verið búið til frumulínumódel með CRISPR/Cas9 erfðatækni sem líkir eftir $BRCA2^{999del5}$ stökkbreytingunni. Markmiðið er að skilgreina betur áhrif arfblendinnar stökkbreytingar og arfhreinnar stökkbreytingar á hæfni frumna í viðgerð tvíþátta brota, stöðugun stöðvaðra eftirmyndunarkvísla og viðhald telomera.

Í síðasta hluta verkefnisins voru sýni úr brjóstakrabbameinum í körlum og eggjastokkakrabbameinum skimuð með Sanger raðgreiningu fyrir þremur algengustu stökkbreytingunum sem tengjast brjósta- og eggjastokkakrabbameini í íslenska þýðinu; $BRCA2^{999del5}$, $BRCA1^{G5193A}$, $BRIP1^{2040}$_2041insTT. Af þeim karlmönnum sem greinst hafa með brjóstakrabbamein á Íslandi frá 1955-2018 bera 32% $BRCA2^{999del5}$ stökkbreytinguna. Tap á arfblendni hafði átt sér stað í 88% æxla. Af þeim konum sem greindust með eggjastokka-, eggjaleiðara- eða lífhimnukrabbamein á árunum 1999-2013 á Íslandi voru 7.3% $BRCA2^{999del5}$ arfberar, 1.8% $BRCA1^{G5193A}$ arfberar og 4.5% báru $BRIP1^{2040}$_2041insTT stökkbreytinguna. Tap á arfblendni þessara gena hafði átt sér stað í miklum meirihluta tilfella eða 90.5% af *BRCA2*, 100% af *BRCA1* og 84.6% af *BRIP1* æxlum. Greining á brjóstakrabbameinum í konum með sömu aðferð sýndi tap á arfblendni í 56.7% æxla frá $BRCA2^{999del5}$ arfberum og 100% æxla frá $BRCA1^{G5193A}$ arfberum.

Lykilorð:

BRCA2, Stakstæð áhrif, Telomerar, DNA viðgerð, Brjóstakrabbamein

Abstract

Germline mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2* confer high risk of developing breast and ovarian cancer. The *BRCA2*^{999del5} (*BRCA2*$^{c.767\_771delCAAAT}$) founder mutation is responsible for almost all BRCA2-related breast and ovarian cancers in the Icelandic population. It leads to a prematurely truncated and non-functional protein product. The BRCA2 protein has a role in homologous recombination repair of DNA double strand breaks and protection of stalled replication forks from nuclease degradation. Loss of BRCA2 function leads to gross chromosomal abnormalities, characteristic of many BRCA-related tumors. BRCA2 is therefore important for maintaining genomic stability.

The *BRCA* genes are considered classical tumor suppressor genes and in mutation carriers, loss of the wild-type allele in tumors is generally assumed. However, a subset of tumors from *BRCA* mutation carriers exhibit retention of the wild-type allele. This is especially evident in the case of female breast cancer where loss of heterozygosity (LOH) is only observed ~50% of tumors from *BRCA2* mutation carriers, suggesting BRCA2 haploinsufficiency.

Tumors and cell lines from *BRCA2* mutation carriers have been shown to have telomere abnormalities. Telomeres, the nucleoprotein structures at the end of linear chromosomes, may be more sensitive to BRCA2 deficiencies than the rest of the genome due to replication problems at highly repetitive telomere sequences. In the first part of this project, the aim was to study telomere length and telomere dysfunction in mutation carriers of the Icelandic *BRCA2*^{999del5} mutation as a possible indicator of BRCA2 haploinsufficiency and/or predictor of breast cancer risk.

Telomere length was measured using a multiplex monochrome qPCR method on blood samples from a well-defined cohort of female *BRCA2* mutation carriers, sporadic breast cancer cases and controls. Telomere length did not differ between *BRCA2* mutation carriers and non-carriers, however shorter telomere length was associated with increased risk of developing breast cancer in the mutation carrier group. This observation was made by exclusively evaluating samples acquired before breast cancer diagnosis and underscores the importance of study design, specifically the availability of samples prior to diagnosis.

Additionally, telomere length was measured in normal FFPE female breast tissue samples using Q-FISH and telomere dysfunction induced foci identified with immunofluorescent co-staining of 53BP1, a marker of DNA double strand breaks. The luminal epithelial cell layer of the breast was shown to have the shortest telomere length and highest levels of DNA double strand breaks. Similar to results from blood samples, the average telomere length in normal breast tissue did not differ between *BRCA2* mutation carriers and non-carriers. Within the *BRCA2* mutation carrier group, short telomere length however correlated with earlier age at breast cancer diagnosis. Collectively, these results indicate that short telomere length is a modifier of breast cancer risk in *BRCA2* mutation carriers which may be indicative of dysfunctional telomere maintenance and BRCA2 haploinsufficiency.

To further elucidate BRCA2 haploinsufficiency at a functional level, an isogenic cell line model mimicking the $BRCA2^{999del5}$ mutation was established using the CRISPR/Cas9 genome editing technology. The aim is to functionally characterize the effect of the mutation in a heterozygous and homozygous state on DNA double strand break efficiency, problems at stalled replication forks and dysfunctional telomere maintenance.

In the final part of this project, male breast tumors and ovarian tumors were screened for the three most prevalent mutations linked to breast and ovarian cancer in the Icelandic population; $BRCA2^{999del5}$, $BRCA1^{G5193A}$, $BRIP1^{2040\_2041insTT}$, using targeted Sanger sequencing. The $BRCA2^{999del5}$ mutation is found in 32% of male breast cancer patients in Iceland diagnosed from 1955-2018. Locus specific loss of heterozygosity of the wild type *BRCA2* allele was present in 88% of tumors. In a collective cohort of ovarian, fallopian tube and peritoneal tumors diagnosed from 1999-2013 in Iceland, the $BRCA2^{999del5}$ mutation was detected in 7.3% of cases, the $BRCA1^{G5193A}$ mutation in 1.8% of cases and the $BRIP1^{2040\_2041insTT}$ mutation in 4.5% of cases. Locus specific LOH was present in majority of cases or 90.5% for *BRCA2*, 100% of *BRCA1* and 84.6% of *BRIP1* tumors. Re-analysis using the same method on female breast cancer samples showed locus specific LOH in 56.7% of tumors from $BRCA2^{999del5}$ mutation carriers and 100% of $BRCA1^{G5193A}$ mutation carriers.

Keywords:

BRCA2, Haploinsufficiency, Telomeres, DNA repair, Breast Cancer

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Contents

List of abbreviations

53BP1	p53 Binding Protein 1
Alt-NHEJ	Alternative Non-Homologous End-joining
ALT	Alternative lengthening of telomeres
AR	Androgen Receptor
ATM	Ataxia telangiectasia mutated protein
ATR	ATM-and-RAD3-related protein
BER	Base excision repair
BFB	Breakage-fusion-bridge
BRCA1 / *BRCA2*	Breast Cancer Susceptibility Gene type 1/2
BRCA1 / BRCA2	Breast Cancer Susceptibility Protein type 1/2
BRIP1	BRCA1-interacting protein C-terminal helicase 1
C-NHEJ	Canonical Non-Homologous End-Joining
CNA	Copy Number Aberrations
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DCIS	Ductal Carcinoma in Situ
DDR	DNA Damage Response
DNA	Deoxyribonucleic acid
DSB	Double Strand Break
ER	Estrogen Receptor
FA	Fanconi Anemia
FANCD1	Fanconi anemia, complementation group D1
FB	Fibroblast
FBC	Female Breast Cancer
FFPE	Formalin-Fixed Paraffin-Embedded
FISH	Fluorescence *in situ* Hybridization
FTC	Fallopian Tube Cancer
HBOC	Hereditary Breast and Ovarian Cancer
HDR	Homology-Directed Repair
HER-2	Human Epidermal Growth Factor Receptor 2
HR	Homologous Recombination
ICL	Inter-strand Cross-Links
IHC	Immunohistochemistry
IF	Immunofluorescence
LEC	Luminal epithelial cell
LOH	Loss of Heterozygosity
MBC	Male Breast Cancer
MEC	Myoepithelial cell
MMEJ	Microhomology Mediated End-Joining
MMqPCR	Multiplex monochrome quantitative PCR
MMR	Mismatch Repair
MRN	MRE1111-RAD50-NSB1

NHEJ	Non-Homologous End-joining
NLS	Nuclear localization signal
OC	Ovarian Cancer
PAM	Protospacer Adjacent Motif
PARP	Poly ADP-Ribose Polymerase
PCR	Polymerase Chain Reaction
PNA	Peptide Nucleic Acid
PR	Progesterone Receptor
PTC	Peritoneal Cancer
qPCR	Quantitative PCR
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid Interference
RPA	Replication Protein A
SEOC	Synchronous Endometrial and Ovarian Cancer
sgRNA	single guided RNA
siRNA	short interference RNA
ssDNA	single-strand DNA
SSA	Single-Strand Annealing
SSB	Single-Strand Break
TCGA	The Cancer Genome Atlas
TDLU	Terminal Duct Lobular Unit
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TIF	Telomere Dysfunction Induced Foci
TMA	Tissue Microarray
TNBC	Triple Negative Breast Cancer
TSG	Tumor Suppressor Gene
wt	wild-type
γ-H2AX	γ-Histone H2AX Phosphorylated on serine-139

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List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. **Telomere Length Is Predictive of Breast Cancer Risk in *BRCA2* Mutation Carriers.** Thorvaldsdottir B, Aradottir M, Stefansson OA, Bodvarsdottir SK, Eyfjörd JE. Cancer Epidemiology, Biomarkers & Prevention. 2017; 26:1248–54.

In addition, some unpublished data is presented.

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Declaration of contribution

Paper I: I participated in project planning and in choosing the study group along with P.I. Jórunn E. Eyfjörð, Sigríður K. Böðvarsdóttir and Margrét Aradóttir. I set up and validated the MMqPCR telomere length measurement method in the laboratory and performed the measurements with the help of Margrét Aradóttir. I performed data and statistical analysis for the paper under advice from Ólafur A. Stefánsson. I wrote the manuscript together with my supervisor Jórunn E. Eyfjörð.

Q-FISH and IF on tissue specimens: Jórunn E. Eyfjörð and Elizabeth H. Blackburn planned the project. I defined the study group with guidance from Jórunn E. Eyfjörð and Ólafur A. Stefánsson. Normal breast tissue slices were selected and prepared by Katrín Ólafsdóttir, Jón G. Jónasson and Kristrún Ólafsdóttir at the department of Pathology, Landspitali. I performed all staining of tissue slices, microscopy and image analyses with guidance from Beth A. Cimini and Morgan E. Diolaiti. I performed statistical analysis and data interpretation.

CRISPR: I planned the project with Þorkell Guðjónsson, Stefán Þ. Sigurðsson and Jórunn E. Eyfjörð. Original design of materials for CRISPR experiments were performed by Tobias R. Richter, some of which I redesigned and modified. Stefán. Þ. Sigurðsson cloned and prepared the gRNA plasmid. I was responsible for all mammalian cell work and established the CRISPR cell lines as well as validating them at a genetic level. Þorkell Guðjónsson validated the cell lines at protein level.

Mutational and LOH analyses: I planned the male breast cancer project and ovarian cancer project with Stefán Þ. Sigurðsson. The study groups were determined and extracted from the Icelandic Cancer Registry by Guðríður H. Ólafsdóttir and Laufey Tryggvadóttir. Tumor samples were selected and prepared by Jón G. Jónasson, Anna M. Jónsdóttir and Helga S.Gunnarsdóttir at the Department of Pathology, Landspitali. I isolated the DNA, performed Sanger sequencing, LOH analysis, all statistical analysis and data interpretation for the thesis. These projects are ongoing with my continued involvement in the capacity of study supervision, statistical analysis and drafting of manuscripts along with P.I. Stefán Þ. Sigurðsson.

1 Introduction

1.1 Breast Gland Morphology

The female breast gland is composed of branching epithelial ductal systems, reaching from the nipple to the terminal duct lobular units, TDLUs. These are the milk producing functional units and consist of acini which empty into ductules and a larger collecting duct. The epithelial ducto-lobular system is composed of two cell layers, the secretory inner luminal epithelial cell (LEC) layer and an outer layer of contractile myoepithelial cells (MECs), separated from the enveloping stroma by a basement membrane (**Figure 1**). A number of cell populations are found in the stroma, including adipose cells, fibroblasts (FBs), endothelial cells and lymphocytes (Sun et al., 2018). The breast gland is highly dynamic, undergoing drastic morphological changes through the reproductive life cycle from puberty to menopause, with each menstrual cycle, pregnancy and lactation. A population of breast epithelial stem cells give rise to both epithelial cell lineages (LECs and MECs) through cycles of proliferation and differentiation (Petersen & Polyak, 2010; Visvader, 2009).

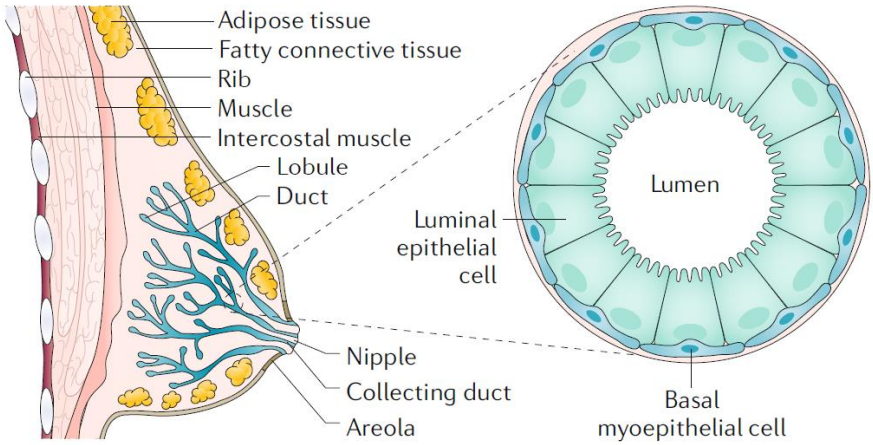

Figure 1. Breast gland morphology. A system of lactiferous ducts branches from the nipple to the terminal duct lobular units (left). A cross-sectional view (right) of a duct showing the dual epithelial layer of luminal epithelial cells (LECs) and myoepithelial cells (MECs) separated by a basement membrane from the surrounding stroma (not depicted). Reproduced with permission from Springer Nature License #4676541127057 (Harbeck et al., 2019)

1.2 Female Breast Cancer

Cancer is one of the major causes of death world-wide. Breast cancer is the most common cancer in women, affecting over 2 million women each year, and among the main causes of cancer-related deaths with over 600.000 estimated deaths in 2018 (Bray et al., 2018). Over 200 women are diagnosed with breast cancer in Iceland each year, accounting for 25-30% of cancer diagnoses in women (Icelandic Cancer Registry, 2019). The single largest risk factor for developing breast cancer is age, with the average age at diagnosis being 62 years (Iceland, 2013-2017). Family history is also an important risk factor. Additionally, high breast density, prolonged exposure to estrogen and reproductive factors such as early menarche, late menopause and late first pregnancy have been linked to increased breast cancer risk (Rojas & Stuckey, 2016). Breast cancer incidence has consistently increased over the last decades while mortality has decreased. In Iceland, the 5-year survival increased from 69% during 1964-1973 to 94% in 2004-2013, presumably owing to improved diagnostic and treatment options (Icelandic Cancer Registry, 2019).

Breast cancer, like all cancers, is a heterogeneous disease reflecting the variable pathways driving tumor formation. Breast tumors are classified based on histological and molecular characteristics that both affect prognosis.

There are two main histological groups of breast cancer, carcinomas of epithelial origin (>95%) and sarcomas arising from stromal cells. Most epithelial carcinomas arise from the luminal epithelial cells of the TDLUs and are further subclassified into invasive ductal carcinomas (80%) and invasive lobular carcinomas (10-15%) (Lakhani, 2012). Tumors arising from myoepithelial cells are extremely rare (Papazian et al., 2016). Conventionally, breast cancer tumorigenesis is considered to go through stages of epithelial cell hyperproliferation, from atypical hyperplasia to ductal or lobular carcinoma *in situ* (DCIS/LCIS) to invasive ductal or lobular carcinomas and eventually distal metastatic disease (Lakhani, 2012). *In situ* carcinomas are however considered a non-obligate precursor as many remain noninvasive and never progress to an infiltrating disease (Gorringe & Fox, 2017).

Breast cancer prognosis and treatment is dependent upon a variety of factors including Tumor-Node-Metastasis (TNM) staging, histological grade and the expression of clinically relevant markers (Harbeck et al., 2019). Breast tumors have been categorized into molecular subtypes based on expression profiles identified by genome wide expression analysis (Perou et

al., 2000; Sorlie et al., 2001). Clinical subgroups based on these molecular subtypes are indexed through immunohistochemical staining (IHC) of representative markers. The subgroups are largely based on expression patterns of estrogen receptors (ER), progesterone receptors (PR), human epidermal growth factor receptor 2 (HER2, also known as ERBB2 or NEU), the epidermal growth factor receptor (EGFR) and cytokeratins 5/6 with the addition of proliferation markers such as Ki-67. The subtypes are luminal-A (ER^+/ PR^+, $HER2^-$, Ki-67 low), luminal-B (ER^+/ PR^+, Ki-67 high, some $HER2^+$), HER2 (ER^- and PR^-, $HER2^+$), basal-like (ER^-/PR^-/$HER2^-$, $CK5/6^+$ or $EGFR^+$) and normal-like (Cheang et al., 2008; Sorlie et al., 2003). Identifying the different subtypes is important for determining treatment options. Tumors of the luminal subtypes are the most abundant (~75% of all cases), respond to hormonal drugs such as tamoxifen and aromatase inhibitors and have generally good prognosis, luminal-A better than luminal-B. Tumors overexpressing the HER2 receptor (15-20% of cases) can specifically be targeted with trastuzumab (Herceptin) but still have worse prognosis than luminal tumors due to fast growth rate and higher relapse risk (Prat et al., 2015). Tumors expressing none of the aforementioned targetable factors (ER^-/PR^-/$HER2^-$) are clinically termed triple-negative (TNBC, 10-15% of cases). The TNBC and basal-like terms are not entirely synonymous, with the latter used in the research setting to refer to the expressional profile rather than IHC staining. Triple negative tumors are generally treated with conventional cytotoxic chemo- or radiotherapy after surgical removal of the tumor or mastectomy and have the worst prognosis of the breast cancer subtypes. Extensive efforts focused on finding treatments for specific molecular targets in triple-negative breast cancer have been largely unsuccessful (Garrido-Castro et al., 2019; Prat et al., 2015). Exceptions to this are new exciting treatment options which apply to a subset of breast cancers, including triple negative, as well as other cancer types; most notably PARP inhibitors and PD-1/PD-L1 immunotherapy. PARP inhibitors target tumors with underlying defects in homologous recombination (HR) DNA repair (Lord & Ashworth, 2017) while PD-1/PD-L1 inhibitors target tumors with high levels of tumor infiltrating lymphocytes or high expression of the PD-1/PD-L1 immuno-checkpoints. TNBCs are often characterized by genomic instability which has been associated with higher production of neoantigens and increased immune infiltration (Kwa & Adams, 2018).

1.3 Male breast cancer

Male breast cancer (MBC) is rare, accounting for less than 1% of total breast cancer diagnoses (Weiss JR et al., 2005). In Iceland, MBC incidence has steadily increased over time, averaging in 4 new cases annually in recent years (Icelandic Cancer Registry, 2019). The ratio between female and male diagnoses is similar to that found in other Western countries (Jonasson et al., 1996). MBC resembles postmenopausal female breast cancer (FBC) in many ways but differs in important aspects. MBC is typically diagnosed at an older age and at a more advanced stage than FBC, possibly due to lack of awareness and screening, which is reflected in worse overall survival outcomes (Fentiman et al., 2006; Greif et al., 2012). The average age at diagnosis in Iceland is 69 years for MBC compared to 62 years for FBC and the 5-year survival is 85% and 94%, respectively (The Icelandic Cancer Registry, 2019).

The vast majority of male breast tumors are invasive ductal carcinomas (80-90%), followed by papillary carcinomas. Lobular carcinomas are rare (Fentiman et al., 2006; Jonasson et al., 1996). Male breast tumors are subjected to the same subtype classification system as described for FBC above. However, male breast carcinomas are almost exclusively hormone receptor positive, expressing ER, PR and the androgen receptor (AR) but HER-2, basal-like or other triple-negative subtypes are rare (Kornegoor et al., 2012; Murphy et al., 2006). Furthermore, male breast tumors have been demonstrated to be somewhat molecularly distinct from female breast tumors (Johansson et al., 2013; Piscuoglio et al., 2016). Due to the rarity of MBC, specialized treatment options have not been developed and rather adjusted from FBC regimens. Evidence suggest that MBC patients could additionally benefit from receiving treatment with AR inhibitors (Severson & Zwart, 2017). The strongest risk factor for developing MBC is family history of the disease (discussed in 1.6.3). Epidemiological risk factors mainly include hormonal imbalances associated with obesity, gynecomastia, testicular disorders, endocrine cancer therapy, liver cirrhosis and Klinefelter's syndrome (47, XXY karyotype) (Weiss JR et al., 2005).

1.4 A brief overview of ovarian cancer

With the main focus of the thesis being on breast cancer, only a brief overview of ovarian cancer will be presented here.

Ovarian cancer is the eighth most common cancer in women worldwide with approximately 300.000 new cases diagnosed annually (Bray et al.,

2018). An average of 18 women are diagnosed with ovarian and fallopian tube cancer in Iceland each year, accounting for 2% of all cancer diagnoses in women. It primarily affects women after menopause with an average age at diagnosis of 66 years. (Icelandic Cancer Registry, 2019). Ovarian cancer carries the worst prognosis and highest mortality rate of all gynecological cancers (Coburn et al., 2017). The disease is typically diagnosed at a late stage due to being largely asymptomatic, reflecting in poor survival rate. The recent 5-year survival of ovarian cancer in Iceland is 52% (Icelandic Cancer Registry, 2019).

Over 90% of ovarian tumors are epithelial in origin but still comprise a heterogenous group of tumors classified into subgroups based on cellular origin, histology and molecular characteristics which vary greatly in clinical presentation and outcome (reviewed in (McCluggage, 2011)). Tumorigenesis and cellular origins of ovarian cancer are not completely understood. Majority of tumors are thought to originate in other gynecological tissues such as fallopian tubes or the endometrium with only secondary involvement of the ovaries (Kurman & Shih, 2010). For this reason, ovarian, fallopian tube and peritoneal tumors are grouped together and treated similarly clinically. First line of treatment for most cases is surgical removal or tumor debulking followed by a combination of taxane- and platinum-based chemotherapy for high-stage/grade tumors. Recurrence risk is high. Promising targeted treatments have been implemented in recent years with many more in development, giving hope of better outcomes for this lethal disease (Ledermann, 2017).

1.5 Tumor molecular evolution

The driving forces behind tumor formation at a cellular level remain a constant research topic. Essentially, for a cell to become cancerous it has to successively accumulate key changes in the function of tumor suppressor genes (TSG) or oncogenes, thereby acquiring hallmark tumor capabilities (Hanahan & Weinberg, 2011). The original 6 hallmarks of carcinogenesis described by Hanahan and Weinberg (Hanahan & Weinberg, 2000) include self-sustaining proliferative signaling, evading growth suppression, evading apoptosis, enabling limitless replicative potential, inducing angiogenesis, and activating invasion and metastasis (Hanahan & Weinberg, 2000). Later, two hallmarks were added; changes in energy metabolism and evading immune destruction (Hanahan & Weinberg, 2011).

Genomic instability is a major driving force in cancers, which are all thought to acquire most if not all of these traits through the accumulation of mutations over time (Hanahan & Weinberg, 2011). Cancer predisposing mutations can be inherited in the germline but most are acquired somatically via multiple mutational processes, both internal to cells and external factors. The types of somatic mutations found in cancer cells range from small scale changes such as base substitutions, small insertions or deletions of DNA (indels), to larger genomic rearrangements like copy number aberrations (CNA) or chromosomal translocations. Additionally, epigenetic changes can affect gene expression by altering chromatin organization and structure. Tumorigenesis can be considered as an evolutionary process where cells acquiring advantageous mutations, so-called driver mutations, are subject to clonal selection over neighboring cells. An abundance of mutations with no obvious advantages to the cancer cell accumulate in parallel, often termed passenger mutations (Stratton et al., 2009). The number of driver mutations needed for tumor formation has been a matter of debate for decades. In a recent study of 7664 tumors across 29 cancer types, numbers ranged from 1-10 driver mutations depending on cancer type and underlying mutational burden (Martincorena et al., 2017).

With the explosion of data available on cancer genomes in the new age of massive parallel sequencing, an ever-longer list of driver mutations has emerged. As an example of the daunting diversity, of over 2000 breast tumors sequenced at the Wellcome Trust Sanger Institute, no two tumors shared the same set of drivers (Nik-Zainal & Morganella, 2017). Identifying actionable drivers in individual tumors for targeted therapy remains a difficult task for many tumor types. Recent endeavors using next generation sequencing methods have identified passenger mutational signatures, providing insight into tumor molecular evolution and revealing the underlying dysfunctional cellular processes (Alexandrov et al., 2013). These signatures can guide treatment by exploiting tumor weaknesses. As an example, breast tumors with defective HR DNA repair are characterized by specific indel mutational patterns (Davies et al., 2017; Nik-Zainal et al., 2016) and can be specifically targeted with platinum-based chemotherapy or PARP inhibitors (discussed in chapter **1.11**). Incorporation of molecular characteristics with current treatment practices is key for the advancement of precision oncology and personalized treatment.

Breast cancer is a heterogenous disease, as previously mentioned, exhibiting great variability between individual tumors. This inter-tumoral heterogeneity is then complicated further with the intra-tumoral heterogeneity,

both morphological and genetic (Turashvili & Brogi, 2017). A wide-range of tumor cells can exist within a single tumor, reflecting clonal expansion of cells with proliferative advantages over neighboring tumor cells (Nik-Zainal et al., 2012). This complicates clinical treatment as targetable traits may not be present in all tumor cells. Multi-regional sequencing of breast tumors has demonstrated treatment resistance and metastatic potential arising within tumor subclones, highlighting the importance of analyzing tumor heterogeneity for improved treatment (Yates et al., 2015).

1.6 The *BRCA* genes and familial cancer

Most cancers are considered sporadic, occurring in individuals with little or no family history of the disease. For female breast cancer, it is estimated that underlying germline mutations account for up to 10% of cases (Daly et al., 2010). The definition of familial breast cancer is vague, but criteria for Hereditary Breast and Ovarian Cancer (HBOC) syndrome include multiple breast and/or ovarian cancers in the same family with a tendency for younger age at diagnosis (<50 years), breast and ovarian cancer in the same patient or male breast cancer. An underlying predisposing genetic mutation is however not always identified (Daly et al., 2010). The most commonly mutated genes in familial breast cancer are ones involved in maintaining genomic stability. Risk contribution varies, with rare mutations in high penetrance genes including *BRCA1*, *BRCA2*, *TP53*, *PTEN*, *CDH1* and *STK11* accounting for majority of identified cases while mutations in *PALB2*, *CHEK2*, *BRIP1* and *ATM* are considered of moderate penetrance. Additionally, low penetrance genetic variants have been identified that may contribute to breast cancer risk, alone or in polygenic fashion (Shiovitz & Korde, 2015).

Of these, mutations in *Breast Cancer Susceptibility gene type 1* and *type 2* (*BRCA1* and *BRCA2*) are by far the most abundant (Daly et al., 2010). They confer highly increased life-time risk of breast and ovarian cancer as well as prostate and pancreatic cancer to a lesser extent (Levy-Lahad & Friedman, 2007). The cumulative breast cancer risk to the age of 80 has been estimated to be 72% for *BRCA1* and 69% for *BRCA2* mutation carriers and 44% and 17%, respectively, for ovarian cancer (Kuchenbaecker et al., 2017). Over a thousand germline mutations are known in each of the *BRCA* genes (Rebbeck et al., 2018), with variable impact on cancer risk (Rebbeck et al., 2015). The world-wide prevalence of *BRCA* mutations is low (ranging from 0.1-0.7%) but numbers vary significantly as founder mutations have been identified in some populations, most notably in individuals of Ashkenazi

Jewish descent and some European populations, including Icelanders (Rebbeck et al., 2018).

The *BRCA* genes were identified in the 1990s through linkage-studies (Hall et al., 1990; Miki et al., 1994; Tavtigian et al., 1996; Wooster et al., 1994) and encode large proteins that bear few structural similarities despite being involved in many of the same cellular processes. Both proteins have multiple roles in maintaining genomic stability, most notably in the DNA damage response and HR repair of DNA double strand breaks (DSBs) (Chen et al., 2018; Roy et al., 2012). BRCA1 is a versatile protein involved in HR repair as well as DNA damage signalling and cell-cycle checkpoint regulation after DNA damage (Rosen, 2013). The original described role of BRCA2 was in HR repair where it facilitates RAD51 recombinase-mediated strand invasion, a crucial step in this repair pathway (Yuan et al., 1999). BRCA2 also stabilizes stalled replication forks by preventing degradation of nascent DNA strands (Schlacher et al., 2011) and has been shown to be involved in R-loop processing (Bhatia et al., 2014). Additionally, BRCA2 has been implicated in cytokinesis (Daniels et al., 2004; Jonsdottir et al., 2009; Mondal et al., 2012), the spindle assembly checkpoint (Choi et al., 2012) and G2/M checkpoint maintenance (Menzel et al., 2011). The BRCA proteins have both been linked to the Fanconi anemia (FA) pathway which functions in removing inter-strand cross-links in DNA (Howlett et al., 2002; Sawyer et al., 2015). Finally, both BRCA1 and BRCA2 have been shown to be important for maintaining telomere homeostasis (Badie et al., 2010; Ballal et al., 2009; Min et al., 2012). These topics will be discussed further later along with the consequences of their dysfunction on genomic stability and cancer.

1.6.1 The BRCA2 protein

Of relevance for the subject of this thesis the focus will now shift to BRCA2. The *BRCA2* gene, located on chromosome 13q12.3, comprises 27 exons encoding for a large protein of 3418 amino acids. The BRCA2 protein contains several important structural elements, binding sites for interaction partners and post-translational modification sites (**Figure 2A**). RAD51 binding is of integral importance for the function of BRCA2 and is achieved via two domains. The BRC repeats are eight conserved motifs located in exon 11 towards the middle of the protein (Bignell et al., 1997). They bind to monomeric RAD51 which is then loaded onto ssDNA at break sites thereby initiating homology search, a key event in HR repair of DNA DSBs (Jensen et al., 2010; Liu et al., 2010; Thorslund et al., 2010). A distinct RAD51 binding domain, TR2, is located at the C-terminus of BRCA2 (Sharan et al., 1997).

This domain is involved in stabilization of RAD51 filaments (Davies & Pellegrini, 2007) which is important for stalled replication fork protection but dismissible for HR repair of DSB (Schlacher et al., 2011). The C-terminal region additionally contains a serine phosphorylation site, which has been shown to regulate RAD51 binding post-translationally (Esashi et al., 2007), as well as nuclear localization signals (NLS) (Yano et al., 2000). A DNA-binding domain (DBD) is located towards the C-terminus (Yang et al., 2002). BRCA2 binds several other proteins reflecting its various functions including PALB2, EMSY, FANCD2, DSS1 and DMC1 (Fradet-Turcotte et al., 2016).

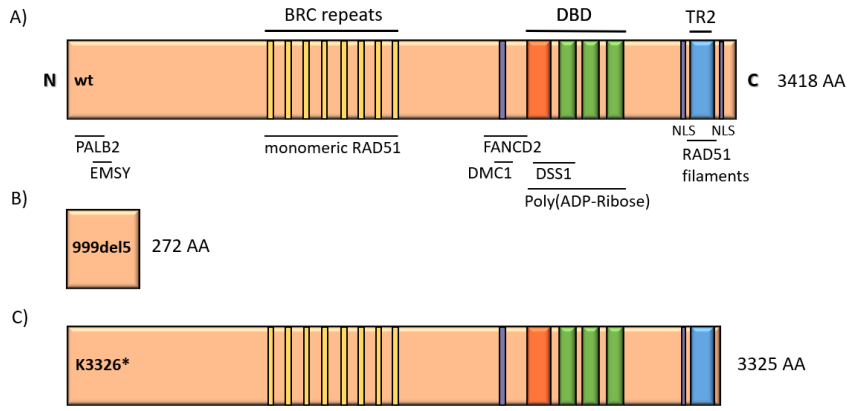

Figure 2. The BRCA2 protein A) BRCA2 functional domains and interaction partners. A N-terminal domain interacts with PALB2 and EMSY. Centrally located BRC repeats bind monomeric RAD51. The DBD domain binds ssDNA, DSS1 and poly(ADP-Ribose). A motif involved in FANCD2 and DMC1 binding is located adjacent to the DNA binding domain (DBD). The C-terminus contains a distinct RAD51 binding domain (TRF2) and two nuclear localization signals (NLS). Image based on and adapted from (Fradet-Turcotte et al., 2016). B) $BRCA2^{999del5}$ protein product. C) $BRCA2^{K3326*}$ protein product.

1.6.2 *BRCA* founder mutations in the Icelandic population

The Icelandic population is unique with regards to mutations in the *BRCA* genes since almost all mutation carriers carry the same few founder mutations. This makes the population feasible for studying the influence of single mutations at a population level.

A single mutation, commonly referred to as $BRCA2^{999del5}$ (rs80359671, NM_000059.3:c.767_771delCAAAT, NP_000050.2:p.Asn257Lysfs) (Thorlacius et al., 1996), is responsible for a majority of HBOC cases in the Icelandic population. It has a population carrier frequency of 0.8%

(Gudbjartsson et al., 2015; Thorlacius et al., 1997) and can be found in 6-7% of female breast cancer patients and 40% of male breast cancer patients in Iceland (Thorlacius et al., 1997). Interestingly, the *BRCA2*^{999del5} mutation was originally found in a male breast cancer family after its location had been identified through linkage analysis (Thorlacius et al., 1995). Penetrance varies and mutation carriers differ with respect to age of onset and severity of disease. In breast cancer patients diagnosed before the age of 40, approximately 25% carry the *999del5* mutation. (Thorlacius et al., 1997). The cumulative risk of developing breast cancer before the age of 70 has been estimated over 70% for mutation carriers and has increased over time (Tryggvadottir et al., 2006). Additionally, the *999del5* mutation confers increased risk of developing ovarian and prostate cancer (Johannesdottir et al., 1996; Rafnar et al., 2004; Thorlacius et al., 1997; Tulinius et al., 2002). The mutation is associated with poor prognosis, both in breast and prostate cancer (Sigurdsson et al., 1997; Thorlacius et al., 1996; Tryggvadóttir et al., 2007). The poor breast cancer outcome has been linked to tumor diploidy (Tryggvadottir et al., 2013), having a luminal subtype (Jonasson et al., 2016; Stefansson et al., 2011a) and overexpression of the Aurora A kinase (Aradottir et al., 2015).

The *BRCA2*^{999del5} mutation is a 5 base pair deletion in exon 9 of the *BRCA2* gene (Thorlacius et al., 1996). It causes a frameshift resulting in a prematurely truncated protein product of 272 amino acids, with the last 16 amino acids out of frame (Mikaelsdottir et al., 2004). Of functional relevance, both RAD51 binding domains are missing from the truncating protein product, as are the C-terminal nuclear localization signals (NLS) (**Figure 2B**). However, even though a mutant mRNA transcript is produced, the protein stub is not detected in the cytoplasm and the mRNA is most likely subject to nonsense mediated decay (Mikaelsdottir et al., 2004).

Another mutation in *BRCA2* is also present in the Icelandic population, the *BRCA2*^{K3326*} (rs11571833, NM_000059.3:c.9976A>T, NP_000050.2: p.Lys3326Ter), found at a allelic frequency of 1.1%. Interestingly, this mutation does not confer risk to breast and ovarian cancer in the population but rather a risk for small cell lung cancer and squamous cell carcinoma of the skin (Rafnar et al., 2018). The *BRCA2*^{K3326*} is a nonsense mutation in the last exon of the *BRCA2* gene, leading to loss of the last 93 amino acids of the protein (**Figure 2C**). The K3326* protein product contains the BRC repeats (RAD51 binding domain) and has been shown to be localized in the nucleus and proficient in DNA DSB repair (Kuznetsov et al., 2008; Wu et al., 2005). The truncation is however in proximity to the C-terminal RAD51 binding

domain of BRCA2 (Davies & Pellegrini, 2007) which may impair its protecting function at stalled replication forks, though this remains to be validated. It has been hypothesized that the tissue specificity in tumorigenesis between *999del5* and *K3326** mutation carriers highlights dependency on separate functions of BRCA2 (Rafnar et al., 2018). While breast tissue might be more dependent on the DSB function of BRCA2, other tissues under stronger environmental genotoxic stress might be more sensitive to replication fork stalling problems. In the case of lung and skin cancer, tobacco smoke and UV radiation are both known to cause bulky DNA adducts resulting in replication fork stalling (Hecht, 2011; Sinha & Häder, 2002).

As an example of the less severe phenotype of *K3326** compared to *999del5*, homozygotes are found in the expected Hardy-Weinberg equilibrium and do not show signs of Fanconi Anemia. This is not the case for *999del5* mutation where cases of homozygosity have never been seen and are considered embryonically lethal (Rafnar et al., 2018).

A founder mutation has also been identified in the *BRCA1* gene in the Icelandic population, the rare *BRCA1*^{G5193A} (rs80187739, also referred to as NM_007294.3:c.5074G>A, NP_009225.1:p.Asp1692Asn) (Bergthorsson et al., 1998). It is a single base substitution disrupting a splice site in exon 17, leading to lack of functional protein (Bergthorsson et al., 1998). Due to the low population frequency of *BRCA1*^{G5193A} it does not contribute substantially to either breast or ovarian cancer risk in Iceland (Arason et al., 1998; Bergthorsson et al., 1998; Rafnar et al., 2004). Other mutations are found in the *BRCA* genes in the Icelandic population but are thought to be rarer still (unpublished data from the Landspitali Genetic Counseling Unit (Stefansdottir et al., 2013)).

1.6.3 Genetic predisposition to male breast cancer

Family history is a strong risk factor for male breast cancer. While 5-10% of FBC are linked to underlying germline mutations, the corresponding ratio for MBC ranges from 4-40%, depending on the population (Fentiman et al., 2006). Results from multi-gene panel testing of MBC show that pathogenic germline mutations in *BRCA2* and *CHEK2* are the most abundant and mutations in *PALB2*, *ATM* and *BRCA1* are less common (Pritzlaff et al., 2017). Contribution of other DNA repair genes associated with HBOC syndrome, such as *BRIP1, PTEN* and *RAD51C,* to male breast cancer risk is less well established (Deb et al., 2016).

Thorough analysis of germline mutations in male breast cancer has not been conducted for the Icelandic population. A previous study (Thorlacius et al., 1997) found the *BRCA2*^{999del5} founder mutation present in 40% of cases diagnosed between 1955-1995, which is much higher than reported for other populations (Rizzolo et al., 2013).

1.6.4 Genetic predisposition to ovarian cancer

One of the strongest risk factors for developing ovarian cancer is family history of the disease. Two familial cancer syndromes are primarily involved; the HBOC and the Hereditary Non-Polyposis Colorectal Cancer Syndrome (HNPCC or Lynch syndrome). The largest portion of genetic predisposition to ovarian cancer is attributable to mutations in the *BRCA* genes, or 5-20% of all cases (Cancer Genome Atlas Research Network, 2011; Daly et al., 2010; Ramus & Gayther, 2009). In a population-based study of ovarian cancers in Iceland diagnosed over the period 1990-2000, the *BRCA1*^{G5193A} mutation was present 1.2% while the *BRCA2*^{999del5} mutation was present in 6% of the patients (Rafnar et al., 2004). Germline mutations in other genes involved in HR DNA-repair, such as *BRIP1*, *PALB2*, *RAD51C* and *RAD51D* to name a few, have also been shown to contribute to ovarian cancer risk (Kanchi et al., 2014; Ramus et al., 2015; Song et al., 2015). A truncating frameshift mutation in *BRIP1* is found in the Icelandic population (*BRIP*$^{2040\_2041insTT}$, rs587778134, NM_032043.3: c.2040_2041insTT, NP_114432.2:p.Leu680fs) at 0.41% allelic frequency (Rafnar et al., 2011). The *BRIP*$^{2040\_2041insTT}$ mutation significantly increases the risk for ovarian cancer in particular and pancreatic and rectal cancer to a lesser extent. Interestingly, the increased risk is not observed for breast cancer (Rafnar et al., 2011). Germline mutations in Lynch Syndrome genes involved in DNA mismatch repair (MMR); *PMS2*, *MLH1*, *MLH2* and *MSH6*, have been shown to increase the risk of developing both ovarian and endometrial cancer (Bonadona et al., 2011; Ten Broeke et al., 2015). In a recent study on the prevalence of Lynch Syndrome in the Icelandic population, deleterious founder mutations were identified in *PMS2* (c.736_741del6ins11, population carrier frequency 0.234% and c.2T4A, population carrier frequency 0.092%) and *MSH6* (c.1754T4C, population carrier frequency 0.080%) which all dramatically increase the risk of endometrial cancer. Both *PMS2* mutations are additionally associated with a significantly increased risk for ovarian cancer. Mutations in *MLH1* and *MSH2* are more common world-wide but are interestingly almost non-existent in the Icelandic population, suggesting a negative founder effect (Haraldsdottir et al., 2017).

1.7 DNA damage and repair of DNA double strand breaks

Cells are constantly exposed to internal and external factors that may cause DNA damage. It is estimated that each human cell is subject to 70.000 DNA lesions per day (Lindahl & Barnes, 2000). Exogenous sources include UV light, cigarette smoke and ionizing radiation but even more common are DNA lesions arising from endogenous sources in cells such as free-oxygen radicals from cell-metabolism and DNA replication errors (Tubbs & Nussenzweig, 2017). Persistent DNA damage interferes with normal cell function such as replication and can cause accumulation of mutations, chromosome abnormalities and genome instability, eventually leading to tumor formation (Kass et al., 2016). Accurate DNA repair is therefore of pivotal importance for proper cell function and maintenance of genome stability. Types of DNA damage include single strand and double strand breaks (SSBs and DSBs) in DNA, mismatched bases, depurination and deamination of bases, and inter-strand cross-links (ICLs). Various pathways have evolved in response to these different types of DNA damage, involving detection, signal transduction and repair, collectively termed the DNA Damage Response (DDR)(Jackson & Bartek, 2009). Defective DDR is found in most tumors and unsurprisingly, inherited or acquired mutations in many DDR genes result in increased cancer risk.

1.7.1 DNA double strand breaks

A DSB is a cleavage of both strands of DNA resulting in loss of continuation of the genome. DSBs can be generated by the many exogenous and endogenous sources described above, often as a result of nucleolytic cleavage of structures in DNA such as stalled or collapsed replication forks, DNA ICLs or R-loops (Berti & Vindigni, 2016; Hanada et al., 2006; Sollier et al., 2014). DSBs are the most cytotoxic of the different types of DNA damage. Failure to repair even a single DSB can cause chromosome translocations or gross genomic rearrangements, eventually leading to senescence, cell death or even tumor initiation (Kass et al., 2016). Due to its importance for genomic stability and tumor suppression, repair of DNA DSBs is employed by a few different pathways. The two main high-fidelity pathways involved are Canonical Non-Homologous-End-Joining (C-NHEJ), which directly ligates DNA ends, and Homologous Recombination (HR) repair, which uses a sister-chromatid as a template for accurate repair. Additionally, two more error-prone pathways, alternative end joining (alt-EJ) and single-strand annealing (SSA) are involved in DSB repair.

1.7.2 Detection of double strand breaks and repair pathway choice

Initial recognition of DNA DSBs is through the MRE11-RAD50-NBS1 (MRN) complex (Petrini & Stracker, 2003) which recruits the Ataxia Telangiectasia Mutated (ATM) kinase, initiating a cascade of signal transduction by phosphorylation and ubiquitination of many proteins involved in DNA repair and cell-cycle checkpoint control (Dantuma & van Attikum, 2016). One of the main targets is histone H2AX which is phosphorylated at serine residue 139 (γH2AX) at the break site with the signal rapidly spreading across the flanking chromatin (Rogakou et al., 1998). γH2AX then recruits various DNA repair factors to the site of damage, culminating in the recruitment of tumor suppressors p53-binding protein 1 (53BP1) and BRCA1 (Dantuma & van Attikum, 2016).

Pathway choice at DSBs is dependent on the phase of the cell cycle and level of DNA end-resection at the break, largely determined by the antagonistic relationship between BRCA1 and 53BP1 (Panier & Boulton, 2013; Zimmermann & de Lange, 2014). Of the repair pathways previously mentioned, only C-NHEJ is independent of end-resection and is available throughout the cell cycle, being favored in G0/G1 when end-resection is blocked. However, end-resection at DSBs in S/G2 commits cells to repair via the other pathways, preferring the accurate HR repair pathway over the intrinsically mutagenic SSA and alt-EJ pathways (Ceccaldi et al., 2016a; Karanam et al., 2012). See overview of DNA DSB break repair pathways in **Figure 3**.

End-resection, the processing of DNA ends to produce 3’ single strand DNA (ssDNA) ends, occurs in the S/G2 phases of the cell cycle and is mediated by cyclin-dependent kinases (CDKs) through phosphorylation of multiple substrates (Ceccaldi et al., 2016a). An initial resection step involves CtBP-interacting protein (CtIP) and the MRN complex (Lamarche et al., 2010). This initial end-processing results in short 3‘ ssDNA-overhangs that can act as intermediates for alt-EJ (Truong et al., 2013). A second phase of extensive end resection then follows, carried out by a number of helicases and exonucleases (including DNA2, EXO1, BLM and WRN) committing cells to repair with SSA or HR (Nimonkar et al., 2011; Sturzenegger et al., 2014). In G1, 53BP1 and RAP-interacting factor 1 (RIF1) localize at DSBs, blocking BRCA1 binding and end-resection, thus promoting C-NHEJ (Feng et al., 2013). The binding of BRCA1 and its interacting partner CtIP to DSBs in G2/S, in turn antagonizes RIF1 accumulation, inhibiting NHEJ (Escribano-Díaz et al., 2013). This model is however overly simplistic since both proteins

have multiple roles in the DDR and seem to regulate pathway choice and promote repair on multiple levels (Chen et al., 2018; Panier & Boulton, 2013; Zimmermann & de Lange, 2014).

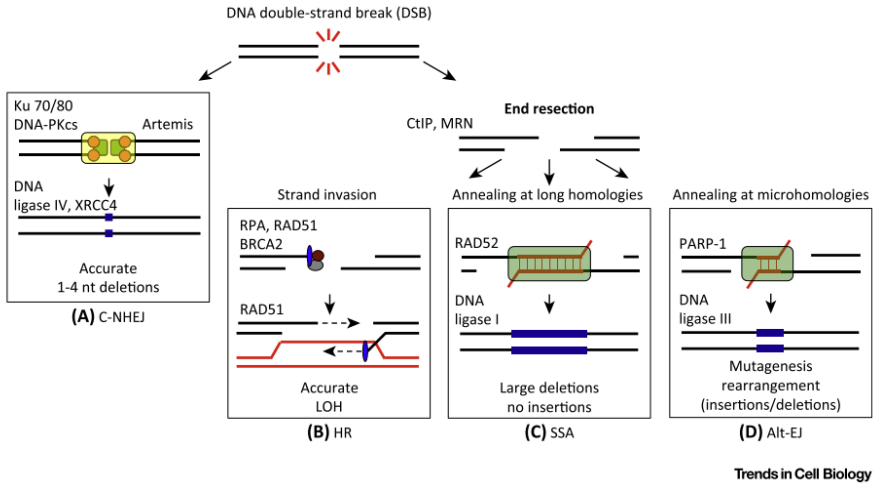

Figure 3. Overview of DNA double strand break repair pathways, main players and genetic consequences. After detection of a DSB, pathway choice is largely determined by the level of DNA end resection at the break site. C-NHEJ (A) is the preferred pathway choice when end-resection is blocked. When end-resection occurs, DSBs are repaired through the accurate HR pathway (B) or error-prone SSA (C) and Alt-EJ (D) pathways. Reproduced with permission from Elsevier License #4654970770208 (Ceccaldi et al., 2016a).

1.7.3 Homologous Recombination repair

HR repair is considered the most precise way to repair DNA DSBs. It is restricted to the mid/late S to G2 phases of the cell cycle when replication is active, due to its requirement for a sister chromatid template (Karanam et al., 2012). Extensive end-resection of DSBs is required for the initial steps of HR repair. The resulting exposed 3'-ssDNA overhangs are rapidly coated with Replication Protein A (RPA), protecting the ends from nuclease degradation and formation of secondary structures (Chen et al., 2013). BRCA1 interacts with PALB2 which in turn recruits BRCA2 to the site (Sy et al., 2009; Zhang et al., 2009). BRCA2 binds RAD51 through its BRC repeats, directs it to the site of damage and then mediates RAD51 loading onto the ssDNA ends, forming a nucleoprotein filament and replacing RPA (Liu et al., 2010; Thorslund et al., 2010; Yang et al., 2002; Yuan et al., 1999). This step commits cells to repairing with HR (Moynahan et al., 2001). The RAD51

nucleoprotein filament catalyzes homology search and strand invasion to the sister-chromatid, forming a displacement loop (D-loop) (Baumann & West, 1998). Repair synthesis then ensues from the invading end by using the sister chromatid as a template (Jasin & Rothstein, 2013).

1.7.4 Single-strand annealing

Apart from HR repair, the other DSB repair mechanism that depends on extensive end-resection in S/G2 is single-strand annealing (SSA). This process does not require a template for repair and is therefore not dependent on replication and the availability of a sister chromatid like HR. After end-resection, homologous sequences flanking the DSB are revealed and annealed together, forming a synapsed intermediate that is subsequently processed for ligation (Bhargava et al., 2016). The processing involves nucleolytic removal of the non-homologous 3'-ssDNA overhangs and is mediated by RAD52 and ERCC1/XPF complex (Bennardo et al., 2008; Motycka et al., 2004). DNA polymerases then fill in the gaps to generate substrates for ligation. This completes the SSA process and inevitably causes loss of genetic information between the interspersed matching sequences, often ranging in hundreds of base pairs (Bhargava et al., 2016).

1.7.5 Canonical Non-Homologous End-Joining

Non-homologous end-joining is thought to play the largest role in DSB repair in humans. The pathway mediates direct ligation of broken DNA ends independent of sequence homology (Lieber et al., 2003). C-NHEJ is therefore available throughout the whole cell cycle but homology-based pathways like HR are favored during mid-S/G2 phases as previously described. NHEJ therefore primarily repairs DSBs arising in G0/G1 and early S phase (Karanam et al., 2012).

The first step in NHEJ repair involves the recruitment of the Ku70/80 heterodimer to the DSB break site where it coats the broken DNA ends and protects them from non-specific processing (Downs & Jackson, 2004). The Ku heterodimer then acts as a scaffold along with DNA-PKcs recruiting the main payers in NHEJ repair; XRCC4, DNA ligase IV and XLF that catalyze synapsis and ligation of the broken DNA ends. In some cases, DNA ends are not compatible for direct ligation and require processing to create ligatable ends. The end-processing can be in the form of removing blocking groups, end-resection and gap filling and is mediated by different DNA end processing enzymes including the nuclease Artemis (reviewed in (Chang et al., 2017)).

NHEJ has historically been considered an error-prone repair pathway since gain or loss of genetic material is generally observed at the DSB break site. New data however indicates that the NHEJ process itself is not inherently error-prone but rather the adaptability of the pathway to repair imperfect non-complementary ends needing end-processing. This is very important for maintaining chromosome integrity by preventing chromosome rearrangements. NHEJ can therefore be considered high-fidelity on a global genomic scale, at the cost of small errors. The bad reputation of NHEJ could also be explained by the lack of distinction between C-NHEJ and the existence of a highly mutagenic alternative NHEJ pathway (Bétermier et al., 2014; Deriano & Roth, 2013).

1.7.6 Alternative Non-Homologous End-Joining

In recent years, highly error-prone alternative end-joining processes have been identified, termed alternative NHEJ or alt-EJ. The molecular mechanism behind alt-EJ is not completely understood but it is likely comprised of a few different mechanisms, including the Microhomology Mediated End-Joining (MMEJ) process. MMEJ requires minimal end-resection and repairs DSBs by annealing short stretches (<25nt) of homologous sequences flanking the DSB, introducing deletions with microhomologies around the break. The process is therefore highly mutagenic around the DSB but also seems to have a tendency for joining DSBs on different chromosomes, creating chromosomal translocations (Mcvey et al., 2017). MMEJ is mediated by PARP1, the XRCC1/DNA ligase III complex, FANCD2 and the error-prone translesion polymerase theta (Polθ) (Audebert et al., 2004; Kais et al., 2016; Mateos-Gomez et al., 2015). Alt-EJ was originally thought to be a back-up mechanism in the case of C-NHEJ absence. It has however been shown that alt-EJ is active in cells proficient in C-NHEJ and HR (Sfeir & Symington, 2015).

1.8 Replication fork stalling and collapse – focus on BRCA2

DNA replication must be completed for the entire genome before a cell can divide. The normal progression of DNA replication forks is frequently challenged by DNA lesions or other obstacles in the genome including secondary structures like R-loops and G-quadruplexes as well as repetitive sequences. This can cause replication fork stalling. Failure to restart these stalled replication forks can result in their collapse and nucleolytic processing, forming a DSB (Berti & Vindigni, 2016). BRCA2 is a key player in HR repair and therefore has a role in maintaining genome stability through repairing

DSBs resulting from collapsed replication forks.

Early studies suggested another separate role for BRCA2 in stabilizing stalled replication forks (Lomonosov et al., 2003). This function has since been shown to happen through prevention of nucleolytic processing (Schlacher et al., 2011; Ying et al., 2012). Stalled replication forks activate the ATR-mediated replication stress response that promotes fork stabilization and restart while halting progression through the cell cycle. In some cases the fork reverses in an attempt to restart, leaving nascent DNA strands exposed to degradation by nucleases such as MRE11 (Zeman & Cimprich, 2014). As previously described, the C-terminal TR2 domain of the BRCA2 protein is involved in stabilizing RAD51 filaments but not the loading of RAD51 onto ssDNA (Davies & Pellegrini, 2007). BRCA2 is thought to prevent degradation of nascent DNA strands by stabilizing RAD51 filaments at the stalled forks. In the absence of BRCA2, stalled replication forks are not protected and lead to chromosomal instability. This protective role of BRCA2 is independent from its role in HR repair (Schlacher et al., 2011).

1.9 Fanconi Anemia and repair of inter-strand cross-links

While heterozygous mutations in the *BRCA* genes predispose to HBOC syndrome, biallelic mutations are linked to Fanconi Anemia (FA), a recessive genetic disorder characterized by childhood bone marrow failure, developmental defects, growth retardation and increased cancer susceptibility. The FA pathway consist of 22 FANC proteins, identified to date, and plays an important role in maintaining genome stability by removing inter-strand crosslinks (ICLs) in DNA (Niraj et al., 2019). An ICL is a covalent connection between nucleotides on opposite strands. ICLs are highly toxic lesions if not repaired since they completely block strand separation and thereby stall replication forks. Cells with defective FA pathway are therefore hypersensitive to known cross-linking agents such as mitomycin C and cisplatin. This is demonstrated by increased chromosomal instability and a characteristic formation of radial chromosomes, along with cell death (Huang & Li, 2013). The mechanism of the FA pathway in ICL repair is complex and will not be covered in detail here. Briefly it relies on FANCM and associated sensor proteins to detect the lesion and recruiting the multisubunit FA core complex. The FA core complex then ubiquitinates the FANCI/FANCD2 heterodimer which then interacts with downstream factors involved in translesion synthesis and HR repair (Niraj et al., 2019).

Individuals with biallelic *BRCA2* mutations are classified to Fanconi Anemia subtype D1 (FANCD1)(Howlett et al., 2002). This subtype is clinically distinct from the other FA subtypes and is especially associated with brain and haematological malignancies in early childhood (Meyer et al., 2014). The identification of BRCA2 as FANCD1, linked the FA and HR pathways. Subsequently, other proteins involved in the BRCA pathway were identified as FANC proteins, PALB2 as FANCN, RAD51C as FANCO, BRIP1 as FANCJ and XRCC2 as FANCU (Ceccaldi et al., 2016b; Park et al., 2016). Recently, a patient with Fanconi Anemia was shown to carry biallelic mutations in BRCA1, leading to the classification of BRCA1 as FANCS (Sawyer et al., 2015). Carrying deleterious null mutations in both alleles of the *BRCA* genes is generally considered embryonically lethal (Moynahan, 2002; Sharan et al., 1997) so in these FA cases, one allele is expected to retain partial function (Howlett et al., 2002).

1.10 Genomic instability in BRCA tumors

Evidence suggests that despite the relatively similar cancer predisposing phenotype, tumorigenesis happens by distinct mechanisms in BRCA1 and BRCA2 tumors. BRCA1-associated breast cancers are primarily basal-like (van ’t Veer et al., 2002) while BRCA2-associated breast cancers have been shown to display predominantly luminal subtypes of high histological grade, more similar to sporadic tumors (Bane et al., 2007; Stefansson et al., 2009). Nevertheless, an important similarity is that *BRCA* tumors typically exhibit extreme genomic instability. BRCA2 associated tumors are characterized by structural chromosomal aberrations such as loss of chromosome arms, chromatid breaks, chromosome end-to end fusions, radial chromosomes and telomere abnormalities (Bodvarsdottir et al., 2012; Gretarsdottir et al., 1998; Tirkkonen et al., 1997). This chromosome instability is usually attributed to the role of BRCA2 in error free HR repair and protection of stalled replication forks but also highlights the multifunctionality of BRCA2 in many processes maintaining genomic stability (listed above in 1.6). Dysfunctional telomere maintenance is a well-known cause for chromosomal instability (discussed below), as are problems in cytokinesis giving rise to breakage-fusion-bridge (BFB) cycles and anaphase bridges (Eyfjord & Bodvarsdottir, 2005; Jonsdottir et al., 2009).

Shifting focus back to dysfunctional DNA repair. When HR repair is absent, cells rely more on alternative and more error-prone DNA repair pathways such as NHEJ, alt-EJ or SSA, leading to accumulation of mutations and chromosomal instability (Ceccaldi et al., 2015; Gudmundsdottir &

Ashworth, 2006; Mateos-Gomez et al., 2015; Venkitaraman, 2002). Reflecting this dependency, mutational signatures have been identified in tumors from *BRCA* mutation carriers, characterized by elevated levels of indels, rearrangements and base substitutions (short deletions with microhomology at the break-point) (Nik-Zainal et al., 2016; Polak et al., 2017). Based on these mutational signatures, multiple HR deficiency scores have been developed to predict the "BRCAness" of tumors (Davies et al., 2017; Maxwell et al., 2017; Telli et al., 2016).

1.11 Targeting BRCAness in cancer treatment

Knowledge of HR repair deficiency in BRCA-related tumors and their inability to repair stalled replication forks has been utilized for treatment. Cells completely lacking BRCA1/2 function are extremely sensitive to DNA damage inducing agents causing replication fork stalling or collapse, such as platinum salts (Mylavarapu et al., 2018), topoisomerase inhibitors (Rahden-Staroń et al., 2003) and PARP inhibitors (Lord & Ashworth, 2017).

Over the last two decades, PARP (Poly(ADP-ribose) polymerase) inhibitors have emerged as an example for exploiting the concept of synthetic lethality in cancer treatment (Lord et al., 2015). Two genes are considered in synthetic lethal relationship if inactivation of both leads to cell death, but the inactivation of either one does not. Originally, the synthetic lethal relationship between PARP1 and the BRCA proteins was thought to be based on the role of PARP1 in SSB repair of DNA through the base excision repair (BER) pathway (Bryant et al., 2005; Farmer et al., 2005). In the absence of PARP1, SSB are not repaired and subsequently converted to DSB during replication (Satoh & Lindahl, 1992). Treatment of BRCA deficient cells with PARP inhibitors would therefore lead to accumulation of irreparable DSB resulting in cell death (Ashworth, 2008). Studies have since elucidated numerous roles of PARP1 in maintaining genomic stability through its function in various DNA repair pathways, stabilization of replication forks and modulation of chromatin structure (Ray Chaudhuri & Nussenzweig, 2017). As an example, PARP1 is involved in alt-EJ (Mansour et al., 2010), a back-up mechanism for HR in DSB repair which if inhibited leads to synthetic lethality in BRCA deficient cells (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015). It has also been shown that PARP inhibition acts as a DNA damaging agent by trapping PARP1 on DNA, blocking replication and creating very cytotoxic lesions (Murai et al., 2012; Pommier et al., 2016).

PARP inhibitors are currently in clinical use for treatment of breast and ovarian cancer in *BRCA* mutation carriers (Kaufman et al., 2015; McCann & Hurvitz, 2018) and have also been shown to benefit patients with a range of different tumor types (Lord & Ashworth, 2017). In this regard, it should be noted that not all tumors arising in *BRCA* mutation carriers, or tumors with somatic *BRCA* mutations, harbor the characteristic HR deficient phenotype. This especially applies to non-BRCA-linked tumor types that seem to be mostly sporadic in nature and would not benefit from targeted treatment (Jonsson et al., 2019). That being said, it is clear that tumors exhibiting BRCAness are not limited to carriers of *BRCA* mutations (Lord & Ashworth, 2016). The HR deficiency mutational signatures have identified a much larger group of breast cancer patients (22%) with an underlying HR deficiency, resulting from germline and somatic inactivation of HR genes, either by deleterious mutations or promoter methylation (Davies et al., 2017). Estimates for ovarian cancer are even higher, or up to 50% of all high-grade serous tumors (Cancer Genome Atlas Research Network, 2011). Identifying this group is important as it would benefit from targeted treatment.

Disappointingly, both primary and acquired resistance to PARP inhibitors (and platinum-based therapy) is common. Simplistically, resistance is acquired by restoration of HR repair and/or stabilization of replication forks. Mechanisms of the former include reverse mutations or reversal of promoter methylation in the *BRCA1/2* or other HR genes (D'Andrea, 2018). Mutational signatures can identify tumors with underlying HR defects, but the signature may still persist after restored HR function. For improved discrimination between tumors truly HR deficient, biomarker assays are currently being developed for clinical use, including RAD51 foci staining as a surrogate marker for proficient HR repair (Castroviejo-Bermejo et al., 2018; Cruz et al., 2018).

The search continues for new therapeutic strategies for exploiting DNA repair deficiencies in tumors, often focusing on their dependency for "back-up" alternative mechanisms. As an example, recent publications have shown that some BRCA deficient tumors upregulate Polθ and FANCD2, both involved in error-prone alt-EJ, as a compensatory mechanism for reduced HR efficiency (Ceccaldi et al., 2015; Kais et al., 2016; Mateos-Gomez et al., 2015). FANCD2 has also been shown to have a role in stabilizing stalled replication forks (Kais et al., 2016; Schlacher et al., 2012). Inhibiting these proteins in BRCA deficient cells causes synthetic lethality and holds promise for new treatment strategies. Similarly, loss of RAD52 function is synthetically lethal with BRCA1, BRCA2 or PALB2 deficiency (Lok et al., 2013). RAD52 is

involved in SSA DSB repair (Bennardo et al., 2008) and also considered a back-up factor for RAD51 in HR (Lok & Powell, 2012). More recently it has been shown to facilitate DNA synthesis during mitosis following replication stress (Bhowmick et al., 2016). BRCA2 suppresses such replication stress through HR (Feng & Jasin, 2017).

1.12 Telomeres, telomere dysfunction and cancer

Telomeres are nucleoprotein structures at the end of linear chromosomes that provide protection against DNA damage and degradation, thereby playing a critical role in maintaining chromosome integrity.

1.12.1 Telomere structure and function

Mammalian telomeres are comprised of repeated nucleotide sequences $(TTAGGG)_n$ ranging from 10-15 kb in humans and terminating in a single stranded 3'-overhang (Moyzis et al., 1988; Wright et al., 1997). This G-rich overhang folds over to form a displacement loop (D-loop) in the double stranded region, creating a larger loop structure (the T-loop) in the process (Griffith et al., 1999). The telomere repeats are bound by the shelterin complex which is composed of six proteins; TRF1, TRF2, TIN2, POT1, TPP1 and RAP1. The shelterin complex aids in the formation of the T-loop, essentially capping the chromosome end and preventing cells from recognizing telomeres as sites of damage (**Figure 4A**) (de Lange, 2005a).

Telomeres pose a challenge for the replication machinery due to their structure. Incomplete lagging strand DNA synthesis results in progressive shortening of telomere sequences with each cell cycle. This is commonly referred to as the end-replication problem (Levy et al., 1992). Telomerase can compensate for telomere shortening by adding new telomeric repeats onto chromosome ends (Blackburn et al., 1989). Telomerase is composed of two subunits, a reverse transcriptase (hTERT) (Nakamura et al., 1997) and an RNA component (TERC) (Feng et al., 1995). The catalytic hTERT subunit of telomerase is however not ubiquitously expressed in humans, being repressed in most somatic cells during gestation and only expressed in germ cells and stem cells (Wright et al., 1996). Telomeres therefore shorten over a lifetime in somatic cells due to the end-replication problem as well as other factors affecting telomere replication, such as replication fork stalling, nucleolytic processing and oxidative stress. This is considered one of the hallmarks of aging (López-Otín et al., 2013).

1.12.2 Dysfunctional telomere maintenance and cancer

Sufficient telomere length is necessary for maintaining the T-loop structure and a protective capped state of the chromosome end. Under normal circumstances in the absence of a telomere length maintaining mechanism, telomere length reaches a certain threshold and triggers the cell's DDR, inducing replicative senescence and cell growth arrest (Levy et al., 1992). Inherited mutations in genes involved in telomere maintenance and capping can accelerate this process, giving rise to premature aging diseases, commonly referred to as telomeropathies (Armanios & Blackburn, 2012; Holohan et al., 2014) or predispose to cancer.

Replicative senescence due to telomere shortening is considered an important tumor suppressor mechanism. However, this state can be bypassed by acquiring mutations in genes involved in cellular checkpoints, leading to further telomere shortening and chromosomal instability, eventually contributing to malignant transformation (Murnane, 2010). Indeed, dysfunctional telomere maintenance and the ensuing genomic instability is considered a driving force and a hallmark of human cancers (Hanahan & Weinberg, 2011). Over 85% of all cancers escape senescence by activating telomerase expression (Shay & Bacchetti, 1997) while ~10-15% rely on a DNA recombination pathway called alternative lengthening of telomeres (ALT) (Bryan et al., 1997), which is characterized by extreme telomere length heterogeneity (Dunham et al., 2000). This is contradictory to the notion that short telomeres predispose to cancer. Studies have however shown that activation of a telomere maintenance mechanism is not required for tumor initiation but critical for cancer progression (Shay, 2014) and that most tumors have previously undergone periods of severe genomic instability due to telomere dysfunction (de Lange, 2005b; Maciejowski & de Lange, 2017). See overview of telomere shortening and dysfunction in senescence and tumorigenesis in **Figure 4B**.

Of relevance to this thesis, telomere shortening has been shown to be an early alteration in epithelial cancers (Artandi et al., 2000; Meeker et al., 2004b), including breast cancers (Chin et al., 2004; Raynaud et al., 2010).

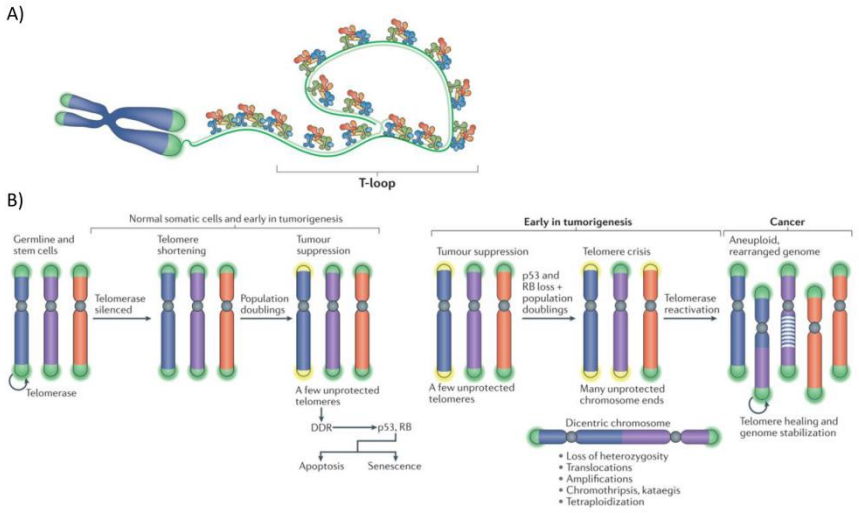

Figure 4. Telomere dysfunction and cancer. A) Telomeric sequences at chromosome ends are bound by the shelterin complex which aids in T-loop formation, capping the end and protecting it from degradation. B) Telomerase is repressed in somatic cells and telomeres shorten with each cell division due to the end-replication problem. Critically short telomeres activate the DDR, inducing replicative arrest and apoptosis. Loss of tumor suppressor pathway functions allow cells to continue to divide, resulting in more telomere shortening and telomere crisis where chromosomes fuse and cause genomic instability. Reactivation of telomerase alleviates the crisis by elongating telomeres, immortalizing cells and reestablishing genomic stability. This results in tumor cells with extensive genomic rearrangements and active telomerase. Reproduced with permission from Springer Nature License #4671621379061 (Maciejowski & de Lange, 2017).

1.12.3 BRCA2 and telomeres

As previously described, BRCA2-associated tumors are characterized by genomic instability linked to defects in HR repair and stalled replication fork protection (Gretarsdottir et al., 1998; Schlacher et al., 2011; Venkitaraman, 2002). These functions are especially important for various aspects of telomere maintenance (Tacconi & Tarsounas, 2015). Telomere sequences are G-rich and frequently form secondary structures such as G-quadruplexes which hinder replication, leading to stalled or collapsed replication forks that need alleviating via HR repair (Gilson & Géli, 2007). Additionally, HR is involved in T-loop formation (Verdun & Karlseder, 2006). With HR playing a key role in both telomere capping and replication, it is not surprising that BRCA2 has been shown to be important for telomere protection and maintenance.

BRCA2 is associated with telomeres in S and G2 phases of the cell cycle where it loads RAD51 onto telomeres (Badie et al., 2010). Deletion of BRCA2 in mouse embryonic fibroblasts (MEFs) leads to telomere shortening and accumulation of dysfunctional telomeres, common fragile sites and telomere sister-chromatid exchanges, suggesting a role for BRCA2 in telomere replication (Badie et al., 2010; Min et al., 2012). Recently it has been shown that BRCA2 facilitates telomere replication across challenging DNA structures such as G4-quadruplexes (Zimmer et al., 2016). This role is most likely mediated through restart of stalled replication forks and repair of replication-associated DSB within telomeres, rather than in dissolving these structures (Zimmer et al., 2016). Tumors from *BRCA2* mutation carriers have short telomeres and show signs of telomere dysfunction, with frequent chromosome end-to end fusions and accumulation of telomere dysfunction induced foci (TIFs) (Badie et al., 2010; Bodvarsdottir et al., 2012; Martinez-Delgado et al., 2013). Chromosomal instability in BRCA2-associated tumors is therefore at least partly due to telomere dysfunction.

1.13 *BRCA* loss of heterozygosity and haploinsufficiency

Knudson's two hit hypothesis of tumorigenesis is a classical theory in the cancer field. It states that one functional allele of a TSG is sufficient to prevent tumor formation, only when the other allele is lost, cancer can develop (Knudson, 2001). The *BRCA* genes are canonical TSGs, as are many other genes involved in maintaining genomic stability. In *BRCA* mutation carriers, it could therefore be considered that one of these "hits" has already occurred in every cell of the body and a somatic second "hit" of the wild-type allele would lead to cancer formation, to some extent explaining the cancer predisposition among mutation carriers over non-carriers where two somatic "hits" would be needed. Based on this, loss of heterozygosity (LOH) of the wild type allele was for long considered essential for tumor formation in *BRCA* mutation carriers (Berger et al., 2011; Gudmundsson et al., 1995). This is indeed supported by the observation that LOH is present in morphologically normal breast epithelial cells, early malignant lesions and DCIS in some mutation carriers (King et al., 2007). However, it is clear that tumorigenesis in *BRCA* mutation carriers is a more complicated process, evident by the varied penetrance and the fact that a subset of tumors exhibit retention of the wild-type allele, raising the question of *BRCA* haploinsufficiency (King et al., 2007; Nik-Zainal et al., 2016; Stefansson et al., 2011a).

1.13.1 LOH in tumors from *BRCA* mutation carriers

Tumors from *BRCA1* and *BRCA2* mutation carriers show different patterns

with regards to LOH. In a recent study combining datasets from the Cancer Genome Atlas (TCGA) and the University of Pennsylvania (Maxwell et al., 2017), somatic LOH of the wild-type allele was found to be present in 90% of *BRCA1* mutation carriers with breast cancer and 93% with ovarian cancer. LOH does not seem to be as abundant among the same tumor types in *BRCA2* mutation carriers, with 84% of ovarian tumors and only 54% of breast tumors exhibiting LOH (Maxwell et al., 2017). Similar results have also been reported from a large-scale exome-wide analysis of ovarian cancer, where LOH was present in 100% of tumors from *BRCA1* mutation carriers and 76% of *BRCA2* mutation carriers (Kanchi et al., 2014). Previous results from Icelandic $BRCA2^{999del5}$ mutation carriers using quantitative allelotyping (and later validated by aCGH analysis) support this, with LOH only detected in 52% of the breast tumors studied (Aradottir et al., 2015; Stefansson et al., 2011a). In contrast, an early study found LOH in 94% of breast tumors from $BRCA2^{999del5}$ mutation carriers (Arason et al., 1998). The few $BRCA1^{G5193A}$ tumors analyzed have all shown LOH (Arason et al., 1998). In a total of 90 breast tumors with germline or somatic mutations in *BRCA1/2* or promoter methylation of *BRCA1* included in the Wellcome Trust Sanger Institute's study of mutational signatures in breast cancer, 80 tumors exhibited LOH of the wild type allele (Nik-Zainal et al., 2016). In both the Sanger and U.Penn/TCGA studies, breast tumors from *BRCA* mutation carriers with LOH have the characteristic high HR deficiency scores while tumors retaining the wild type allele do not. These tumors lack typical *BRCA*-related tumor characteristics such as high mutational burden and their HR deficiency scores rather resemble those seen in HR proficient tumors from non-carriers (Davies et al., 2017; Maxwell et al., 2017; Nik-Zainal et al., 2016). It is however unlikely that all these tumors are sporadic in nature given the early age of onset in many cases, suggesting a mechanism of tumorigenesis distinct from sporadic tumors (Maxwell et al., 2017).

Similar studies on LOH in MBC from *BRCA* mutation carriers are lacking but *BRCA2* LOH in prostate cancer has been reported in 67-100% of malignant cases, although in small cohorts (Annala et al., 2017; Castro et al., 2015; Edwards et al., 2010; Willems et al., 2008). Analysis of 7 pancreatic tumors from $BRCA2^{999del5}$ carriers additionally revealed that LOH is not necessary for pancreatic tumor formation in the background of *KRAS* mutations. Furthermore, *BRCA2* LOH is associated with different histological subtypes of pancreatic cancer, a finding supported by studies in murine models (Skoulidis et al., 2010).

Collectively, these observations indicate that LOH is an important event in *BRCA1* tumors, but is more variable between tumor types in *BRCA2* mutation carriers. The apparent difference in LOH dependency between

BRCA1 and *BRCA2* and tissue specific carcinogenesis is intriguing. The lack of LOH and the accompanying HR deficient phenotype in a subset of breast tumors has important clinical implications. These tumors are not likely to respond to treatment with PARP inhibitors as they by principle selectively kill cells completely deficient in BRCA2 function but not heterozygous cells (Bryant et al., 2005; Farmer et al., 2005).

1.13.2 Possible *BRCA2* haploinsufficiency

The evidence outlined above calls into question the widely accepted notion that LOH is necessary for the onset of tumorigenesis in *BRCA2* mutation carriers. There are indications that *BRCA2* heterozygosity suffices to promote tumor development on the background of other key driver mutations, and that LOH may then be a later event (Berger et al., 2011; Skoulidis et al., 2010; Stefansson et al., 2011a). A possible explanation for retention of the wild type allele could be intolerance for complete LOH in a normal genetic background, as is the case for *BRCA2* in murine development and normal breast epithelial cell lines (Feng & Jasin, 2017; Sharan et al., 1997). This ties in with the idea of haploinsufficiency, a term referring to when a single functional copy of a gene does not sufficiently maintain protein function. A reduction in specific cell function may cause underlying weaknesses in the cell, gradually predisposing it to become cancerous, but only leading to tumor formation when accompanied with other driving events (Berger et al., 2011). In the case of *BRCA2* mutation carriers, minimal defects in HR repair and replication fork protection due to lowered expression affecting function in a dose-dependent manner, may cause accumulation of mutations over time contributing to cancer risk (Jackson & Bartek, 2009; Tubbs & Nussenzweig, 2017), with or without loss of the wild type allele.

Carriers of *BRCA* mutations show no obvious signs of haploinsufficiency, appearing completely normal aside from the increased cancer risk in certain tissues. BRCA1 haploinsufficiency is nevertheless reasonably well established with regards to HR, replication fork protection, telomeres and general genomic instability (Konishi et al., 2011; Pathania et al., 2014; Sedic et al., 2015), whereas BRCA2 haploinsufficiency is not and requires more thorough research.

Recent studies on BRCA2 haploinsufficiency indicate that if existent, it is very mild or a case of conditional haploinsufficiency (Tan et al., 2017; Zámborszky et al., 2017). A recent study described aldehyde-induced haploinsufficiency in *BRCA2* heterozygous cells, by stalling and destabilizing replication forks. Other genotoxic agents tested did not show the same effect

nor cause any DNA repair defects in heterozygous cells (Tan et al., 2017). Another study described very little to no increase in the accumulation rate of base substitutions and indels in *BRCA*2 heterozygous cells compared to *BRCA2* null cells, neither spontaneous nor after treatment mimicking endogenous alkylating damage (Zámborszky et al., 2017).

Telomeres might be more sensitive to minor BRCA2 defects in HR repair and replication fork stalling protection than the rest of the genome, due to their repetitive nature which is notorious for replication problems (Badie et al., 2010; Min et al., 2012). Accelerated telomere attrition and accumulation of telomere defects over time would lead to chromosomal instability, increasing cancer risk. Data on possible BRCA2 haploinsufficiency effect on telomeres is scarce. Results from telomere length measurements in normal tissues have been conflicting (Killick et al., 2014; Martinez-Delgado et al., 2011; Pooley et al., 2014) but heterozygous cell lines from *BRCA2* mutation carriers have been shown to have increased chromosome instability and telomere dysfunction (Bodvarsdottir et al., 2012; Rubner Fridriksdottir et al., 2005). Recent studies on BRCA2 haploinsufficiency have not been focused on telomeres.

As previously mentioned, cells completely lacking BRCA2 protein function are HR deficient and rely on alternative DNA repair mechanisms, in some cases upregulating genes with overlapping function, including FANCD2 and POLθ (Ceccaldi et al., 2015; Kais et al., 2016). A recent publication has shown that in heterozygous *BRCA1* cells, a redundant DNA repair pathway mediated by RNF168 compensates for DNA repair haploinsufficiency (Zong et al., 2019). This back-up mechanism masks the haploinsufficiency effect and is necessary for maintaining genomic stability and prevent tumorigenesis in *BRCA1* heterozygous mice. The reliance of *BRCA2* heterozygous cells on alternative repair mechanisms is currently unknown. The identification of other compensatory mechanisms in *BRCA* heterozygous cells may lead to discovery of new drug targets based on the concept of synthetic lethality for *BRCA* deficient tumors (Zong et al., 2019).

1.14 Recent advancements in genome editing

Genome editing methods are essential for studying gene and protein function. The term refers to the practice of permanently editing the DNA sequence of cells via insertions or deletions of genetic material. Through the years, the most common way of studying loss or gain of gene function has been to use RNA interference (RNAi) and transgenesis, respectively. These methods have the limitation of only transiently exerting the intended effect, with RNAi downregulating the gene of interest rather than knock-out and in the case of transgenesis, expressing an exogenous gene either transiently or

randomly integrated into the genome, often with undesired side-effects. Targeted genome editing overcomes this by introducing stable genetic alterations at the endogenous level.

In 1995, Maria Jasin demonstrated that targeted DNA DSBs could be induced in mammalian mitotic cells with the use of the I-SceI meganuclease (Smih et al., 1995). The DSB could then be repaired with HDR from a provided template, enabling precise edits at the target site. With the subsequent emergence of other homing engineered nucleases such as Zink Finger Nucleases (ZFNs) (Urnov et al., 2010) and Transcription Activator-Like Effector Nucleases (TALENs) (Joung & Sander, 2013), a new era began in genome editing. These methods use bioengineered non-specific endonucleases coupled with DNA-binding proteins that are designed to target specific sequences, providing high target specificity. The newest addition to the field is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and associated protein 9 technology (CRISPR/Cas9), first described in 2013 (Cho et al., 2013; Cong et al., 2013; Mali et al., 2013; Martin Jinek et al., 2012). This system uses RNA-DNA interaction instead of protein-DNA interaction to guide the nuclease activity. CRISPR/Cas9 quickly gained popularity for its advantages over pre-existing methods including ease of design, increased accuracy, higher editing efficiency and low cost. It has since dominated the field.

1.14.1 Principles of CRISPR/Cas9 genome editing

The CRISPR/Cas system is a prokaryotic antiviral RNA-mediated adaptive defense system (Wiedenheft et al., 2012) which has been exploited for genome editing in eukaryotes (Cho et al., 2013; Cong et al., 2013; Mali et al., 2013; Martin Jinek et al., 2012). The system consists of a short ~20bp single guided RNA (sgRNA or gRNA) forming a ribonucleoprotein complex with a Cas endonuclease. The gRNA is composed of the crRNA (CRISPR RNA) sequence, designed to recognize a target sequence within the genome, and the tracrRNA (trans-activating crRNA) which guides Cas to the locus (Martin Jinek et al., 2012). The Cas endonuclease recognizes so-called protospacer adjacent motifs or PAM (NGG in the case of spCas9 from *Streptococcus pyogenes*) and cleaves the DNA if the PAM is next to a sgRNA target sequence (Martin Jinek et al., 2012) (**Figure 5**). The Cas-mediated DSB activates the cell's DDR machinery and is most often repaired with the NHEJ pathway, randomly causing small insertions and deletions at the cut site. These small mutations can disrupt gene function if they cause a frame-shift in the reading frame, generating premature stop codons and a non-functional protein product. Alternatively, the NHEJ pathway can be transiently inhibited in order to favor the high-fidelity HR pathway (Maruyama et al., 2015). This

allows for more specific editing by providing a single strand donor DNA oligo with the desired edits for homology (**Figure 5**).

The fast development of genome editing tools in the last decade has raised ethical questions on their use for gene therapy of heritable diseases. That discussion is outside of the scope of this thesis but one major area of concern are the undesired off-target effects of these methods. In the case of CRISPR/Cas9, it has been shown that Watson-Crick base-pairing of the sgRNA and the genomic target sequence can tolerate several mismatches (Fu et al., 2013). This poses a hazard for clinical applications and can confound results in functional studies. Efforts are constantly being made to increase the precision and efficiency of the CRISPR technology and it holds great promise for the future.

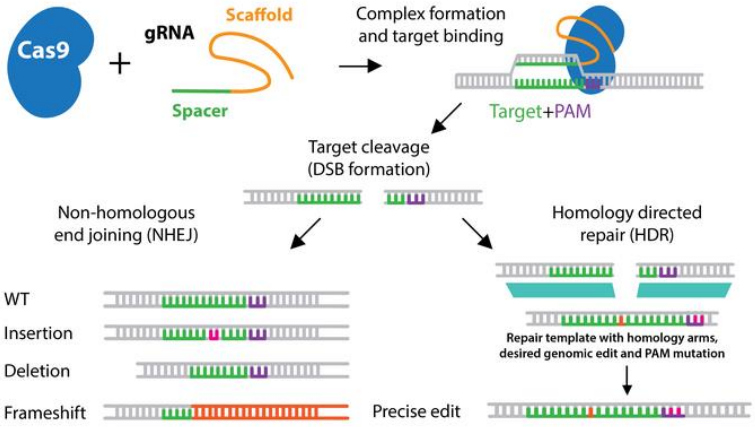

Figure 5. Overview of the CRISPR/Cas9 genome editing process. The system is composed of two components, a single guide RNA (gRNA) and Cas9 endonuclease, forming a ribonucleoprotein complex. The gRNA contains a ~20 nucleotide spacer sequence (crRNA) that recognizes a target sequence in the genome and a scaffold sequence (tracrRNA) that binds Cas9 and directs it to the target sequence. Cas9 recognises a Protospacer Adjacent Motif (PAM) and mediates DNA cleavage if the PAM is located immediately adjacant to the target sequence. The resulting double-strand break is then repaired by either the more error-prone NHEJ or the high-fedility HR repair pathway. Repair via NHEJ frequently causes small indels, often resulting in frameshift events leading to premature stop-codons and a non-functional protein. More precise edits can be made by introducing a repair template with the desired genomic edit, flanked by homology arms, which can be incorporated into the genome during HR repair. To prevent repeated cutting by Cas9 at the target site, silent mutations are typically integrated into the PAM sequence or the 3‘-seed sequence of the template. Adapted from Addgene. CRISPR Guide. https://www.addgene.org/crispr/guide/

2 Aims

BRCA2 is involved in maintaining genomic stability through its roles in DNA double strand break (DSB) repair and protection of stalled replication forks. It has for long been considered a classical tumor suppressor. The fact that a significant proportion of breast tumors from *BRCA2* mutation carriers retain the wild-type allele contradicts this notion and indicates there might be BRCA2 haploinsufficiency at play. Even if mild, slight repair deficiency might result in accumulation of mutations over a lifetime and contribute to cancer formation in these individuals. Tumors and cell lines derived from *BRCA2* heterozygous mutation carriers have frequent telomere abnormalities. Telomeres might be sensitive to BRCA2 deficiencies, more so than the rest of the genome, due to replication problems at highly repetitive telomere sequences. The level of telomere maintenance in *BRCA2* mutation carriers may therefore be reduced. Disruptions in telomere homeostasis can result in excessive telomere shortening and drive chromosome instability, a hallmark of *BRCA2*-related cancers.

A)

The project's original main objective was to study telomere length and telomere dysfunction in *BRCA2*^{999del5} mutation carriers and BRCA2-associated breast cancer in the Icelandic population. The specific aims were:

1) Measure telomere length in blood isolated DNA from *BRCA2* mutation carriers with and without a breast cancer diagnosis, matching sporadic cancer patients and healthy controls to assess the association between blood telomere length, cancer risk and clinical outcomes. (Paper I)

2) Measure telomere length and telomere dysfunction on tissue samples, both tumor and normal tissue, from *BRCA2* mutation carriers diagnosed with breast cancer and matched sporadic breast cancer patients in relation to cancer risk and clinical outcomes.

B)

Previous cell-line studies on *BRCA2* heterozygosity have lacked proper controls, often comparing results from different cell lines which may have very different genetic profiles. With the emergence of the CRISPR/Cas9 genetic editing technology, stable cell lines with a desired mutation can now be generated in a more controlled manner. The specific aims for this part of the project were the following:

3) Establish an isogenic cell line model for the Icelandic *BRCA2*^{999del5} mutation, constituting of *BRCA2*$^{+/+}$, *BRCA2*$^{999del5/+}$ and *BRCA2*$^{999del5/999del5}$ cell lines of the same origin, using homology directed repair variation of CRISPR/Cas9 genetic editing system.

4) Functionally characterize the newly established cell lines with regards to DNA DSB repair efficiency, problems at stalled replication forks and dysfunctional telomere maintenance.

C)

A subset of tumors from *BRCA2* mutation carriers exhibit retention of the wild-type allele, supporting the idea of BRCA2 haploinsufficiency. The aim was to verify and expand on previous results on this topic in Icelandic *BRCA2*^{999del5} mutation carriers, specifically:

5) Assess the frequency of *BRCA2* wild type allele loss in breast (female and male) and ovarian tumors from *BRCA2*^{999del5} mutation carriers using targeted Sanger sequencing.

3 Materials and methods

3.1 Study groups and sample acquisition

The work in this thesis is largely based on several different study groups, described below.

3.1.1 Archived DNA isolated from blood samples, breast tumors and tumor adjacent normal tissue

All blood samples were collected between the years 1988-2006 and the isolated DNA stored at the Cancer Research Laboratory, University of Iceland. This study group is described in further detail in Paper I. The work was carried out based on permits from the Icelandic Data Protection Commission (2006050307) and Bioethics Committee (VSNb2006050001/03-16). Written informed consent was obtained from all participants.

DNA samples from breast tumors (ICD-10 C50.9) and tumor adjacent normal tissue were also available from the laboratories previous studies and had been extracted from either formalin fixed paraffin-embedded (FFPE) or fresh-frozen tissue using standard phenol-chloroform and Proteinase K DNA isolation methods. The samples were provided by the Department of Pathology at Landspitali University Hospital. All samples had previously been analyzed for the *BRCA2*^{999del5} mutation (Stefansson et al., 2009; Tryggvadottir et al., 2006).

Information on all female breast cancer cases (3.1.1 and 3.1.3) was obtained from the Icelandic Cancer Registry or from patient files and included date of birth, primary tumor location, date of diagnosis, age at diagnosis, side (left, right), tumor size (cm), histological grade, clinical staging, surgery (mastectomy, lumpectomy, bilateral mastectomy), chemotherapy regimen, distant metastasis, other cancers (site, date), date of death and cause of death.

3.1.2 Newly acquired blood samples

Newly acquired blood samples were collected from 23 volunteers. Written informed consent was obtained from all participants according to a permit from the Bioethics Committee (VSN-17-117). No personally identifiable data was gathered. Two 5 ml vials of blood were drawn from each volunteer, a whole blood vial and a vial separated into granulocytes and lymphocytes

using Histopaque® 1077 solution (Sigma-Aldrich, 10771) following manufacturer instructions. DNA was isolated from whole blood and separated blood parts using a standard phenol-chloroform plus Proteinase K extraction.

3.1.3 Female breast cancer and normal breast tissue samples

Tissue microarrays (TMAs) from Icelandic FBC patients had been prepared for previous studies (Stefansson et al., 2009, 2011a) and were accessible at the Department of Pathology of Landspitali University Hospital. The arrays contained ~450 tumor samples, each in triplicate, chosen as two sporadic cases matched on age and year of diagnosis with a breast tumor from a *BRCA2* mutation carrier. These TMAs had previously been characterized for commonly studied breast cancer biomarkers, including ER, PR, Ki67, HER-2, CK5/6, CK8 and CK18 (Stefansson et al., 2009).

Tumor adjacent normal breast tissue samples for this project were selected based on availability from the TMA group described above, one *BRCA2* mutation carrier matched with a sporadic control based on age and year of diagnosis, total of 180 samples (90 pairs). Hematoxylin and eosin (H&E) stained slides from each patient were reviewed by a pathologist to select areas of normal breast tissue histology for sectioning. These samples were prepared as 5 µm thick paraffin embedded tissue sections on Superfrost™ Plus microscope slides (ThermoScientific). All tissue samples were prepared at the Department of Pathology of Landspitali University Hospital. The work was carried out according to permits from the Icelandic Data Protection Commission (2006050307) and Bioethics Committee (VSNb2006050001/03-16).

3.1.4 Male breast cancer samples

The study group consisted of all men diagnosed with breast cancer (C50.9) in Iceland since the establishment of the Icelandic Cancer Registry in 1955 and through 2018, 82 cases in total (Bioethics Committee permit: VSNb2018100019/03.01). The tissue samples were obtained from the Department of Pathology of Landspitali University Hospital. Tumor tissue FFPE blocks were available for 76 men and normal tissue for 38 men. Additional archived DNA from blood samples was available at the Cancer Research Laboratory for 26 of the 82 men in the study group. H&E stained tumor slides were microscopically examined by a pathologist to choose the most appropriate area for DNA isolation (ensuring high tumor content, preferably over 70-80%). Respective FFPE tumor blocks were sectioned into 3x10 µm rolls for DNA isolation. DNA was extracted using the Zymo

Research Quick-DNA FFPE miniprep kit (D3067). All DNA samples were quantified with Qubit fluorometric quantification (Qubit 3.0) which accurately estimates low DNA quantities from degraded or low-quality samples, characteristic for DNA extracted from paraffin. DNA in sufficient amount and quality for downstream analyses was obtained from all 76 tumor samples and 30 normal tissue samples.

Information on date of birth, date and age at diagnosis, side (left, right), contralateral breast cancer, other cancers (site, date), date of death and cause of death was obtained from the Icelandic Cancer Registry. Data on tumor pathological characteristics and other clinical parameters described in 3.1.1 is currently being gathered for the MBC group.

3.1.5 Ovarian cancer samples

A pilot study was performed with 130 women diagnosed with ovarian cancer (C56*) between 1955-2018 who had given a biological sample to the UI Cancer Research Laboratory under a broad informed consent for previous studies, thereby within the confines of existing permits from the Icelandic Data Protection Commission (2006050307) and Bioethics Committee (VSNb2006050001/03-16). Tumor samples were provided by the Department of Pathology of Landspitali University Hospital and DNA extracted as previously described for the MBC samples. FFPE tumor blocks were available for 118 women and sufficient-quality DNA obtained for 100 samples.

Expanding on the pilot study, a new group was retrieved from the National Cancer Registry which included all women diagnosed with ovarian (C56*), fallopian tube (C57*) and peritoneal (C48.1, C48.2) cancers in Iceland over the 15-year period 1999-2013 or 361 cases in total (Bioethics Committee permit: VSNb2018060019/03.01). Tumor samples were provided by the Department of Pathology, Landspitali University Hospital and DNA extracted as previously described. Tumor FFPE blocks were available for 288 samples and high-quality DNA in sufficient amount was obtained from all 288 samples with an overlap of 29 samples from the pilot study.

Information on ovarian cancer cases from the pilot study was obtained from the Icelandic Cancer Registry or from patient files and included date of birth, primary tumor location, date of diagnosis, age at diagnosis, tumor size (cm), histological grade, clinical staging, surgery (oophorectomy), chemotherapy (yes/no, regimen), distant metastasis, other cancers (site, date), date of death, cause of death and status at the end of study (alive with/without recurrence or signs of active disease). Clinical data is currently being gathered for the expanded study group.

3.2 MMqPCR telomere length measurements

Multiplex monochrome quantitative PCR (MMqPCR) measurements, based on the method developed by (Cawthon, 2009), were performed with slight modifications as described in Paper I.

3.3 IF and Q-FISH staining of paraffin embedded tissue

3.3.1 Deparaffinization

Tissue slices and TMAs (described in 3.1.3) were deparaffinized in Xylenes (Sigma 534056), twice for 5 minutes, followed by rehydration by ethanol series (100%, 90%, 70%, 50%), 5 minutes each. Slides were then washed briefly in water. Antigen retrieval was performed by immersing slides in Sodium Citrate Buffer (pH6.0) and boiling in microwave for 10-15 minutes. Slides were then washed in PBST (Phosphate Buffer Saline (PBS) w/0.05% Tween-20 (Sigma P1379)) twice for two minutes.

3.3.2 Immunofluorescent staining (IF)

Tissue was permeabilized in PBS containing 0.5% NP-40 (Abcam, ab142227) for 10 minutes followed by a wash in PBS for 5 minutes. Slides were then blocked in PBG (0.2% (w/v) cold water fish gelatin (Sigma G-7765), 0.5% (w/v) BSA (Sigma A-2153) in PBS) for 20 minutes before incubation with primary antibody against 53BP1 (anti 53BP1, Novus Biochemicals NB 100-304, 1:500 dilution in PBG) overnight (O/N) at 4°C. Slides were then washed thrice in PBG before incubation with secondary antibody (AlexaFluor 488, Molecular Probes, 1:750 dilution in PBG) for one hour at room temperature (RT) and washed again thrice in PBG. Slides were then fixed with 2% paraformaldehyde (16% PFA, EMS cat no. 15710) in PBS for 10 minutes followed by two washes in PBS, 5 minutes each. Slides were treated with 0.1mg/ml RNase A (Qiagen cat. no. 158922) in PBS for 10 minutes at 37°C, washed twice with PBS for 5 minutes each and finally dehydrated through ethanol series (70%, 95%, 100%) for five minutes each before being air dried.

3.3.3 Q-FISH

Telomere and centromere Fluorescent in situ Hybridization (FISH) staining was performed after IF as described (Diolaiti et al., 2013), without pepsin treatment. The peptide nucleic acid (PNA) probes (Panagene) used were:

FAM-OO-ccctaaccctaaccctaa (0.5µg/ml) for telomeres and
Cy5-OO-aatcaacccgagtgcaat (0.5µg/ml) for centromere 9.

3.3.4 Microscopy and image analysis

Slides were imaged using a DeltaVision RT deconvolution microscope (Applied Precision) with the 100x/1.4N PlanApo objective (Olympus). Images were acquired in 0.5 µM increments, deconvoluted and Z-projected in Softworx (Applied Precision). Telomeric, centromeric and 53BP1 foci were identified and counted and their integrated intensity measured with CellProfiler 3.0 image analysis software (Broad Institute, www.cellprofiler.org). Relative telomere length was calculated by averaging integrated intensities of individual telomere foci per nucleus corrected for the mean centromere 9 foci integrated intensity value for that nucleus. Telomere dysfunction induced foci (TIFs) were detected where co-localization of 53BP1 foci and telomere foci occurred.

3.4 Tissue culture

Two commercially available cell lines were used in this study, both obtained from the American Type Culture Collection (ATCC).

MCF10A (ATCC® CRL-10317) is a mammary epithelial breast cell line derived from fibrocystic disease. MCF10A cells were cultured in Dulbecco's Modified Eagle's Medium, DMEM/F-12 (Gibco 3133038), with 1.5 µg/mL Penicillin/Streptomycin (Gibco 15070063), 5% horse serum (Gibco 16050130) and supplemented with growth factors listed in **Table 1**.

Table 1. Growth factors for MCF10A growth media

Growth factor	Final concentration	Manufacturer	Catalog no.
Insulin	10 µg/ml	SigmaAldrich	I-1882
Epidermal Growth Factor (EGF)	20 ng/ml	PeproTech	AF-100-15
Hydrocortisone	0,5 µg/ml	SigmaAldrich	H-0888

HeLa (Kyoto) cells (ATCC®CCL-2™), derived from cervical adenocarcinoma, were cultured in DMEM GlutaMax™ (Gibco 31966047) with 10% Fetal Bovine Serum (FBS, Gibco 10270-106) and 1.5 µg/mL Penicillin/Streptomycin.

Additionally, primary normal epithelial breast cell lines derived from a *BRCA2*^{999del5} mutation carriers, A176 (*BRCA2-999del5-1N*) and A240 (*BRCA2-999del5-1N*), were used. These cell lines were established from tumor adjacent normal breast epithelium using HPV-16 E6/E7 transformation

(Rubner Fridriksdottir et al., 2005). A176 and A240 were cultured in H14 media that is composed of DMEM/F-12 with 1.5 µg/mL Penicillin/Streptomycin and additional growth factors listed in **Table 2**.

All cell lines were cultured at 37°C with 5% CO_2 and routinely checked for mycoplasma infections.

Table 2. H14 growth media formulation

Growth factor	Final concentration	Manufacturer	Catalog no.
Insulin	250 ng/ml	SigmaAldrich	I-1882
Transferrin	10 µg/ml	SigmaAldrich	T-1147
Epidermal Growth Factor (EGF)	10 ng/ml	PeproTech	AF-100-15
Na-Selenite	2,6 ng/ml	SigmaAldrich	S-5261
Estradiol	10^{-10} M	SigmaAldrich	E-2758
Hydrocortisone	0,5 µg/ml	SigmaAldrich	H-0888
Prolactin	5 µg/ml	SigmaAldrich	L-6520

3.5 CRISPR genome editing

CRISPR/Cas9 genome editing technology was used to generate *BRCA2* heterozygous and *BRCA2* knock-out cell lines.

3.5.1 gRNA and plasmid constructs

For expressing Cas9 the pST1374-NLS-flag-linker-Cas9 plasmid (Addgene plasmid #44758, gift from Xingxu Huang) was used, containing a Blasticidin S. selection cassette.

A guide RNA oligo (gRNA) was designed for targeting exon 9 of the *BRCA2* gene, with an expected cutting site ~20 bases downstream of the *BRCA2 999del5* mutation locus:

BRCA2 4 forward: ACACCG CAAAGAGAAGCTGCAAGTCA G

BRCA2 4 reverse: AAAAC TGACTTGCAGCTTCTCTTTG CG

The gRNA was cloned into the MLM3636 gRNA expression vector (Addgene plasmid #43860, gift from Keith Young) using BsmBI restriction digestion (NEB, R0580S). Annealing was performed by heating the annealing reaction mixture (1ul of forward and reverse gRNA oligos (diluted to 100uM), 5ul

NEBuffer2 (NEB, B7002S) and 47ul H2O) to 95°C and cooling slowly to 4°C. For ligation, 1ul of BsmBI cut MLM3636 plasmid was mixed with 3ul of annealing mix, 1ul of water and 5ul of Instant Sticky end ligase Master mix (NEB, M0370S). Transfection was done by adding 2uL of ligation mixture to 50uL of competent E. coli DH5α cells which were then plated onto LB-Amp plates and incubated at 37°C O/N. The plasmid sequence was validated with sanger sequencing at Genewiz® with the OS280 primer: CAGGGTTATTGTCTCATGAGCGG

3.5.2 ssDNA oligo design

A single strand DNA oligo (ssDNA oligo) for introduction of the *BRCA2 999del5* mutation into the genome was designed with 30bp homology arms around the inserted mutation and two phosphorothioate bonds on each end of the oligo to inhibit exonuclease degradation. To prevent further cutting of Cas9 after the incorporation of the oligo, three silent mutations (based on the resulting mutated sequence) were introduced in the gRNA seed sequence (the 12nt closest to the PAM motif) to mask the gRNA recognition site (**Figure 6**). These silent mutations were selected to form a restriction site for BstNI to facilitate pre-screening of clones with enzymatic digestion before Sanger sequencing (see.3.5.5).

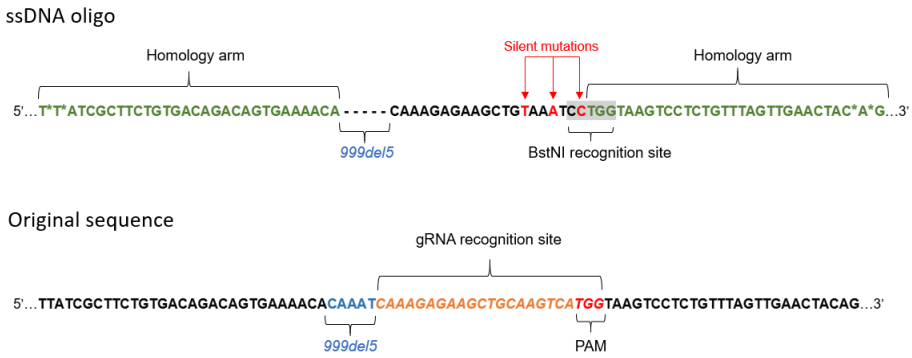

Figure 6. ssDNA oligo design for introducing the *BRCA2 999del5* mutation into the genome via HDR mediated CRISPR genome editing.

3.5.3 Transfection and selection

Cells were seeded into a 24 well plate 24 hours prior to transfection to be at ~60-70% confluency at the time of transfection. For co-delivering of Cas9 plasmid and gRNA plasmid along with the ssDNA oligo, cells were transfected using Lipofectamine™ LTX Reagent with PLUS™ Reagent

(Thermo Scientific 15338100). 500ng of both the Cas9 plasmid and the gRNA plasmid were added to 50µl of Opti-MEM (Thermo Scientific 31985062) along with the ssDNA oligo (for a final concentration of 5nM in 500 µl). To an equal volume of Opti-MEM were added 2µl LTX and 1µl of PLUS Reagent. These two solutions were then mixed thoroughly together and incubated at RT for 15 min. Then the L755507 NHEJ inhibitor was added (for a final concentration of 5 µM in 500µl) when the mixture was added to the wells with 400µl fresh media. After 24h cells were trypsinized, spun down and seeded sparsely onto 10cm dishes (1-5% of total cells) for drug selection. Cells were then treated with Blasticidin S. (Thermo Scientific A1113903) at 5µg/ml for 72h, media changed once during that time. After 72h of selection, plates were rinsed thoroughly with PBS and normal media added. Media was changed every 2-3 days until single cell clone colonies had grown sufficiently to be transferred to a 24well plate using 1/10 diluted Trypsin/EDTA and a sterile filter tip.

3.5.4 DNA extraction

Cells were harvested when they reached confluency in a 24 well-plate, genomic DNA was obtained from half of the cells (~$1{,}2 \times 10^5$ cells) while the other half was kept in culture for further expansion. For DNA isolation the cells were trypsinized and spun down. The pellet was then resuspended in 25µl Igepal buffer (50 mM Tris-HCl, 50 mM KCl, 3.15 mM MgCl2, 0.25% (v/v) Igepal (I8896-IGEPAL® CA-630, Sigma-Aldrich), 0.5% (v/v) Tween 20, pH 8.0) containing 1 mg/ml Proteinase K (NEB #P8107S) and incubated at 60°C for 90 min followed by an incubation at 95°C for 15 min. Then, the mixture was centrifuged at 16000 x g for 15 min. Supernatant was used for downstream analysis.

3.5.5 Pre-analysis of clones

Specific DNA regions were amplified using Polymerase Chain Reaction (PCR) in total volume of 12 µl containing 1X final concentration of 10X*Taq* standard buffer (NEB M0273L), 200 µM dNTPs (NEB, N0447S), 0.2 µM of each primer (Sigma, **Table 3**), 1.25 U of *Taq* polymerase (NEB M0273L) and 2 µl of supernatant from Igepal DNA isolation, with nuclease free H_2O to final volume. The thermal cycling profile was as follows; incubation at 95°C for 10 minutes, then 40 cycles of 95°C for 30 seconds, variable°C for 30 seconds and 72°C for 30 seconds, and finally 72°C for 10 minutes.

Table 3. PCR primers for analysis of CRISPR clones

Target	Primer name	Sequence (5'-3')	Annea-ling T (°C)	Amplicon size (bp)
BRCA2	BRCA2_Anlz_Fwd	CAAGTGGGATGGAGCAAGAT	67	815
	BRCA2_Anlz_Rev	CTAACTTGGTGTGCCTGTGA		
RIC8A	Off_target_1_Fwd	GTGAAGCTCACAGAGCGTGT	60	235
	Off_target_1._Rev	GCTCAGTCTCTTGGGAAGGA		
TNFRSF21	Off_target_2_Fwd	TGCTTCCCTCTCTTCTCTGC	60	197
	Off_target_2_Rev	TCCTGGCTCTTGACTCCAAT		
NLRP5	Off_target_3_Fwd	GTCTGCTGAGGAAGGTCCTG	60	191
	Off_target_3_Rev	ACGCAACCCTTGTGTCTTCT		
TXK	Off_target_4_Fwd	GGTTTTCACAGTCCAACACG	60	221
	Off_target_4_Rev	TTACCCCAAACGGTCTCTTG		

For identification of incorporation of the ssDNA oligo, the BRCA2 PCR product was digested with BstNI (**Figure 7**) following manufacturer's instructions (NEB, cat. R0168S) and run on 1.2% agarose gel at 90V for 25 minutes for analysis. The expected product sizes are shown in **Table 4**.

Figure 7. BstNI restriction sites in *BRCA2* PCR amplicon. A) BstNI recognition sequence (W is either A or T). B) Sequence of the *BRCA2* PCR amplicon, showing the ssDNA oligo in green and BstNI restriction sites in gray.

Table 4. Expected product sizes after BstNI enzymatic digestion

Incorporation of ssDNA oligo	Total amplicon length (bp)	No. of BstNI restriction sites	Product sizes (bp)
None (WT)	815	1	632 – 183
Heterozygous	815 / 810	1 / 2	632 – 395 – 232 - 183
Homozygous	810	2	395 – 232 - 183

3.5.6 Sanger sequencing and analysis

Selected clones were sent to sequencing to Genewiz ® after PCR clean-up with Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up Kit (Macherey-Nagel 740609.50) according to manufacturer's instructions. Results on FASTA and .ab1 file formats were analyzed in Benchling (https://benchling.com) and ICE (https://ice.synthego.com), an online CRISPR editing analysis tool provided by SYNTHEGO.

3.6 Western blot

For detection of the BRCA2 protein with western blot, cells were grown to confluency in 60mm culture dishes. Cells were washed once with cold PBS, dishes put on ice and all liquid removed. Samples were then extracted by scraping with 200 µL of loading buffer containing NuPAGE™ LDS Sample Buffer (4X) (Thermo Scientific NP0007) and 10X dithiothreitol (DTT) reducing agent, both diluted to 1X in ddH2O. 0.2µL of Benzonase Nuclease (Santa Cruz, sc-202391) was then added to each sample to remove all nucleic acids and incubated at RT for 3 minutes. Samples were then placed in a heat block at 95°C for 10 minutes and spun down at highest speed for 15 minutes. The supernatant was stored at -20°C and used for downstream steps.

40-50 µL of each sample and 5 µL of Color Prestained Protein Standard (NEB, P7712S) were loaded onto a 1.5mm thick 6% SDS-polyacrylamide gel with a stacking gel (**Table 5**). The gel was run in 1xRunning Buffer (10x: 25mM Tris base, 190mM glycine and 0.1% SDS diluted in H_2O, pH 8.3) for 3-4 hours at 110V.

Table 5. Recipes for western blot gels

Component	Polyacrylamide gel/separating solution (6%, 10ml)	Stacking gel (5ml)
ddH2O	5.3 ml	3.4 ml
30% Acrylamide mix	2.0 ml	830 ul
1.5M Tris (pH 8.8)	2.5 ml	-
1.0M Tris (pH 6.8)	-	630 ul
10% SDS	100 ul	50 ul
10% APS	100 ul	50 ul
TEMED	8 ul	5 ul

A buffer for transfer was prepared from 10X Transfer buffer (25mM Tris, 190 mM glycine) diluted to 1X in H_2O with the addition of 20% methanol and 0.5% SDS. Whatmann filter paper and sponges were wet in the buffer prior to the transfer onto a Nitrocellulose Pure Transfer membrane (Santa Cruz, sc-3724). Transfers were run at 200 mA (per membrane) for 180 minutes in chilled transfer chamber.

The membrane was blocked for one hour at RT in blocking buffer composed of 5% skimmed milk powder dissolved in PBS and then rinsed three times with PBST, 5 minutes each. Primary antibodies were diluted in blocking buffer (see **Table 6),** added to membrane and left at 4°C O/N. The membrane was then washed 3 times for 5 minutes in PBST on shaker before secondary antibodies were added (diluted in blocking buffer, **Table 6**) for one hour on shaker. This was followed by 3 washes for 5 minutes in PBST and finally with ddH_2O. The membrane was drained of excess liquid before adding Western Blotting Luminol Reagent (Santa Cruz, sc-2048) for 1 minute, the membrane was kept in the dark during this time. The membrane was imaged using the Biorad Universal Hood II Gel Doc System and Image Lab Software, taking an image every 30 seconds for 15 minutes.

Table 6. Antibodies used for western blot

Antibody	Manufacturer	Catalog no.	Source	Dilution
BRCA2	Calbiochem/Merck	OP95	Mouse	1:250
SMC1	Abcam	Ab9262	Rabbit	1:5000
°2	SantaCruzBiotechnology	sc-2096	Mouse	1:10000
°2	SantaCruzBiotechnology	sc-2313	Rabbit	1:10000

3.7 Mutational analysis with targeted Sanger sequencing

Tumor samples were screened for germline mutations known to be prevalent in the Icelandic population and linked to breast and ovarian cancers; *BRCA1*^{G5193A}, *BRCA2*^{999del5} and *BRIP1*$^{2040\_2041insTT}$, and to detect wild type allele loss at these loci. PCR primers were designed with the Primer 3 Plus software (Untergasser et al., 2007) to amplify short stretches, PCR products <200 bp, suitable for DNA isolated from paraffin (**Table 7**). The PCR reaction was as described in chapter 3.5.5 with the adjustments of higher concentration of dNTPs and longer annealing time; 1X final concentration of 10X*Taq* standard buffer (NEB M0273L), 400 µM dNTP (NEB, N0447S), 0.2 µM of each primer, 1.25 U of *Taq* polymerase (NEB M0273L) and 1 µl of sample, with nuclease free H_2O to a final volume of 12 µl. Cycling conditions were as follows; incubation at 95°C for 10 minutes, then 40 cycles of 95°C for 30 seconds, variable°C for 1 minute and 72°C for 30 seconds, and finally 72°C for 10 minutes.

Table 7. PCR primers for amplification and Sanger sequencing

Target gene	Germline mutation	Sequence (5'-3')	Annea-ling T (°C)	Ampli-con size (bp)
BRCA2	*g.999del5*	*F: AATTTTTGCAGAATGTGAAAAGC	58	156
	c.767_771delCAAAT	R: AAAACCTGTAGTTCAACTAAACAG		
BRCA1	*c.5193G>A*	*F: CAAAGTGCTGCGATTACAGG	62	169
		R: GTTTGCCAGAAAACACCACA		
BRIP1	*c.2040_2041insTT*	F: TGGCAAGAAACACAAAATTCC	62	154
		*R: GGTTTGGGTTGGTACCATTG		

*Sequencing primers labelled with an asterisk

Clean-up of PCR products before Sanger sequencing was performed by adding 1 µl USB® Shrimp Alkaline Phosphatase (Affymetrix, 70092X) and 0.1µl Exonuclease I (NEB, M0293S) per 10 µl of PCR product and incubate at 37°C for 30 minutes followed by heat inactivation at 75°C for 15 minutes.

Cycle sequencing reactions were performed on MJ Research PTC-225 thermal cyclers, using the Big Dye Terminator Cycle Sequencing Kit v3.1 (Life Technologies). PCR primers were also used as sequencing primers (labelled with asterisk in **Table 7**). Sequencing products were loaded onto the 3730 XL Genetic Analyser (Applied Biosystems) and results on ab1. format analyzed in Benchling, an online sequencing analysis software (https://benchling.com).

Loss of heterozygosity (LOH) in tumors was estimated based on peak height of wild type and mutated alleles, measured in Chromas (free program supplied by Technelysium Pty Ltd). Tumors were classified as having LOH if the wild type allele proportion was less than 40% of the total combined peak heights for wild type and mutant alleles. Previous *BRCA2* LOH measurements on FBC samples were performed by TaqMan allele-specific qPCR as described in (Aradottir et al., 2015; Skoulidis et al., 2010; Stefansson et al., 2011a).

3.8 Statistical analysis

All statistical analysis was carried out using R (CRAN). Statistical analysis for TL measurements in blood is described in paper I. Statistical methods for other experiments are outlined in respective chapters. Generally, differences between two categorical variables were examined the Chi-squared test or the Fisher's exact test. Independent groups of numerical variables were compared with the student's t-test or Mann-Whitney test. Comparisons of more than two groups were carried out using one-way Analysis of Variance (ANOVA) or a Kruskal-Wallis test, followed by appropriate post-hoc tests. Correlations between two numerical variables were studied using Spearman's correlation and linear regression analysis. Survival analyses were performed with Kaplan-Meier with log-rank hypothesis testing and multivariate Cox proportional hazards models.

4 Results

4.1 Telomere length measurements in blood

In the first part of the project, telomere length (TL) was measured in DNA isolated from whole blood from *BRCA2*^{999del5} mutation carriers (n = 169), sporadic breast cancer patients (n = 561) and healthy controls (n = 537). The association between TL and *BRCA2* mutation status, breast cancer risk, onset of breast cancer (age at diagnosis) and breast cancer specific survival was assessed. Here, I will outline the main results from Paper I, entitled "Telomere length is predictive of breast cancer risk in *BRCA2* mutation carriers", and present additional unpublished data.

4.1.1 Characteristics of the study group (Paper I)

Summary statistics for the study group are described in **Table 8**. Some participants had been diagnosed with breast cancer before blood sampling, while others were diagnosed during follow-up.

Table 8. Summary statistics for the study group for blood TL measurements

	Noncarriers		*BRCA2* mutation carriers	
	Unaffected	Affected	Unaffected	Affected
N	537	561	68	101
Age at blood draw (mean, SE)	49.9 (0.9)	59.8 (0.6)	42.4 (1.5)	53.8 (1.3)
Sample acquisition time (n)				
Before breast cancer diagnosis	—	76	—	18
After breast cancer diagnosis	—	485	—	83
Age at breast cancer diagnosis (mean, SE)	—	52.7 (0.5)	—	49.7 (1.1)
Follow-up time to breast cancer diagnosis in years (mean, SE)	13.3 (0.1)	7.5 (0.4)	13.7 (0.8)	7.7 (0.9)
Survival follow-up time in years (mean, SE)	—	19.2 (0.5)	—	15.5 (0.9)

TL is known to shorten with increased age. The association of TL with age at blood draw was evaluated in an evenly age-distributed control group (n = 537). The control group was defined as women not carrying a *BRCA* mutation who were cancer-free at the time of study. The expected negative association between age and TL was observed ($p = 1.27x10^{-8}$, **Figure 8**). All TL measurements were subsequently adjusted for age at blood sampling using the line of best fit for controls.

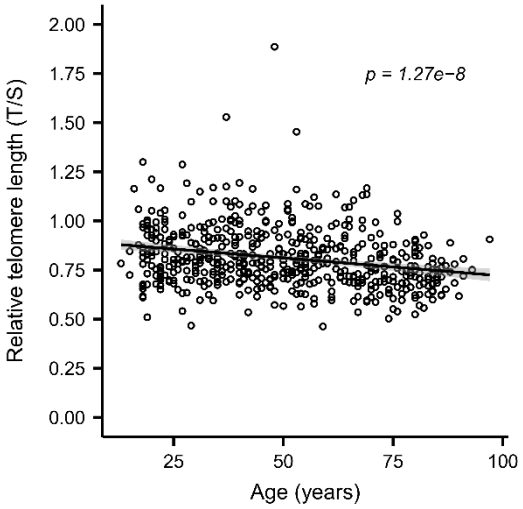

Figure 8. Age-related telomere shortening. Association between relative TL of the control group (n=537) and age age at blood draw.

4.1.2 *BRCA2* mutation status in association with blood telomere length (Paper I)

Breast cancer affected women had significantly shorter TL than unaffected women (student's t-test, $p < 0.0001$), both in *BRCA2* mutation carriers ($p = 0.0097$, **Figure 9A**) and non-carriers ($p = 0.00006$, **Figure 9B**).

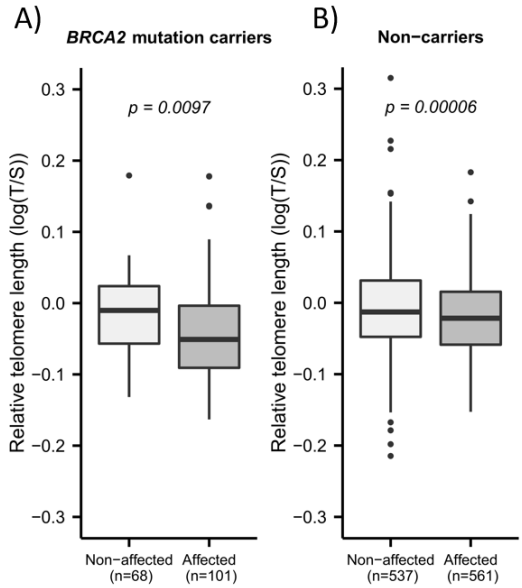

Figure 9. Comparison of blood TL between breast cancer affected and nonaffected women in A) *BRCA2* mutation carriers B) and non-carriers. Data represented through boxplots showing the median and interquartile distance for each group. All TL values have been age-adjusted. The p-values shown on the figure were derived from the Student t test.

No significant differences were found in TL between *BRCA2* mutation carriers and non-carriers who were unaffected by breast cancer (student's t-test, p = 0.22 **Figure 10A**), Stratifying the study group based on sample acquisition time relative to breast cancer diagnosis and exclusively focusing on women who were cancer free at the time of blood extraction but were later affected by breast cancer, no statistically significant difference in TL was found between *BRCA2* mutation carriers and non-carriers (p = 0.31, **Figure 10B**). However, *BRCA2* mutation carriers were shown to have significantly shorter TL than non-carriers (p = 0.01, **Figure 10C**) when comparing women who had already been diagnosed with breast cancer at the time of blood sampling.

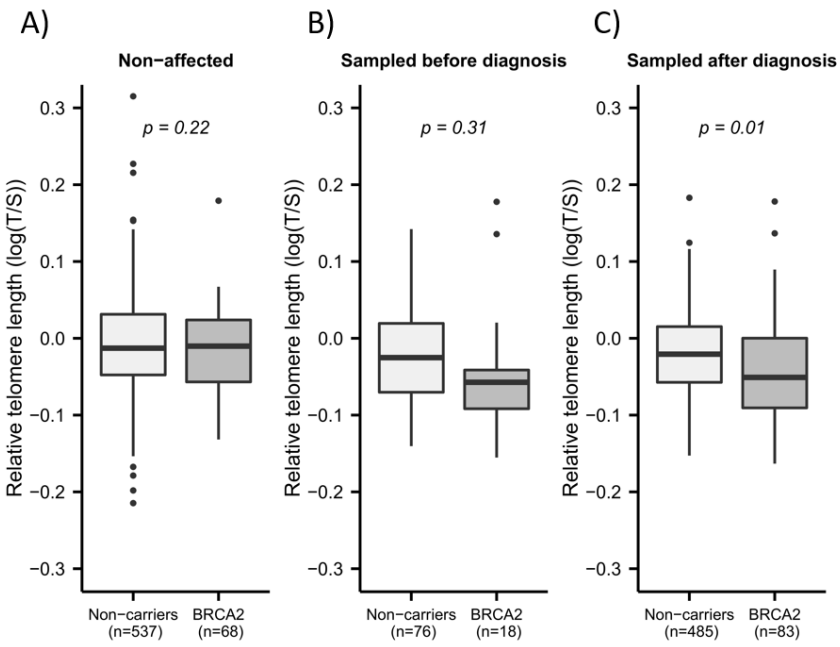

Figure 10. Comparison of TL between *BRCA2* mutation carriers and non-carriers, stratified by time of blood sampling with regards to breast cancer diagnosis. A) Non-affected women B) women sampled before breast cancer diagnosis and C) women sampled after breast cancer diagnosis. Data represented through box-plots showing the median and interquartile distance for each group. All TL values have been age-adjusted. The p-values shown on the figure were derived from the Student t-test.

4.1.3 Telomere length in blood is a modifier of breast cancer risk in *BRCA2* mutation carriers (Paper I)

In a multivariate Cox proportional hazards regression model for time to breast cancer diagnosis analyzed with respect to TL, we found shorter TL to be significantly associated with increased risk for developing breast cancer in the *BRCA2* mutation carrier group (HR, 3.60; 95% CI, 1.17–11.28; p =0.025, **Figure 11A**) but not among non-carriers (HR, 1.40; 95% CI, 0.89–2.22, p = 0.15; **Figure 11B**).

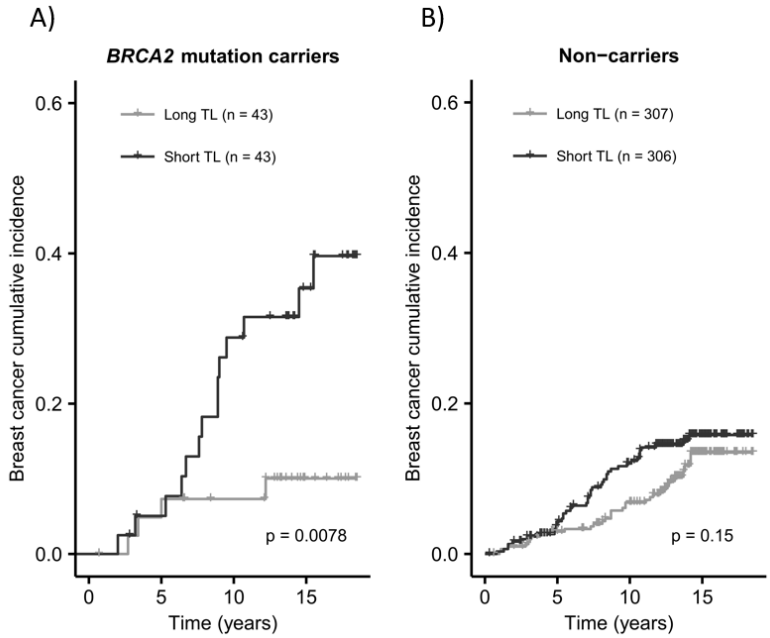

Figure 11. Breast cancer-specific cumulative incidence according to analysis of TL. A) BRCA2 mutation carriers (n = 86) and B) noncarriers (n = 613). The p-values shown on the figure were derived from log-rank hypothesis testing for differences in times to breast cancer diagnosis between groups; that is, long and short TL (divided around the median). Cox proportional hazards model corrected for the year and age at blood sampling; BRCA2 mutation carriers (HR, 3.60; 95% CI, 1.17–11.28; P= 0.025) and noncarriers (HR, 1.40; 95% CI, 0.89–2.22; P= 0.15)

4.1.4 Blood telomere length shows no association with breast cancer prognosis (Paper I)

No significant associations were found between TL and breast cancer–specific survival in this study group, independent of *BRCA2* mutation status and time of blood sampling with regards to breast cancer diagnosis (**Table 9**).

Table 9. Breast cancer-specific survival analysis according to blood TL in *BRCA2* mutatin carriers and non-carriers

	Univariate			Multivariate[a]		
	HR (95% CI)	*P*	*n*	HR (95% CI)	*P*	*n*
BRCA2	1.02 (0.44–2.36)	0.96	101	1.03 (0.38–2.81)	0.95	83
Sporadic	0.92 (0.60–1.43)	0.72	561	0.96 (0.57–1.62)	0.88	400

NOTE: Breast cancer–specific survival analyses using Cox proportional hazard models according to blood TL divided into groups of shorter and longer TL around the median. Associations are presented as HR for short TL with 95% CI.

[a]Analyses adjusted for age and year at breast cancer diagnosis, sample acquisition timing, ER and Ki67 receptor status.

4.1.5 Discrepancies in blood telomere length over time periods

In the original study group for this part of the project were all available archived DNA samples from blood collected over a long time period at the Cancer Research Laboratory in collaboration with the Icelandic Cancer Society and Landspitali University Hospital. Most of the samples were acquired as a part of a large-scale sample acquisition starting in 1998 and all of those samples were treated in the exact same manner from blood draw to storage and DNA extraction (using phenol-chloroform/proteinase K DNA isolation method on whole blood). Before that time, some DNA was isolated from whole blood while the rest was isolated from granulocytes (after removal of lymphocytes for storage). During data analysis, an observation was made that there was a difference in average telomere length between samples acquired before (longer TL) and after (shorter TL) the year of 1998 (**Figure 12**).

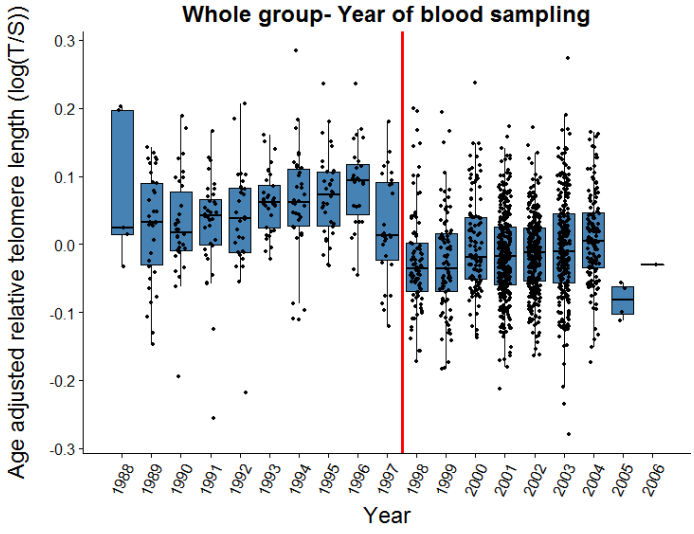

Figure 12. Telomere length in blood samples by year of sample acquisition.

These observations are in concordance with previously published results on telomere length in different cell types in blood, i.e. lymphocytes having shorter telomeres than other blood cell types of the myeloid lineage (Weng, 2001). It would therefore be expected that DNA isolated from whole blood (including lymphocytes) would show shorter TL than DNA isolated from granulocytes only.

As a proof of principle, we validated this finding by drawing new blood samples from 23 individuals and performed paired TL analyses on DNA isolated from both whole blood, granulocytes and lymphocytes from the same individual. The results indeed showed that lymphocytes had the shortest telomere length, followed by whole blood and granulocytes having the longest telomeres (**Figure 13**).

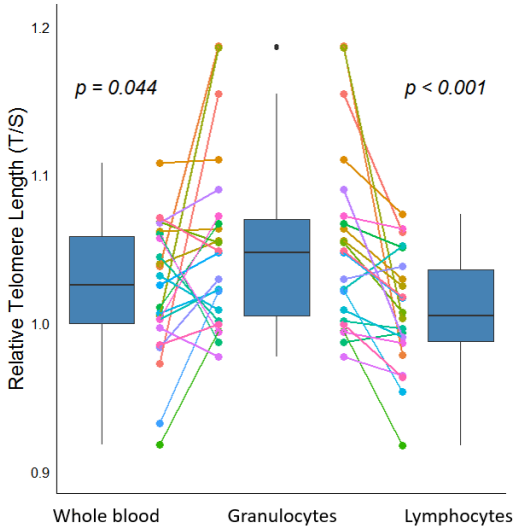

Figure 13. Telomere length in different cell types in blood. Paired TL analyses on DNA isolated from whole blood, granulocytes and lymphocytes from the same individual (n=23), p-values derived from paired Student's t-test. Comparison between whole blood and lymhocytes = ns.

As reliable information on the cell population of origin for DNA extraction was not available for all samples, the study group was consequently censored to only include samples acquired after 1998 in the final analysis, thereby guaranteeing identical sample preparation (Paper I).

4.2 Studying telomere length and dysfunction in normal breast tissue

The aim for the next part of the project was to measure TL and assess telomere dysfunction on paraffin embedded tissue samples from breast tumors (n = 450) and adjacent normal tissue (n = 180) in relation to *BRCA2* mutation status and cancer clinical outcomes. The study group consisted of breast cancer patients carrying the *BRCA2*^{999del5} Icelandic founder mutation and sporadic cases, matched on age and year of diagnosis.

Telomere length was assessed with Q-FISH and IF staining for 53BP1 used as a marker for DNA DSBs. TIFs were identified where co-localization of telomere and 53BP1 signals occurred. Fluorescent staining of paraffin embedded tissue can be problematic as a result of its innate autofluorescence masking the staining signal. That was the case with the TMAs stained in this study which were extremely autofluorescent, making identification and quantification of TL staining signals impossible. These images were subsequently not analysed. We continued with the normal tissue which did not show autofluorescence to the same extent and managed to get reliable TL measurements from 78% of the samples (n = 141/180).

4.2.1 Telomere length and levels of DNA damage differ between cell types in normal breast tissue

Normal breast tissue is composed of a branching ducto-lobular system of two epithelial cell layers, an inner layer of luminal epithelial cells (LECs) and outer layer of myoepithelial cells (MECs). Many cell types are then found in the surrounding stroma, including fibroblasts (FBs). We observed visible differences with regards to telomere length and DNA damage between cell types in normal breast tissue, with LECs having the shortest TL and highest amount of DSBs. To confirm this, 20 samples (10 from *BRCA2* mutation carriers and 10 from non-carriers) were selected and quantitatively analyzed in CellProfiler (**Figure 14**). LECs had the shortest average TL, followed by MEC and FBs had the longest telomeres ($p < 0.0001$, Kruskal-Wallis followed by Dunn's test for multiple comparisons, **Figure 14B**). TL correlated between cell-types within individuals (**Figure 14C**). LECs had higher levels of DNA damage than both MECs and FBs, measured by the number of 53BP1 foci ($p < 0.001$, one-way ANOVA, **Figure 15**). No difference was detected in the number of TIFs between cell types in normal breast tissue.

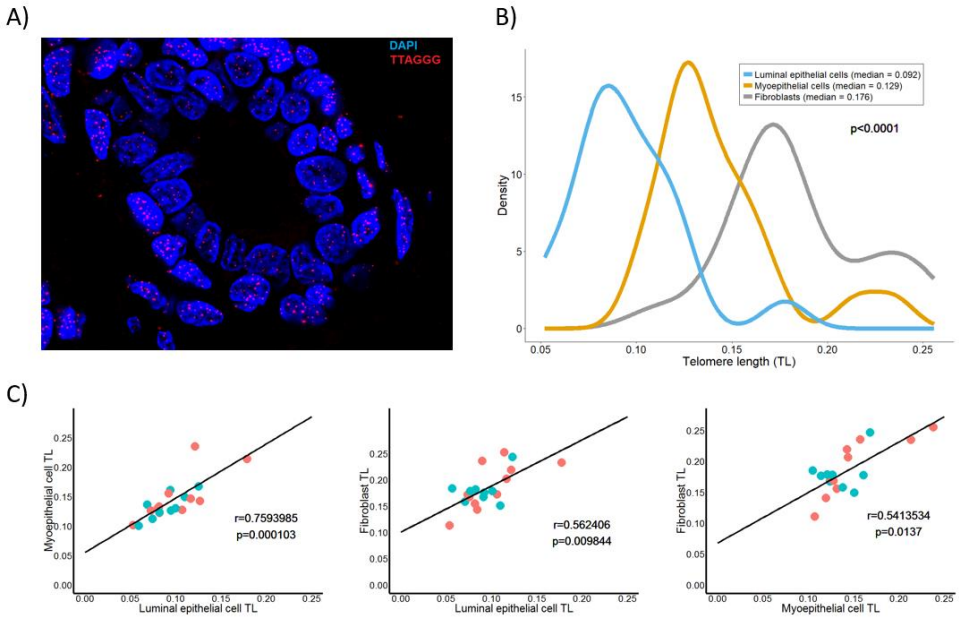

Figure 14. Q-FISH TL measurements of different cell types in normal breast tissue. A) Representative image of Q-FISH staining of normal breast tissue: luminal cells, myoepithelial cells and surrounding fibroblasts. Telomeres stained with a Cy3-labeled telomere-specific PNA probe (red), centromeres stained with a Cy5 PNA probe (omitted for clarity) and DNA stained with DAPI (blue). B) TL measurements in different cell types of the normal breast derived from Q-FISH staining. Telomere and centromere foci were counted and quantified using CellProfiler; integrated intensities of individual telomere foci per nucleus were corrected for the mean centromere foci integrated intensity value for that nucleus. Analysis of 20 cases examined is shown, more than 100 nuclei of each cell type were analyzed per case. Significant differences were seen in TL between cell types ($p<0.0001$, derived from Kruskal-Wallis rank sum test) with TL decreasing in the order fibroblasts > myoepithelial cells > luminal epithelial cells. C) High correlation of TL was found among the 3 cell types within each individual (*BRCA2* mutation carriers presented in pink and non-carriers in blue), p-values derived from Spearman's correlation test.

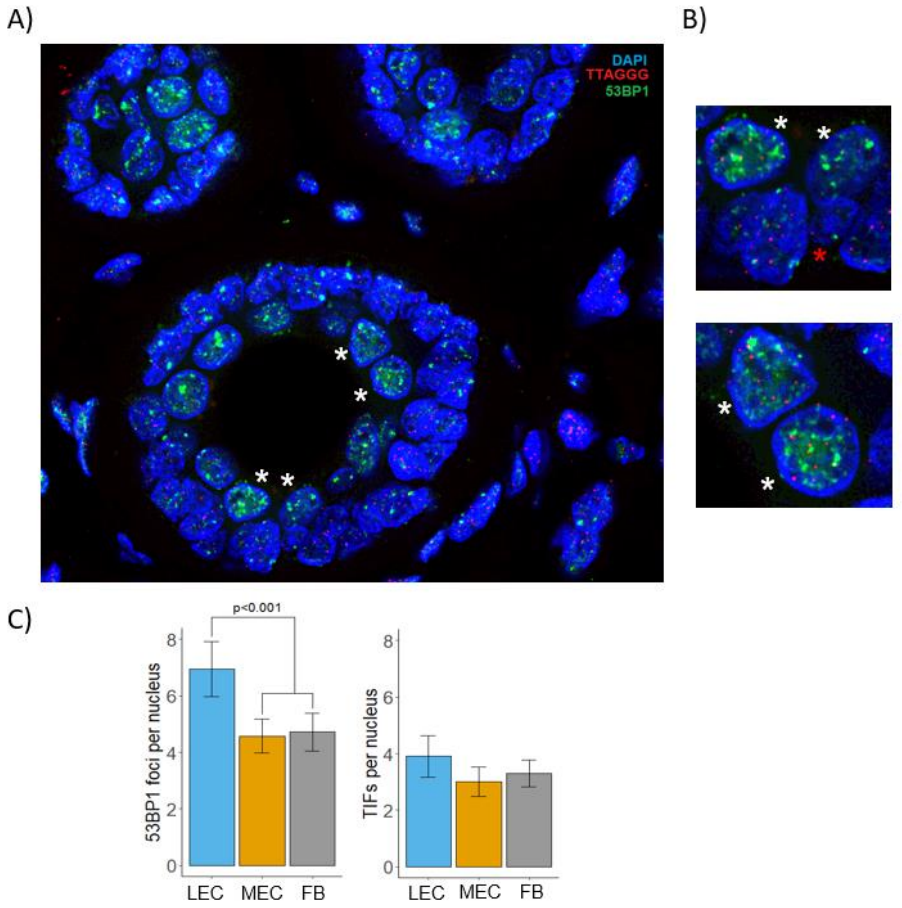

Figure 15. 53BP1 foci and TIFs in different cell types in normal breast tissue. A) Normal breast tissue stained for the double strand break marker 53BP1 (green), cy3 telomere-specific PNA probe (red) and DNA stained with DAPI (blue). B) Enlarged sections of the images from A), luminal cells (white asterisk) and myoepithelial cells (red asterisk). C) Number of DNA DSB foci (53BP1) and TIFs in luminal epithelial cells (LEC), myoepithelial cells (MEC) and fibroblasts (FB) in normal breast tissue. Analysis of 20 cases examined is shown, more than 100 nuclei of each cell type were analyzed per sample. TIFs identified where co-localization of telomere and 53BP1 signals occurs, represented as the percentage of TIFs per telomere per nucleus to correct for variable number of telomere foci between nuclei. p-values derived from one-way ANOVA.

4.2.2 Shorter TL in normal breast tissue is correlated with younger age at breast cancer diagnosis in *BRCA2* mutation carriers

Based on the differences observed in TL and DNA damage between cell layers it is necessary to separate them for following analysis. It was therefore decided to focus on the LECs based on the fact that most breast tumors originate from this cell layer. No significant differences in TL were seen between the *BRCA2* mutation carrier group and non-carriers, neither in median TL (p = 0.81, Mann-Whitney-Wilcoxon test) nor TL variability (p = 0.48, Mann-Whitney-Wilcoxon test) (**Figure 16**). However, a significant correlation between shorter TL and earlier age at breast cancer diagnosis was observed within the *BRCA2* mutation carrier group (p = 0.001, Spearman's correlation) but not among non-carriers (p = 0.38, Spearman's correlation) (**Figure 17**). Of note, the TL measurements in tissue samples have not been age-corrected due to lack of a control group.

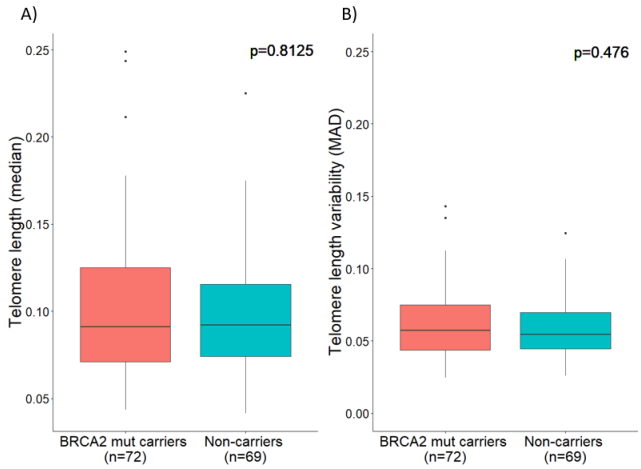

Figure 16. Luminal epithelial cell TL measurements. Comparison between *BRCA2* mutation carriers (n = 72) and non-carriers (n = 69) in A) median luminal epithelial cell TL and B) TL variability measured as Median Absolute Deviation (MAD). p-values derived from Wilcoxon rank-sum test.

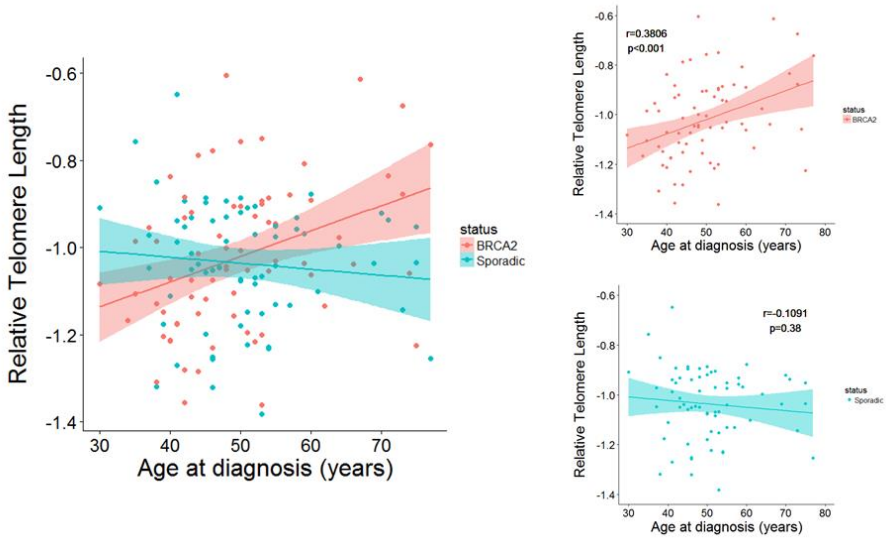

Figure 17. Correlation between normal breast luminal epithelial cell TL and age at breast cancer diagnosis. *BRCA2* mutation carriers (n = 72) (above) and non-carriers (n = 69) (below). p-values derived from Spearman's correlation test.

4.2.3 High levels of DNA damage in non-malignant breast tissue cells

When staining for 53BP1 in cells from culture, distinct foci are expected where DNA DSBs occur. In similar staining on paraffin embedded tissue sections, this is not always the case, instead of the distinct foci, a pan-nuclear signal is often observed (**Figure 18**). Therefore, counting of DNA damage foci and co-localization analysis of telomeres and 53BP foci is problematic and often not representative of the levels of DNA damage. Similarly, it is difficult to count the number of foci in a reliable manner between samples.

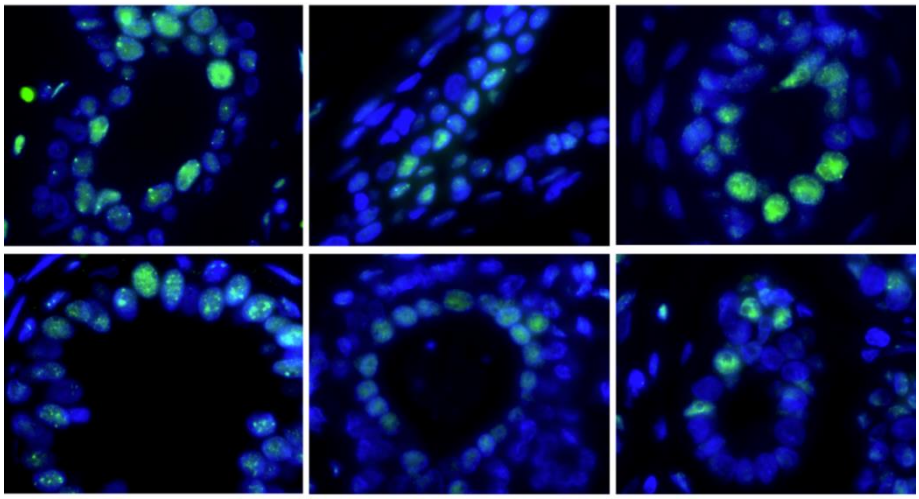

Figure 18. 53BP1 staining patterns in paraffin embedded normal breast tissue sections.

To try to circumvent this problem, a subset of 60 normal breast tissue samples were scored for the level of 53BP1 staining using a simplified scoring system of low, moderate and high staining signal intensity (**Figure 19**). The results show no differences in levels of DNA damage between *BRCA2* mutation carriers and non-carriers and no correlation with TL or age at diagnosis.

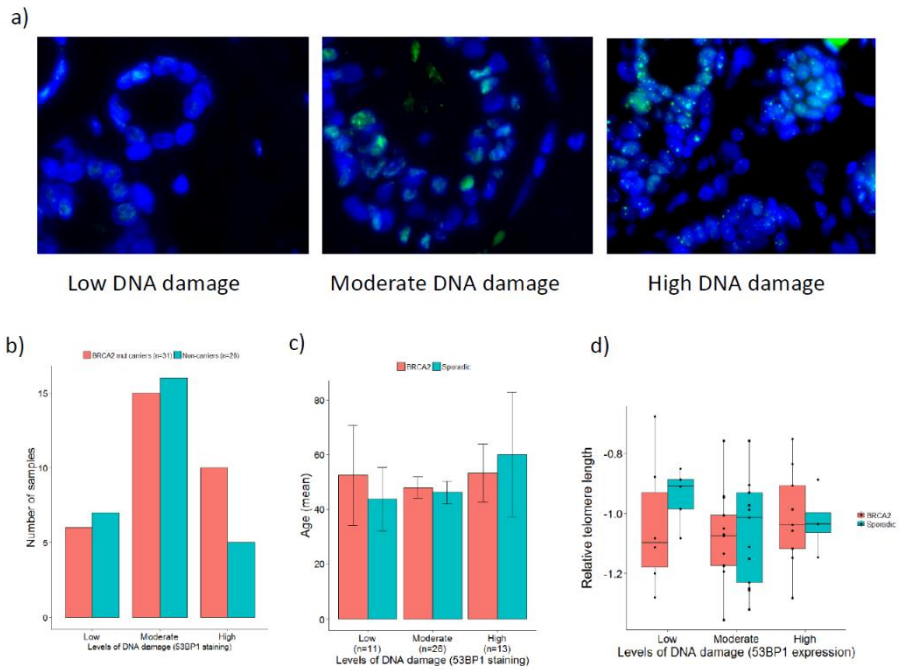

Figure 19. Levels of DNA damage in normal breast LECs. A) Representative images of a simplified scoring system for levels of DNA damage (53BP1 staining, green). B) Levels of DNA damage in LECs between *BRCA2* mutation carriers and non-carriers. C) Association between age at diagnosis and DNA damage in normal tissue. C) Comparisons of levels of DNA damage and TL in *BRCA2* mutation carriers and non-carriers.

4.2.4 TL in normal tissue shows no association BRCA2 wt LOH in the tumor

Previous data was available on wt LOH percentage in breast tumors for 52 *BRCA2* mutation carriers (discussed in detail in chapter 4.4.1) of which 33 overlapped with the group in this study. We tested the theory whether TL in tumor-adjacent normal tissue correlated with the presence/absence of BRCA2 wt LOH in the tumor and found no such association (**Figure 20**).

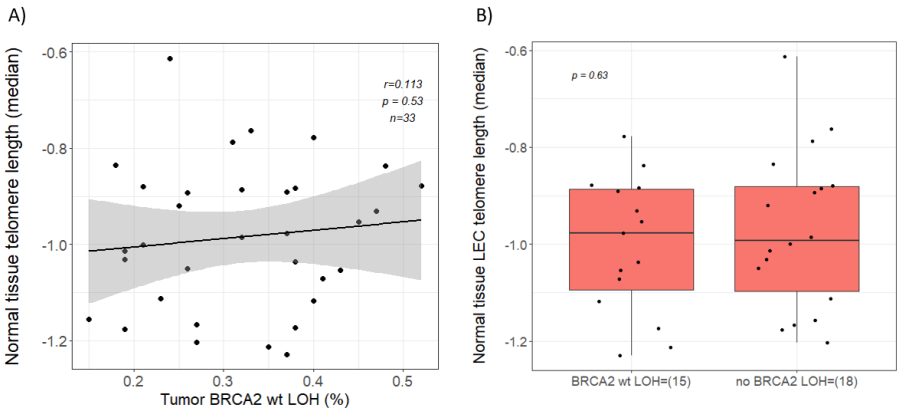

Figure 20. Association between normal tissue TL and *BRCA2* LOH in adjacent breast tumor. A) Correlation between TL length in normal breast tissue LECs and *BRCA2* wt percentage in the breast tumor. P-value derived from Spearman's correlation test. B) Normal tissue LEC telomere length compared based on whether the adjacent breast tumor had *BRCA2* wt LOH. P-value derived from Wilcoxon rank-sum test.

4.3 Generation of *BRCA2*^{999del5} cell line model using CRISPR/Cas9 genome editing technology

Results from our laboratory have shown that *BRCA2*$^{+/-}$ lymphocyte cell lines (various mutations) show marked increase in telomere abnormalities compared to wt cell lines (Hörður Bjarnason Master's Thesis, University of Iceland 2015). Additionally, normal breast epithelial cell lines heterozygous for the *BRCA2*^{999del5} mutation, show a high number of TIFs and other telomere abnormalities compared to a *BRCA2*^{wt} cell line, MCF10A (Bodvarsdottir et al., 2012). Earlier results from these cell lines indicate competent, yet decreased HR ability in *BRCA2*$^{+/-}$ cells (Stefán Hermanowicz Master's thesis, University of Iceland 2015). These results suggest haploinsufficiency with regards to BRCA2 function in heterozygous breast epithelial cells.

A good experimental research model is of vital importance for the quality and reliability of a functional study. Previous cell-line studies on BRCA2 in our lab and others have frequently lacked proper controls, often comparing results from different cell lines which can have very different genetic profiles. With the emergence of the CRISPR/Cas9 genetic editing technology, stable cell lines with a desired mutation can now be generated in a more controlled and potentially easier manner than before. Using this approach makes it possible to generate stable *BRCA2* wild type, heterozygous and homozygous mutated cell lines derived from the same parental cell line (isogenic), thus eliminating inaccurate findings due to cell line discrepancies.

A logical next step in the project was therefore to establish an isogenic cell line model for the Icelandic *BRCA2*^{999del5} mutation, constituting of *BRCA2*$^{+/+}$, *BRCA2*$^{999del5/+}$ and *BRCA2*$^{999del5/999del5}$ cell lines of the same origin, using the CRISPR/Cas9 genetic editing system. Once established, the cell lines would be used as an experimental model to study BRCA2 haploinsufficiency with regards to telomere maintenance, DNA double strand break (DSB) repair and stalled replication fork stabilization.

4.3.1 CRISPR/Cas9 genomic editing in breast epithelial cell lines

Based on my previous results from measurements of telomere length and DNA damage in normal breast tissue, the LEC layer showed the most abnormalities. It was therefore decided to focus on normal breast epithelial cells for this part of the project.

The original plan was to use primary normal epithelial breast cell lines derived from Icelandic *BRCA2*^{999del5} mutation carriers, A176 and A240 (Rubner Fridriksdottir et al., 2005), utilizing HDR CRISPR/Cas9 for both a knock-in, reintroducing the five-base-pairs into the non-functional allele restoring function, and knock-out of the remaining wt allele creating a double knock-out. These cell lines however proved ill-suited for CRISPR/Cas9 editing due to being sensitive to growth conditions and not growing well as single-cell clones. Even if not impossible, the slow-growing nature of these cell lines is likely to lead to very low HDR rates, as indicated by no observed HDR events in the clones analyzed.

We then switched to MCF10A, a near-diploid commercial epithelial breast cell line with no *BRCA2* mutations. MCF10A is the most commonly used model for normal breast epithelial cells in functional breast cancer studies, it is spontaneously immortalized from fibrocystic breast disease (Soule et al., 1990). No positive clones with ssDNA oligo insertion and the *999del5* mutation were obtained in MCF10A, indicating very low CRISPR HDR efficiency. Without inhibiting the NHEJ pathway, editing events were however detected in 38.5% of clones analyzed. Using this approach, two clones were obtained with heterozygous mutations in *BRCA2* causing the same truncating stop codon as the *999del5* mutation. In both cases, a 2bp deletion had occurred around the Cas9-induced break-site (**Figure 21B**), 20-21 bp downstream of the *999del5* mutation locus. This causes a frameshift leading to a prematurely truncated protein product of 273 amino acids, with the last 10 out of frame (**Figure 22**). The *BRCA2*^{999del5} mutation leads to a truncated protein product of 272 amino acids with the last 16 out of frame. Additionally, a knock-out (KO) clone was obtained with a 2bp deletion of both alleles along

with a point mutation of A or G to a T around the break site (**Figure 21C**), resulting in the same 273 amino acid truncated protein product. These cells should be functionally and phenotypically identical to cells carrying the *BRCA2*^{999del5} mutation, in particular since the mutated protein product is not detectable and most likely subject to nonsense-mediated decay (Mikaelsdottir et al., 2004). In addition to the edited clones, three wt cell lines were established for control purposes.

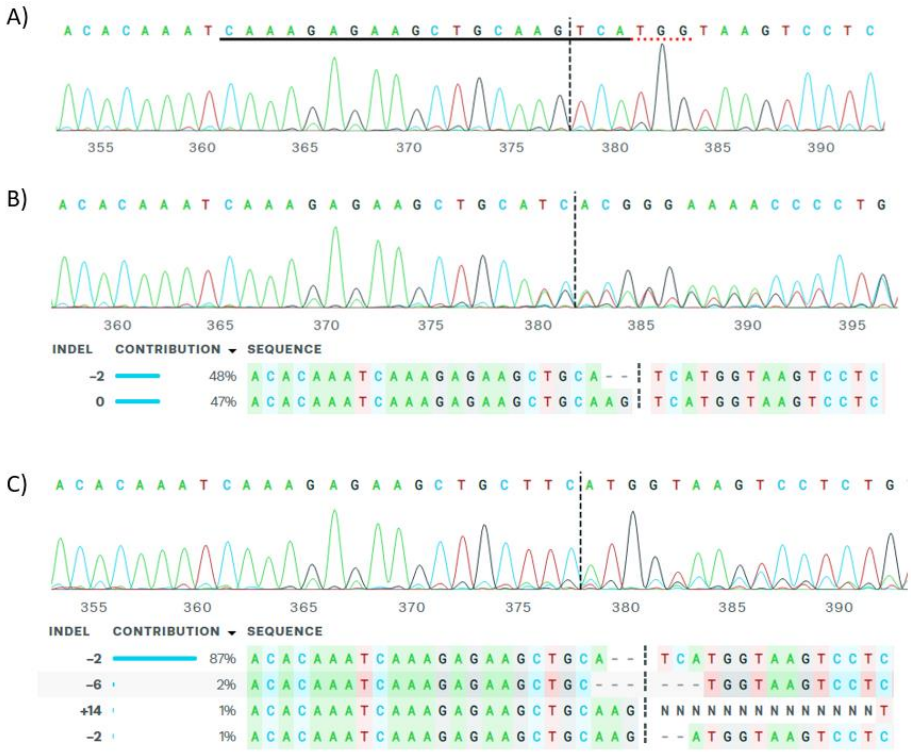

Figure 21. CRISPR/Cas9 genome editing of *BRCA2* in MCF10A cells. A) Sanger sequencing of *wt BRCA2* around the guideRNA recognition sequence, represented by black horizontal underline. Dotted red horizontal underline is the PAM site. Vertical dotted line represents the expected Cas9 cut site. B) Clone with a heterozygous 2bp deletion around the cut-site. C) Clone with a 2bp deletion on both alleles around the cut-site. Alignments and images obtained using the ICE SYNTHEGO online CRISPR analysis tool.

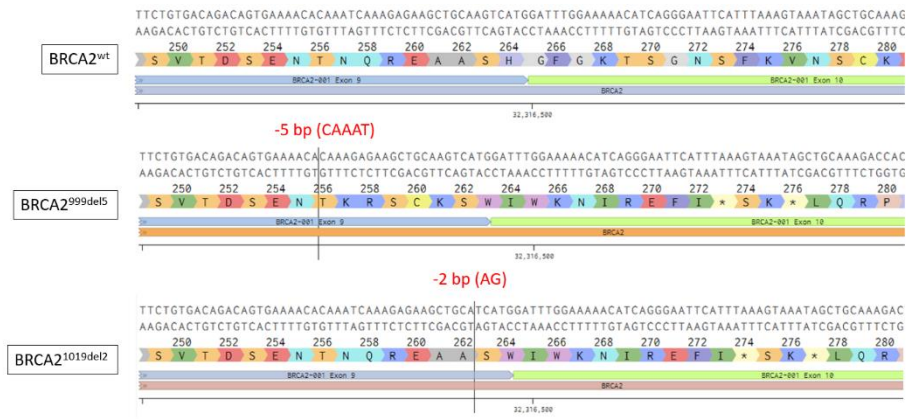

Figure 22. BRCA2 protein product after genome editing. Schematic representation of wt BRCA2 amino acid sequence (top), the BRCA2^{999del5} truncated protein product (middle) and predicted protein product of a CRISPR/Cas9 edited clone line with 2bp deletion around the cut site, leading to the same stop codon.

Unfortunately, the MCF10A^{BRCA2KO} clone quickly died and the established MCF10A^{BRCA2_het} CRISPR cell lines had very slow growth rates and showed morphological signs of senescence (**Figure 23**). It was therefor decided to switch to yet another cell line, HeLa Kyoto, since it had been successfully used for *BRCA2* HDR CRISPR at the time (Tan et al., 2017) and is easy to work with.

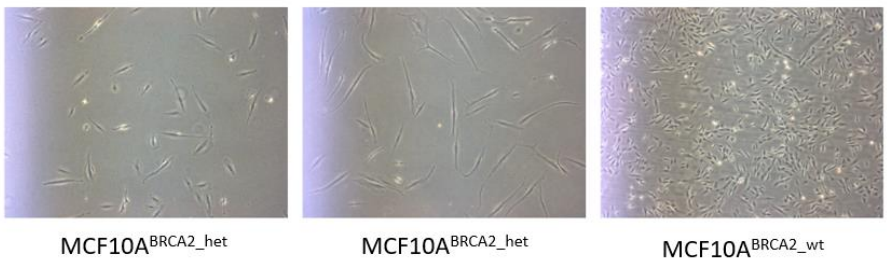

Figure 23. Morphological signs of senescence in MCF10A heterozygous BRCA2 CRISPR cell lines.

4.3.1.1 Generation of HeLa BRCA2 KO model

Using the experimental setup described in chapter 3.5 above, ~10% of analyzed clones showed signs of ssDNA oligo incorporation, identifiable by silent mutations in the ssDNA oligo, resulting in a BstNI restriction site (**Figure 24A&B** and **Figure 7**). However, in most cases the ssDNA oligo incorporation did not extend to the 5 base pair deletion 17-21 bases upstream from the expected Cas9 cut-site of the wt (example in **Figure 24D**). Two clones with heterozygous insertion of the *999del5* mutation had NHEJ events on the other allele and were therefore non-usable. One clone was obtained that had homozygous insertion of the *999del5* mutation (**Figure 24E**) but it turned out to be non-viable.

As a contingency plan, the same CRISPR genome editing process was performed without blocking the NHEJ pathway. In the same manner as described for MCF10A above, clones with editing events causing the same stop codon as the *999del5* mutation, or closely downstream, were obtained in HeLa cells (**Figure 25**). Two heterozygous clones were established into cell lines, one with a 5bp deletion around the Cas9 cut-site causing a truncated protein product of 272 amino acids as described above, and another with a heterozygous 2bp insertion leading to a frameshift and a truncated 276 amino acid protein product, the last 14 out of frame (**Figure 25B**). Two homozygous KO clones were established into cell lines, one with a 2bp deletion on both alleles and the another with a 2bp deletion on one allele and 5bp deletion on the other, all leading to the same protein truncation. Additionally, three clones without modifications, as well as two null clones not transfected with the gRNA plasmid, were established into cell lines as wt controls. The gRNA used had described off-targets. The established clones were screened for the top four highest scoring off-target sites within genes and did not have any editing events.

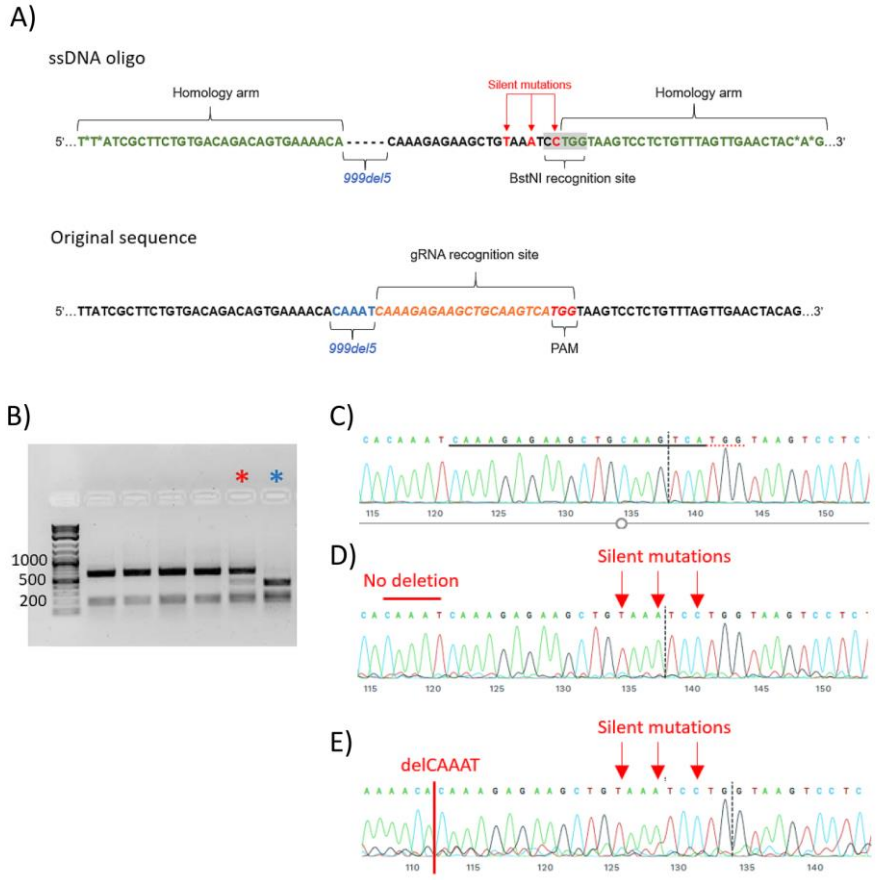

Figure 24. Insertion of *BRCA2*^{999del5} via CRSIPR/Cas9 editing with ssDNA oligo. A) ssDNA oligo design for incorporation of the *BRCA2*^{999del5} mutation and the original wt sequence B) Restriction enzymatic digestion with BstNI showing heterozygous (red asterisk) and homozygous (blue asterisk) incorporation of restriction site C) Sanger sequencing of wt *BRCA2* around the gRNA recognition sequence, represented by black horizontal underline. Dotted red horizontal underline is the PAM site. D) Partial insertion of oligo, including silent mutations around the Cas9 cut site (vertical dotted line) but not reaching to the *999del5* mutation. E) Sanger sequencing of a clone with homozygous incorporation of the ssDNA oligo reaching the *999del5* mutation.

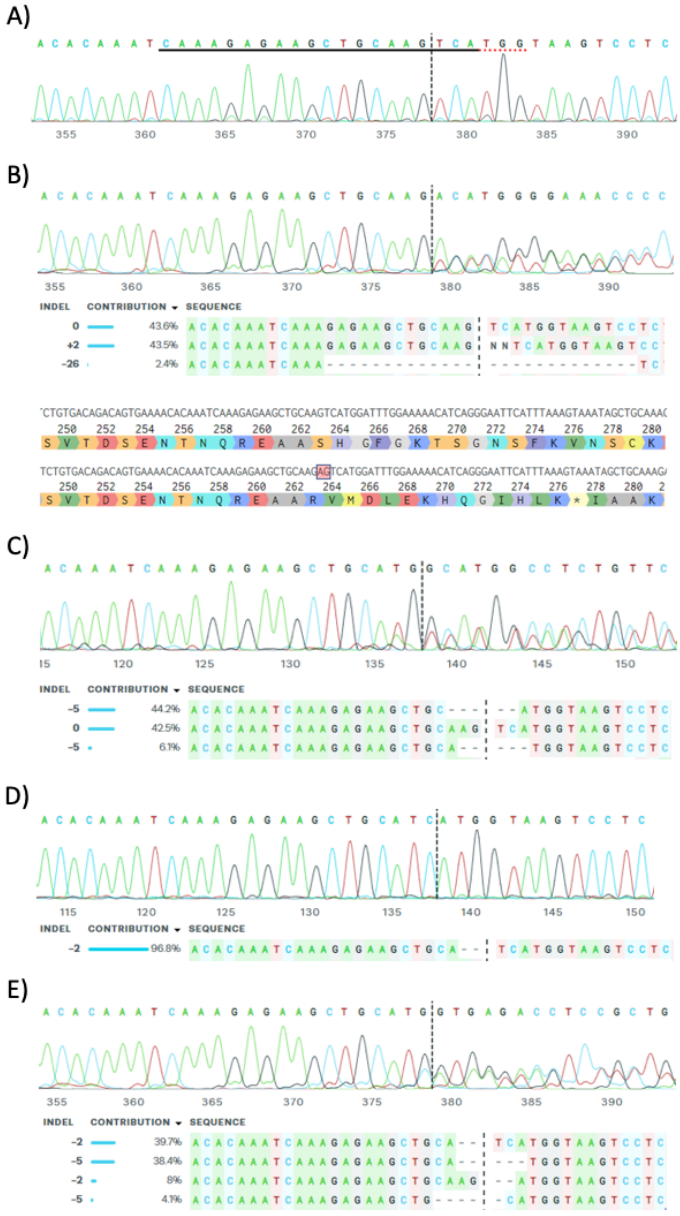

Figure 25. CRISPR/Cas9 editing of *BRCA2* in HeLa Kyoto. A) Sanger sequencing of *wt BRCA2* around the guideRNA recognition sequence, represented by black horizontal underline. Dotted red horizontal underline is the PAM site. Vertical dotted line represents the expected Cas9 cut site. B) Clone with a heterozygous 2bp insertion around the cut-site (top) and predicted truncated protein product of 276 amino acids compared to the wt sequence (below) C) Clone with a heterozygous 5bp deletion around the cut-site D) Clone with a 2bp deletion on both alleles around the cut-site. E) Clone with a 2bp deletion and one allele and 5bp deletion on the other allele. Alignments and images obtained using the ICE SYNTHEGO online CRISPR analysis tool.

4.3.1.2 Validation of loss of BRCA2 expression

Preliminary analysis of BRCA2 protein expression by western blot indicates loss of expression in the two HeLa^{BRCA2_KO} cell lines (**Figure 26**). Expression levels in the heterozygous cell lines remain to be repeated and further validated.

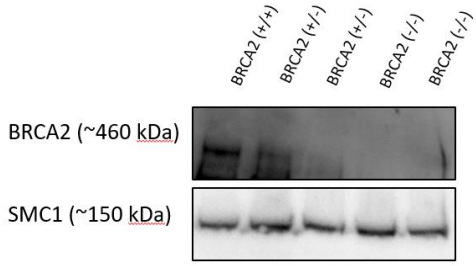

Figure 26. BRCA2 protein expression in HeLa BRCA2 KO and heterozygous CRISPR cell lines. SMC1 used as loading control.

4.4 Loss of heterozygosity in tumors from *BRCA1/2* and *BRIP1* germline mutation carriers in the Icelandic population

Retention of the wild type (wt) *BRCA2* allele in tumors is an important research topic for understanding tumor formation and progression as well as predicting treatment response in mutation carriers. A subset of female breast tumors from *BRCA2*^{999del5} mutation carriers have previously been examined for LOH (Arason et al., 1998; Bodvarsdottir et al., 2012; Davies et al., 2017; Nik-Zainal et al., 2016; Stefansson et al., 2011a), as well as pancreatic tumor samples (Skoulidis et al., 2010) but other tumor types have not been studied in detail. Here, I present validation of the previous results on female breast cancer samples and new analyses of male breast cancer and ovarian cancer cases from mutation carriers of the three most prominent mutations linked to HBOC syndrome in the Icelandic population, namely *BRCA1*^{G5193A}, *BRCA2*^{999del5} and *BRIP1*$^{2040\_2041insTT}$.

Of note, the male breast cancer and ovarian cancer cohorts described here are both a part of recently started larger collaborative efforts focusing on extensive mutational and epigenetic analysis of these tumors in relation to cancer treatment and various clinical factors. As these projects are ongoing, sample analysis and data gathering are in different stages of completion. Of relevance to the thesis topic, the targeted mutational analysis will be covered here and linked to the currently available clinical data. Other aspects of these projects will not be covered in detail in this thesis.

4.4.1 Re-analysis of *BRCA1/2* LOH in female breast cancer patients

Breast tumor DNA samples from 30 women known to carry the *BRCA2*^{999del5} mutation were available and analyzed for locus specific LOH using targeted Sanger sequencing (**Figure 27**). Tumors with wild-type allele frequency below 40% were classified as having LOH. Out of 30 tumors analyzed, 17 had LOH at this locus (56.7%).

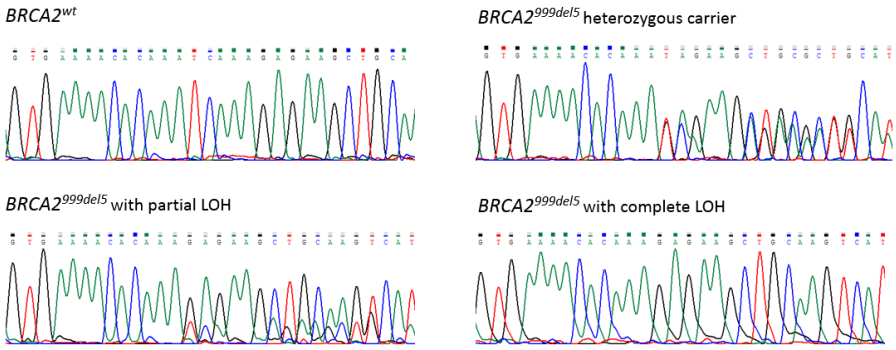

Figure 27. Targeted Sanger sequencing over the *BRCA2*^{999del5} locus. A) Wild type *BRCA2* sequence. B) Heterozygous *BRCA2*^{999del5} sequence C) partial LOH and D) complete LOH over the *BRCA2*^{999del5} locus.

Breast tumors from 52 female *BRCA2*^{999del5} mutation carriers had previously been analyzed for LOH using TaqMan allele-specific quantitative PCR (Aradottir et al., 2015). Of those, 52% (n = 27/52) were classified as having LOH using the criteria of less than 35% of normal allele retained. These results have been validated for a subset of 32 tumors in aCGH analysis (Stefansson et al., 2011a). For method validation purposes, estimates of wt allele proportion in the 30 tumor samples analyzed with Sanger sequencing above and Taqman qPCR allelotyping were compared (**Figure 28**) and shown to significantly correlate (Pearson correlation r = 0.797, p < 0.0001). Analysis of clinical parameters in relation to LOH for these female breast tumors has been described by others (Aradottir et al., 2015; Stefansson et al., 2011a).

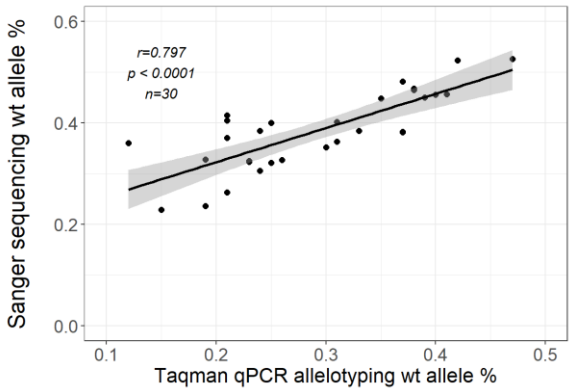

Figure 28. Correlation between LOH estimates in female breast tumor samples with Taqman qPCR allelotyping and targeted Sanger sequencing.

Breast tumor samples from 3 women carrying the *BRCA1*^{G5193A} mutation were available and analyzed for locus specific LOH (**Figure 29**), all displayed partial loss of the wild type allele and classified as having LOH (100%). Screening of the *BRIP1*$^{2040\_2041insTT}$ mutation has not been performed for samples from female breast tumors available at the University of Iceland Cancer Research Laboratory.

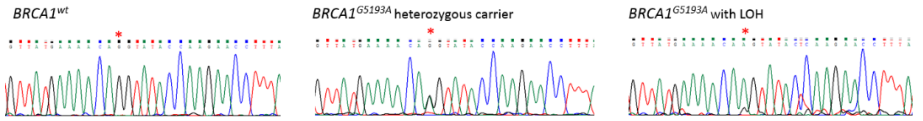

Figure 29. Targeted Sanger sequencing over the *BRCA1*^{G5193A} locus. A) Wild type *BRCA1* sequence. B) heterozygous *BRCA1*^{G5193A} mutation carrier C) LOH over the *BRCA1*^{G5193A} locus

4.4.2 Screening for the *BRCA2*^{999del5} mutation in male breast cancer patients

A total of 82 men have been diagnosed with invasive breast cancer (C50.9) in Iceland since recording began at the Icelandic Cancer Registry in 1955 and through 2018. Few cases are diagnosed annually (**Figure 30A**), averaging in 3 cases per year in the last ten years, 2009-2018. The mean age at diagnosis was 66.8 years (range 44-91 years) (**Figure 30B**).

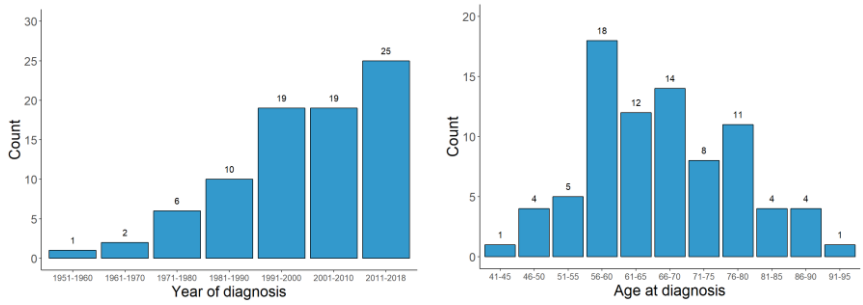

Figure 30. Overview of the male breast cancer cohort. A) Year of diagnosis. B) Age at diagnosis

Samples from 78 men were available for analysis. The *BRCA2*^{999del5} germline mutation is present in 32% of male breast cancer patients in Iceland (n = 25/78). Complete or partial LOH of the wt allele was seen in 88% of tumors (n = 22/25). Two men, cases 16 and 73, had asynchronous bilateral breast cancer (distinct primary breast cancers in each breast separated by > 5 years). Both were carriers of the *BRCA2*^{999del5} mutation and all four breast tumors displayed partial or complete LOH of the *BRCA2* wild type allele.

4.4.2.1 Other germline mutations detected in the male breast cancer cohort

The *BRCA1*^{G5193A} germline mutation was not detected in any patient. Two men carried the *BRIP1*$^{2040\_2041insTT}$ germline mutation (general overview of *BRIP1* sequencing in **Figure 31**), cases 16 and 52. Both of them also carried the *BRCA2*^{999del5} mutation and had retained the *BRIP1* wt allele while losing the *BRCA2* wt allele in the tumor. Case 16 had bilateral breast cancer in which this was the case for both tumors.

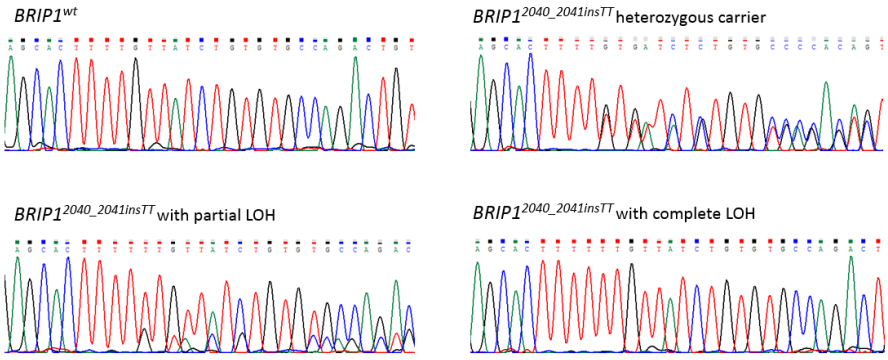

Figure 31. Targeted Sanger sequencing over the *BRIP1*$^{2040\_2041insTT}$ locus. A) Wild type *BRIP1* sequence. B) heterozygous *BRIP1*$^{2040\_2041insTT}$ mutation carrier C) Partial and D) complete LOH over the *BRIP1*$^{2040\_2041insTT}$ locus.

4.4.2.2 Clinical implications – male breast cancer cohort

An overview and comparison of available data on clinical characteristics between non-carriers and *BRCA2*^{999del5} mutation carriers in the MBC cohort is shown in **Table 10**. The average age at breast cancer diagnosis among male *BRCA2* mutation carriers was 64.5 years compared to 67.7 years for non-carriers (p = 0.20, two-tailed student's t-test).

Ten men had a previous cancer diagnosis to their breast cancer diagnosis, thereof three with prostate cancer, while 12 patients had been diagnosed with another cancer after their breast cancer, thereof 6 with prostate cancer. In total, 9 men were diagnosed with prostate cancer in addition to their breast cancer diagnosis, of whom 5 carried the *BRCA2*^{999del5} mutation (**Table 10**). No significant difference was detected in the proportion of *BRCA2* mutation carriers with more than one cancer diagnosis compared to non-carriers (40% and 19% respectively, p = 0.06, Fisher's exact test), or when testing for prostate cancer in particular (20% and 8%, p = 0.14, Fisher's exact test).

Table 10. Clinical characteristics of available male breast cancer cases. Comparison between *BRCA2*^{999del5} mutation carriers and non-carriers.

	Total	Non-carriers	*BRCA2*^{999del5} mutation carriers
Number of cases (%)	78	53 (68%)*	25 (32%)*
Tumor histology (%)			
Infiltrating ductal carcinoma	62 (80%)	43 (81%)	19 (76%)
Papillary adenocarcinoma	4 (5%)	4 (8%)	-
Other†	12 (15%)	6 (11%)	6 (24%)
Average age at diagnosis (range)	66.7 (42-91)	67.7 (42-91)	64.5 (46-80)
Breast tumor classification (%)			
First cancer diagnosis	68 (87%)	47 (89%)	21 (84%)
Second cancer diagnosis	10 (13%)	6 (11%)	4 (16%)
Bilateral breast cancer (%)	2 (2.5%)	-	2 (8%)
Other cancer diagnosis (%)	20 (26%)	10 (19%)	10 (40%)
Prostate cancer	9 (12%)	4 (8%)	5 (20%)
Median follow-up time in years‡ (range)	12.7 (0.08-28.5)	15.9 (0.08-28.5)	10.7 (0.7-27.9)
Median survival time in years§ (95% CI)			
Overall survival	7.8 (5.7-13.1)	6.8 (4.7-13.1)	8.8 (5.9-NA)
Breast cancer specific survival	NR (7.8-NA)	13.1 (7.32-NA)	NR (6.53-NA)

*Percentages are of total number of cases
†Includes adenocarcinoma not otherwise specified
‡Reversed Kaplan-Meier estimate
§Kaplan-Meier estimate. Median survival time is NR (not reported) if survival remains above 50%

No significant difference in 10-year overall (**Figure 32A**) or breast cancer specific survival (**Figure 32B**) was detected between male breast cancer patients carrying the *BRCA2*^{999del5} germline mutation and non-carriers (multivariate Cox proportional hazards model corrected for age and year at diagnosis, overall survival, HR: 0.59, 95% CI 0.29-1.21, p = 0.15, and breast cancer specific survival, HR: 0.49, 95% CI 0.18-1.34, p = 0.16). Similarly, no significant difference in 10-year survival (**Figure 32C**) or breast cancer specific survival (**Figure 32D**) was observed when comparing tumors with *BRCA2* LOH to carriers with no LOH and non-carriers in a multivariate Cox proportional hazards model after correcting for age and year at diagnosis (overall survival, HR: 0.70, 95% CI 0.34-1.44, p = 0.34 and breast cancer specific survival, HR: 0.74, 95% CI 0.29-1.89, p = 0.52). Focusing specifically on primary breast tumors or correcting for other cancer diagnoses did not change the results (data not shown). Clinical data on pathological tumor characteristics and cancer treatment is not available for the whole cohort at the time of writing.

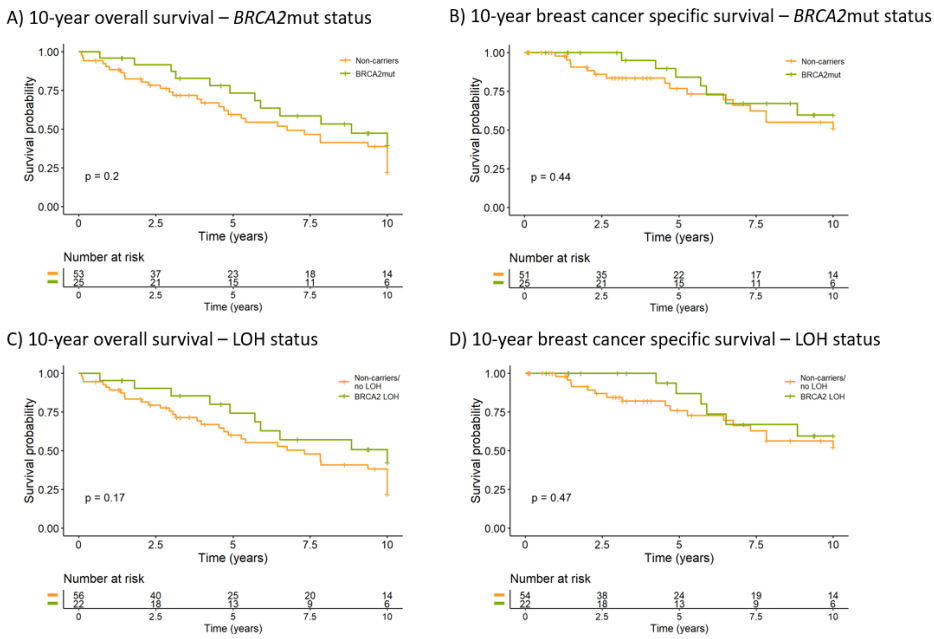

Figure 32. 10-year overall- and breast cancer specific survival in male breast cancer according to *BRCA2* mutation status (A and C) and tumor LOH (B and D). P-values shown are derived from log-rank comparisons of survival proportions. Multivariate Cox proportional hazards models corrected for year and age at diagnosis: A) HR: 0.59, 95% CI 0.29-1.21, p = 0.15. B) HR: 0.49, 95% CI 0.18-1.34, p = 0.16. C) HR: 0.70, 95% CI 0.34-1.44, p = 0.34 and D) HR: 0.74, 95% CI 0.29-1.89, p = 0.52. Data on breast cancer specific survival was not available for two patients.

4.4.3 Ovarian cancer cohort

A total of 488 cases of ovarian (C56*), peritoneal (C48.1, C48.2) and fallopian tube (C57*) cancer were diagnosed in Iceland during the 15-year period 1999-2013. Of those, 127 had borderline malignancy and were not included in the study. 361 cases were invasive carcinomas of ovarian (n = 273), peritoneal (n = 73) and fallopian tube (n = 15) cancer, averaging at 24 cases diagnosed per year (**Figure 33A**). Mean age at diagnosis was 64.4 years (range (15-98 years), see age distribution in **Figure 33B**).

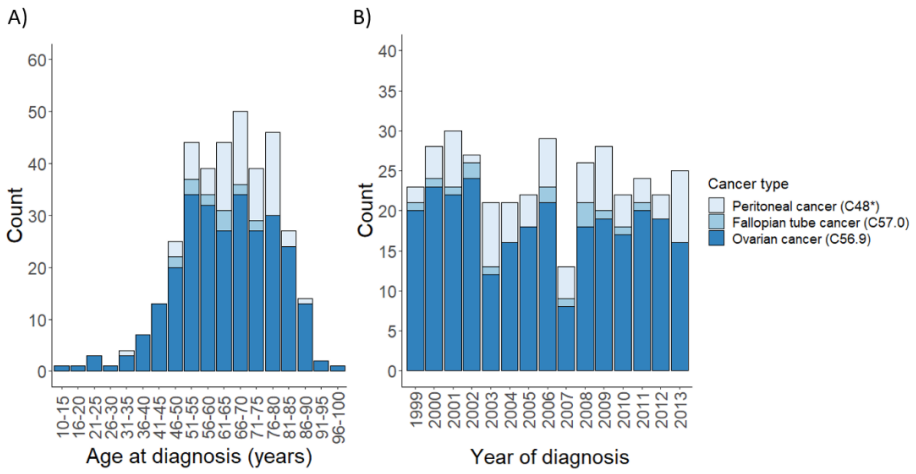

Figure 33. Overview of invasive ovarian, peritoneal and fallopian tube cancers diagnosed 1999-2013 in Iceland (n = 361). A) Age at diagnosis B) Year at diagnosis. Colors represent different tumor types.

Tumor samples were available for 288 patients; 215 ovarian tumors, 13 fallopian tube tumors and 60 peritoneal tumors. These will collectively be referred to as the ovarian cancer cohort unless otherwise specified. Of analyzed tumors, 7.3% carried the *BRCA2*^{999del5} mutation (n=21/288), 1.8% carried the *BRCA1*^{G5193A} mutation (5/280) and 4.5% carried the *BRIP1*$^{2040\_2041insTT}$ mutation (n=13/287). Locus specific LOH was present in 90.5% tumors from *BRCA2* mutation carriers (n=19/21), 100% of tumors from *BRCA1* mutation carriers (n=5/5) and 84.6% of *BRIP1* mutation carriers (n=11/13).

Two women carried both the *BRCA2*^{999del5} mutation and the *BRIP1*$^{2040\_2041insTT}$ mutation, cases 199 and 356. Interestingly, ovarian cancer case 356 displayed LOH of both genes while peritoneal cancer case 199 retained both the *BRCA2* and *BRIP1* wt alleles. Case 199 had been previously diagnosed with breast cancer that has been deep-sequenced and shown to have LOH of *BRCA2* but not *BRIP1* and a high HRDetect score (Davies et al., 2017). Case 356 had two previous diagnoses of DCIS that have not been analyzed for LOH of either gene.

In non-overlapping cases (n = 71) from a pilot study on ovarian cancer, we identified 17 carriers of the *BRCA2*^{999del5} mutation, one carrier of the *BRCA1*^{G5193A} mutation and 3 carriers of the *BRIP1*$^{2040\_2041insTT}$ mutation. Of analyzed tumors, 93.3% of *BRCA2* tumors displayed full or partial loss of the wt allele (n=14/15). LOH was present in the *BRCA1* tumor and 33.3% of tumors from *BRIP1* mutation carriers (n=1/3).

In summary, locus specific LOH was observed in 91.7% of *BRCA2* cases (n=33/36), 100% of *BRCA1* cases (n=6/6) and 75% of *BRIP1* cases (n=12/16) in ovarian cancer (**Table 11**).

Table 11. Overview of tumor LOH in *BRCA1/BRCA2/BRIP1* mutation carriers in both ovarian cancer cohorts

	Pilot study		1999-2013 cohort		Total	
	n	LOH (%)	n	LOH (%)	n	LOH (%)
BRCA1^{G5193A}	1	1 (100%)	5	5 (100%)	6	6 (100%)
BRCA2^{999del5}	15	14 (93%)	21	19 (91%)	36	33 (92%)
BRIP1$^{2040\_2041insTT}$	3	1 (33%)	13	11 (85%)	16	12 (75%)

4.4.3.1 Clinical relevance – ovarian cancer cohort

As for the male breast cancer cohort, clinical data on pathological tumor characteristics and cancer treatment is not currently available for the whole ovarian cancer cohort. For the sake of simplicity and consistency in clinical care practice over time, this preliminary analysis on the clinical relevance of germline *BRCA1/2* and *BRIP1* mutations in ovarian cancer in Iceland will focus on the 1999-2013 cohort (n = 288). See overview of available information in relation to germline mutation carrier status of *BRCA1/BRCA2/BRIP1* in **Table 12**.

Average age at diagnosis for the samples analyzed was 62.1 years and did not differ significantly between non-carriers (61.8 years) and carriers of *BRCA2* (59.8 years) and *BRCA1* mutations (64 years). Carriers of the *BRIP1* mutation were however diagnosed at a significantly higher age (73.6 years) than both non-carriers and *BRCA2* mutation carriers (Kruskal-Wallis test p = 0.03, followed by Dunn's multiple comparisons, *BRIP1* vs *BRCA2* p = 0.02 and *BRIP1* vs non-carriers p = 0.02, all other comparisons were ns).

Data on cancer diagnoses other than ovarian cancer was obtained from the National Cancer Registry. 38 women had been diagnosed with cancer prior to their ovarian cancer diagnoses, thereof 17 with breast cancer. 34 women were diagnosed with another cancer after their ovarian cancer diagnosis, thereof 8 with breast cancer. Seven women had synchronous endometrial and ovarian cancer (SEOC) and were all non-carriers of the analyzed mutations. Carriers of *BRCA1/BRCA2/BRIP1* mutations collectively had significantly higher incidence of other cancer diagnoses (p = 0.005, χ^2 = 7.84, chi-squared test) and of breast cancer in particular (p = 0.0003, Fisher's exact test). The only three *BRCA2* mutation carriers without wt allele LOH in their ovarian tumor, all had a prior breast cancer diagnosis.

Table 12. Overview of the 1999-2013 ovarian cancer cohort

	Total	Non-carriers	*BRCA1*^{G5193A} mutation carriers	*BRCA2*^{999del5} mutation carriers	*BRIP1*$^{2040\_2041insTT}$ mutation carriers	Dual *BRCA2/BRIP1* mutation carriers
Number of cases (%)	288	251 (87.2%)*	5 (1.7 %)*	19 (6.6%)*	11 (3.8%)*	2 (0.7%)*
Tumor type† (%)						
OC (C56.1)	215 (75%)	188 (75%)	5 (100%)	12 (63%)	9 (82%)	1 (50%)
FTC (C57.0)	13 (5%)	12 (5%)		1 (5%)		
PC (C48.1, C48.2)	60 (20%)	51 (20%)		6 (32%)	2 (18%)	1 (50%)
Average age at diagnosis (range)	62.1 (16-89)	61.8 (16-89)	64 (44-86)	59.8 (42-84)	73.6 (61-88)	62.5 (54-71)
OC (C56.1)	61 (16-89)	60.4 (16-89)	64 (44-86)	59.6 (42-84)	74.3 (66-88)	54 (NA)
FTC (C57.0)	60.7 (49-74)	60.5 (49-74)		63 (NA)		
PC (C48.1, C48.2)	66.7 (32-82)	67.2 (32-82)		59.8 (55-65)	70.5 (61-80)	71 (NA)
Tumor classification (%)						
First cancer diagnosis	250 (87%)	224 (89%)	1 (20%)	15 (79%)	9 (82%)	1 (50%)
Later cancer diagnosis	38 (13%)	27 (11%)	4 (80%)	4 (21%)	2 (18%)	1 (50%)
Other cancer diagnosis (%)	62 (22%)	47 (19%)	4 (80%)	7 (37%)	3 (27%)	1 (50%)
Breast cancer	25 (9%)	15 (6%)	3 (60%)	5 (26%)	1 (9%)	1 (50%)
SEOC‡	7 (2.4%)	7 (2.8%)				
Median survival time in years§ (95% CI)						
Overall survival (whole cohort†)	3.5 (2.8-4.6)	3.5 (2.8-4.7)	2.4 (1.8-NA)	5.7 (4.0-NA)	1.6 (1.3-NA)	1.2 (1.2-NA)
Cancer specific survival (whole cohort†)	4.1 (3.2-5.3)	4.1 (3.2-5.7)	2.9 (1.8-NA)	5.7 (4.0-NA)	2.0 (1.3-NA)	1.2 (1.2-NA)

*Percentages are of total number of cases

†Ovarian cancer (OC), Fallopian tube cancer (FTC), Peritoneal cancer (PC)

‡Synchronous endometrial and ovarian cancer (SEOC)

§Kaplan-Meier estimate

No significant difference was seen in 5-year or 10-year overall survival between carriers of *BRCA1/BRCA2/BRIP1* mutation and non-carriers, neither pooled (multivariate Cox proportional hazards models corrected for tumor type, year and age at diagnosis, the same corrections apply to all following Cox models, 5-year overall survival, HR: 1.14, 95% CI 0.77-1.67, p = 0.51 and 10-year overall survival, HR: 1.16, 95% CI 0.79-1.70, p = 0.45, **Figure 34A**) nor separately for each gene (**Figure 35**). Similarly, no significant association was seen between tumor LOH (*BRCA1/BRCA2/BRIP1* pooled*)* and 5-year overall survival (HR: 1.18, 95% CI 0.79-1.76, p = 0.41) or 10-year overall survival (HR: 1.21, 95% CI 0.81-1.80, p = 0.36, **Figure 34B**).

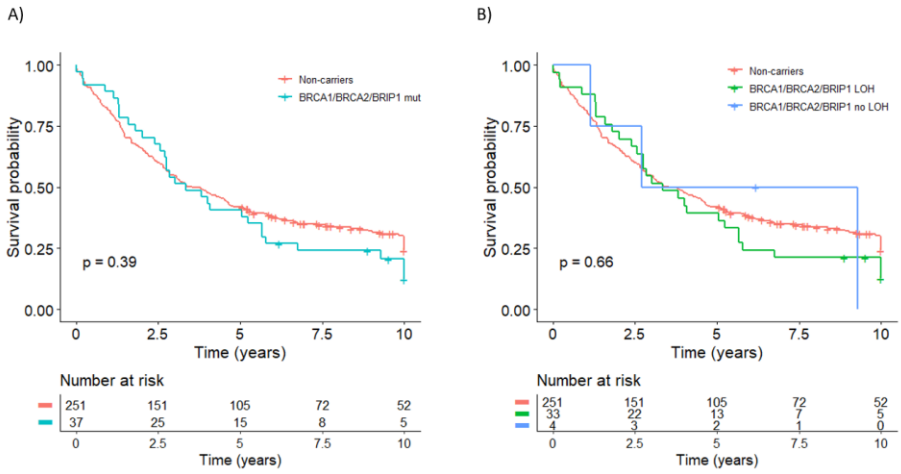

Figure 34. 10-year overall survival in the ovarian cancer cohort. Kaplan-Meier survival curves stratified by A) mutation carrier status (pooled *BRCA1*, *BRCA2* and *BRIP1*) vs non-carriers B) tumor LOH status, non-carriers vs pooled *BRCA1,BRCA2 or BRIP1* mutation carriers with or without LOH in the tumor. P-values shown derived from log-rank comparisons of survival proportions.. Multivariate Cox proportional hazards models corrected for year and age at diagnosis and tumor type (ovarian, fallopian tube, peritoneal cancer): A) HR: 1.16, 95% CI 0.79-1.70, p = 0.45 . B) for LOH pos, HR: 1.21, 95% CI 0.81-1.80, p = 0.36

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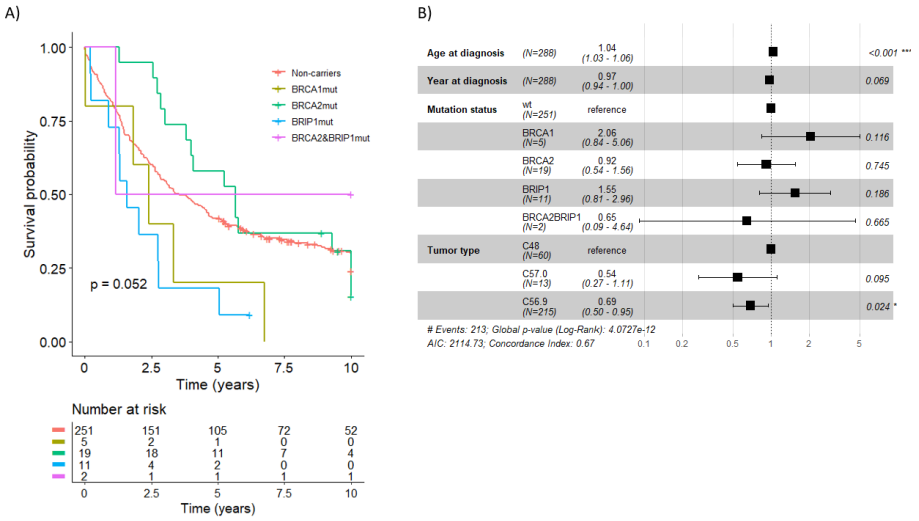

Figure 35. 10-year overall survival in the ovarian cancer cohort based on mutation carrier status. A) Kaplan-Meier overall 10-year survival estimates according to *BRCA1*, *BRCA2*, *BRIP1* and dual *BRCA2&BRIP1* mutation status. P-value derived from log-rank comparisons of survival proportions. B) Results from a multivariate Cox proportional hazards model for mutation carrier status with adjustment for tumor type (ovarian cancer (C56.9), fallopian tube cancer (C57.0), peritoneal cancer (C48)), age and year at diagnosis. * refers to p-value < 0.05, *** refers to p-value < 0.001.

Stratifying the analysis by LOH for each gene did not show any significant associations with 5-year and 10-year overall survival (data not shown). The same analysis was performed for ovarian/fallopian tube/peritoneal cancer specific survival as the end-point and for primary tumors only (n = 250) and in a similar fashion did not show differences in survival between carriers of *BRCA1/BRCA2/BRIP1* mutations or tumor LOH (data not shown).

5 Discussion

5.1 Telomere length measurements in blood samples (paper I)

A relationship between whole blood/leukocyte telomere length and various diseases has been reported, including cancer (Wentzensen et al., 2011; Zhu et al., 2016). Results from numerous studies on the association between blood TL and breast cancer incidence, progression or prognosis have been inconclusive (examples: (De Vivo et al., 2009; Ennour-Idrissi et al., 2017; Samavat et al., 2019; Shen et al., 2009, 2007)) and the same applies for studies in *BRCA* mutation carriers (Killick et al., 2014; Martinez-Delgado et al., 2011; Pavanello et al., 2018; Pooley et al., 2014).

In the first part of this project, whole blood TL was measured in a well-defined Icelandic cohort of female *BRCA2*^{999del5} mutation carriers, sporadic breast cancer patients, and healthy controls with the aim of determining if TL measurements could be used as a stratification method for breast cancer risk and/or prognosis. Blood telomere length did not differ significantly between *BRCA2* mutation carriers and non-carriers among unaffected women or cases sampled before diagnosis. This is in agreement with some published results (Killick et al., 2014; Pavanello et al., 2018) while other studies have described shorter telomeres (Martinez-Delgado et al., 2011) or even longer telomeres (Pooley et al., 2014) in *BRCA* mutation carriers compared to non-carriers. We however observed significantly shorter telomeres in *BRCA2* mutation carriers compared to non-carriers among women who were sampled after breast cancer diagnosis. This may indicate that *BRCA2* mutation carriers are more sensitive to cancer treatment than non-carriers and underscores possible differences in results due to study design, i.e. prospective vs retrospective studies. Comparisons of case-control studies for blood TL and cancer have shown that cases frequently have shorter TL than controls when collected retrospectively but not in prospective studies (Pooley et al., 2010). Most studies are retrospective, with blood samples acquired after breast cancer diagnosis. Both chemotherapy and radiotherapy have been shown to affect telomere length (Benitez-Buelga et al., 2015; Diker-Cohen et al., 2013; Maeda et al., 2013) but few studies take this into consideration. The shorter TL in breast cancer affected women compared to unaffected women in our cohort may be explained by the effects of treatment

since the majority of samples were acquired after breast cancer diagnosis. Blood TL did not affect breast cancer specific survival in our cohort.

Using a semi-prospective approach, looking exclusively at blood samples from women who were unaffected at the time of sampling, shorter telomere length was associated with increased risk for developing breast cancer in *BRCA2* mutation carriers but not in non-carriers. This suggests that TL is a modifier of breast cancer risk in *BRCA2* mutation carriers, possibly indicative of BRCA2 haploinsufficiency with regards to telomere maintenance.

5.2 Telomere length and DNA DSB damage in normal breast tissue

Telomeres may be particularly sensitive to BRCA2 deficiencies, more so than the rest of the genome, due to their repeated structure. In that case, highly proliferating cells such as breast epithelial cells would be expected to show the highest abundance of abnormalities in *BRCA2* mutation carriers, assuming haploinsufficiency. Very short telomeres have been shown to be a common alteration in premalignant lesions (Meeker et al., 2004a, 2004b), and cells transition through telomere crisis in early breast cancer tumorigenesis (Chin et al., 2004; Raynaud et al., 2010). Thus, BRCA2 haploinsufficiency effects at telomeres may contribute to breast cancer initiation in mutation carriers.

Our TL measurements in tumor adjacent normal breast tissue showed the luminal epithelial cell layer having the shortest telomere length of the measured cell types, followed by myoepithelial cells and fibroblasts. This is in agreement with previous published data (Kurabayashi et al., 2008; Meeker et al., 2004a) and likely reflects the high proliferating rates of these cells. Interestingly, the same cell layer also presented with surprisingly high levels of DNA DSB damage, identified by 53BP1 foci staining, some of which co-localized at telomeres. This is highly relevant as most breast cancers originate from the luminal epithelial cell layer. In a similar vein, Kannan et al. have reported that luminal progenitor cells of the breast are characterized by extremely short telomeres and telomere dysfunction (Kannan et al., 2013).

Comparisons of TL in breast luminal epithelial cells between *BRCA2* mutation carriers and non-carriers showed no differences in average TL or TL variation. However, shorter TL in these cells was significantly correlated with earlier age at breast cancer diagnosis in *BRCA2* mutation carriers. This was not the case in samples from non-carriers where no significant association was seen. This indicates that shorter telomeres in normal breast tissue of

BRCA2 mutation carriers increase the risk of breast cancer, which is in concordance with our results from qPCR TL measurements in blood (Paper I) and further suggest TL as a modifying factor of breast cancer risk in *BRCA2* mutation carriers. What causes the shorter TL in some mutation carriers rather than others is unknown.

Critically short, or uncapped telomeres are recognized as DNA damage and are associated with DDR factors such as 53BP1 and γ-H2AX. Co-localization of these DDR factors at telomeres is recognized as TIFs, a marker for telomere dysfunction (Takai et al., 2003). Difficulties with staining patterns in the FFPE study material did not allow for quantitative assessment of TIFs between *BRCA2* mutation carriers and non-carriers for the whole study group. A qualitative approach did not reveal any differences in overall DNA damage levels or association with age at breast cancer diagnosis or TL. The level of telomere dysfunction between normal breast tissues from *BRCA2* mutation carriers and non-carriers remains to be further validated.

Measurements of TL and telomere dysfunction in tumor tissue were not included in this thesis. Previous studies have reported an association with shorter TL in breast tumors and higher TNM stage and worse prognosis (Fordyce et al., 2006). Telomere length in breast tumors has additionally been associated with certain tumor subtypes, specifically short telomere length in luminal B, HER2 and triple-negative tumors (Heaphy et al., 2011). Breast tumors from *BRCA* mutation carriers have been shown to have short telomeres and this is associated with higher tumor grade (Martinez-Delgado et al., 2013). Multiple studies have measured TL in breast tumors in relation to prognosis, often with conflicting results (reviewed in (Ennour-Idrissi et al., 2017)).

5.2.1 Limitations of telomere length measurement methods

Telomere length and rate of telomere shortening in blood is correlated with that of other tissues (Daniali et al., 2013) and is therefore frequently used as proxy for less accessible cell types. It is however debatable whether TL measurements in blood are generalizable to other tissue types as it presumes dysfunctional telomere maintenance to be systemic rather than cell type specific.

All methods have their limitations. qPCR measurements of TL have been shown to be highly affected by pre-analytical steps including different DNA isolation methods and sample storage conditions (Dagnall et al., 2017). Another factor to consider is the composition of the blood sample as cell

types in blood have different telomere lengths (Weng, 2001). The effects this can have on the outcome are highlighted by the bias introduced into our original dataset by using DNA isolated from different blood parts, eventually leading to changes in sample inclusion criteria for Paper I. Even in samples from whole blood only, the different percentages of each cell type at the time of sampling may lead to inconsistent results.

The length of the shortest telomere, or the presence of a few very short telomeres, is well established as a key biomarker for replicative senescence (Hemann et al., 2001; Zou et al., 2004). High-throughput methods, like qPCR-based measurements, are however typically based on averages. FISH-based techniques are better suited for capturing the range of individual telomere lengths at a single cell level but the quality of study material can affect the results. Some FFPE tissue samples can be highly autofluorescent, leading to the faintest telomere signals being masked by the background. This can affect both interpretation of TL measurements and TIF co-localization analyses. A limitation to our analyses on normal breast tissue FFPE samples is the long study period as differences in practice over time as well as prolonged storage may affect probe hybridization efficiency. Also, the normal tissue studied was adjacent to tumor and therefore cancer field effects, which have been shown to include telomere shortening and genomic instability in histologically normal tissues (Heaphy et al., 2006), cannot be ruled out.

Despite the methodological limitations, similar results were observed in our studies using two different TL measurement methods of distinct principles in two different tissue types in samples from the same cohort, leading us to believe that the results are reliable.

5.2.2 BRCA2 haploinsufficiency and tissue specificity

Earlier studies have reported increase in DNA DSBs, impaired DNA repair, chromosomal aberrations, telomere dysfunction and altered gene expression in heterozygous cells derived from *BRCA2* mutation carriers (Arnold et al., 2006; Bellacosa et al., 2010; Bodvarsdottir et al., 2012; Kim et al., 2004), indicating BRCA2 haploinsufficiency. Mutation carriers are however completely normal apart from increased cancer risk in certain tissues and if existent, the haploinsufficiency effects appear mild. A possible mechanism for mild haploinsufficiency in HR for promoting initiation of tumorigenesis is through chronic reliance on alternative more error-prone DNA repair mechanisms resulting in accumulation of mutations, ultimately leading to tumor formation. A recent study by Zámborszky et al. (2017) does not

support this idea, as they report no marked increase in spontaneous accumulation of mutations characteristic of BRCA2 null phenotype in DT40 heterozygous *BRCA2* cells under unstressed normal growth conditions. Recent studies have pointed to another model where heterozygous cells maintain sufficient BRCA2 function under normal circumstances but may be subject to transient impairment of BRCA2 function, i.e. induced haploinsufficiency. Tan et al. (2017) showed that exposure to naturally occurring concentrations of formaldehyde and acetaldehyde induces haploinsufficiency in *BRCA2* heterozygous cells by selectively depleting BRCA2 via proteasomal degradation. This causes accumulation of replication-associated DNA damage and chromosomal aberrations. Gruber et al. (2019) have then recently demonstrated that such transient depletion of BRCA2 (which they term “BRCA2-crisis”) in non-transformed mammary epithelial cells (MCF10A), causes epigenetic and transcriptional changes thereby increasing tumorigenic potential. Signs of these changes were subsequently detected in pre-malignant tissues from *BRCA2* mutation carriers.

Tissue specific conditions leading to transient BRCA2 depletion, such as high levels of endogenous metabolites, could explain why tumors mostly arise in certain tissues in mutation carriers. Research on heterozygous *BRCA1* cells has begun to elucidate tissue specific haploinsufficiency (Sedic & Kuperwasser, 2016). Breast epithelial cells seem to rely more on the function of BRCA1 in DNA damage repair, telomere maintenance and cell differentiation than other cell types (Pathania et al., 2014; Sedic et al., 2015). It has been hypothesized that this can partly be explained by the fast hormonally driven proliferation in breast epithelial cells causing oxidative damage and replication stress, requiring the HR pathway for repair (Fridlich et al., 2015). Whether the same trends apply for BRCA2 remains to be seen and requires studies comparing heterozygous cells from multiple tissue backgrounds.

5.3 Establishing CRISPR cell lines and future functional studies on BRCA2 haploinsufficiency

Functional analysis of possible haploinsufficiency and telomere dysfunction in *BRCA2* heterozygous cells was not an original aim of this PhD project. The results from previous experiments on telomere length and DNA damage in tumor adjacent normal tissue from *BRCA2*^{999del5} mutation carriers however sparked interest in performing functional follow-up experiments. Telomere abnormalities, in particular, had been well characterized in all our

heterozygous breast epithelial cell lines available at the time (Bodvarsdottir et al., 2012). DNA damage repair efficiency and response to PARP inhibitors had also been studied for these same cell lines (Stefán Þór Hermanowicz Master's thesis, University of Iceland, 2015). However, with the emergence of the CRISPR/Cas9 genome editing technology, the generation of an isogenic cell line model with the *BRCA2*^{999del5} mutation became feasible and a logical next step moving forward.

5.3.1 HDR-mediated CRISPR/Cas9 genome editing – struggles and considerations

To establish an isogenic cell line model with the *BRCA2*^{999del5} mutation, we opted for the HDR CRISPR/Cas9 approach. The CRISPR/Cas9 technology is a powerful tool for genome editing. The method was originally employed to knock-out genes, for which it is extremely effective and scalable for large KO screening studies. The technology quickly evolved and is now used for a variety of genome engineering purposes, for example introducing precise edits into the genome. This is most often achieved using a homology-directed repair (HDR) variation of the CRISPR/Cas9 system (see Figure 5), where templates with the desired changes and flanking homology arms are supplied for incorporation via homologous recombination. A drawback of HDR-based editing is its low efficiency compared to NHEJ events. Due to the low efficiency, isolation and analysis of single cell-derived clones is necessary to confirm the presence of the desired edits. To enhance HDR efficiency, measures are typically taken to synchronize cells in the cell cycle (Lin et al., 2014) or inhibiting components of the NHEJ pathway (Maruyama et al., 2015), as well as introducing silent mutations into the PAM sequence or the proximal gRNA seed sequence to block re-cutting by Cas9 (Paquet et al., 2016).

One requirement for CRISPR HDR editing is for the induced DSB to be as close as possible to the desired mutation. When designing gRNAs for a CRISPR study they should ideally have high on-target specificity and a low off-target score. This is usually easily achieved in knock-out experiments since gRNA target sites are abundant in the genome. Editing specific genomic sites is however restricting in the choice of possible gRNAs and compromises may have to be made. Few gRNA target sites (for spCas9 PAM NGG/NAG) are located in close proximity (<50 bp) to the *999del5* mutation in *BRCA2* and the closest ones had high-off target scores but good predicted on-target efficiencies. We observed incorporation of the ssDNA oligo around the cut site in ~10% of clones analyzed in Hela Kyoto cells. However, even if

decent HDR efficiency is achieved, detected by incorporated silent blocking mutations around the cut site, the edited cells do not always contain the desired editing event. This partial oligo insertion is a known phenomenon. It has been shown that distance from the Cas9 cut site to the mutation being introduced severely affects incorporation efficiency as well as directing zygosity. For optimal overall efficiency and homozygous incorporation of the desired edit, the distance should be 10 bp or less. Heterozygous edits are more frequently obtained with distances between 5-25 bp due to lowered efficiency and distances over 30 bp are not considered feasible for clone picking (Paquet et al., 2016). For the highest likelihood of incorporation, the gRNA closest to the *999del5* mutation was chosen, with an expected Cas9 cut site 17-21 bp from the *999del5* mutation. This distance to cut-site should favor heterozygous incorporation but the ssDNA oligo was almost exclusively only partially inserted (**Figure 24**).

We have established a cell line model in HeLa Kyoto cells with wt, heterozygous and homozygous NHEJ-derived edits resulting in protein products truncated at the same amino acid as caused by the *BRCA2*^{999del5} mutation (**Figure 25**). Previous analysis of the *999del5* mutation has shown that the mutant allele is transcribed but the truncated protein product cannot be detected. The mRNA is most likely degraded via nonsense mediated decay (Mikaelsdottir et al., 2004). It can be assumed that this also applies to the newly established cell lines. We observed clones with incorporation of the ssDNA oligo reaching the *BRCA2*^{999del5} mutation validating the experimental setup. In order to obtain the exact *999del5* mutation, the same process could simply be repeated and higher number of clones picked. We are currently repeating this with the Alt-R® CRISPR-Cas9 System (Integrated DNA Technologies), designed to minimize off-target edits. Even though the gRNA used here has high probability scores for off-target editing, the cell lines established did not have edits of the four genomic loci screened (highest scoring off-target loci within known genes). Other off-target edits cannot be ruled-out. Another way to improve target-specificity is to use a double-nickase or dCas9-FokI approach, but the scarcity of gRNA target sites suitable for Cas9 around the *BRCA2*^{999del5} mutation is limiting. Increased availability of Cas variants recognizing other PAM sequences might offer new options. Finally, the design of the ssDNA oligo is important and the best practice guidelines are ever-changing. Homology arm length of the ssDNA oligo has been shown to affect HDR efficiency (Richardson et al., 2016) and altering arm lengths could be explored.

5.3.2 Choosing a fitting research model

Choosing an appropriate research model for functional experiments is key for obtaining reliable results. Previous studies on *BRCA2* mutations have often been based on suboptimal models, comparing results from cell lines with significantly different genetic/epigenetic backgrounds. Establishing isogenic cell line models with the CRISPR/Cas9 technology minimizes the risk of inaccurate findings resulting from such differences.

The choice of parental cell line for CRISPR/Cas9 editing is equally important. Many studies on BRCA2 are performed in tumor cell lines but for studying subtle differences due to possible haploinsufficiency in *BRCA2* heterozygous cells, a non-tumorous background is more appropriate. As outlined previously (chapter 4.3.1), the initial intention was to make use of normal breast epithelial cell lines derived from two carriers of the $BRCA2^{999del5}$ mutation, A176 and A240 (Rubner Fridriksdottir et al., 2005), and use HDR CRISPR/Cas9 to both correct the mutation and to create knock-out cell lines. When these cell lines proved near-unsuitable for single-cell cloning we switched to the commercially available normal-like breast epithelial line MCF10A. We were unsuccessful in introducing the $BRCA2^{999del5}$ mutation into MCF10A cells and upon creating a BRCA2 KO clone, the cells quickly died. A study published around the same time (Feng & Jasin, 2017) showed that complete loss of BRCA2 is lethal for MCF10A cells, confirming our observation. MCF10A is a non-transformed cell line, spontaneously immortalized from fibrocystic disease of the breast, but has been shown to have a relatively stable genome (Soule et al., 1990). Complete loss of BRCA2 is therefore non-viable for normal breast epithelial cells and this could also apply to the A176/A240 cell lines. These cell lines were however immortalized using HPV-16 E6/E7 transformation, which is known to affect cell-cycle checkpoints by inactivating the p53 and Rb tumor suppressors (Hebner & Laimins, 2006), and could therefore be more tolerant to complete BRCA2 loss. The consequences of complete BRCA2 loss have been extensively studied and for our purposes the comparison of the heterozygous and wt are the most important. Total loss of BRCA2 to complete the model can be achieved by transient methods, such as siRNA knock-downs, or more elegant inducible knock-out systems (Feng & Jasin, 2017).

Since our HDR CRISPR/Cas9 endeavors in breast epithelial cell lines were unsuccessful, we decided to verify the experimental setup in HeLa Kyoto cells. A recent publication at the time had described successful

insertion of specific *BRCA2* mutations in HeLa using HDR CRISPR/Cas9 genome editing (Tan et al., 2017). The HeLa cell line is derived from cervical cancer and is notorious for its unstable genome and aneuploidy. Importantly, the HeLa Kyoto lineage has two copies of *BRCA2* (Adey et al., 2013). HeLa is obviously not the best fit for modelling functions of normal tissues or carcinogenesis but has the advantages of being extremely well studied and easy to work with. In the context of an isogenic cell line model, many important functional questions can still be addressed, keeping the limitations of the model in mind. It should be noted for example that studies on telomeres and telomere length in HeLa cells are limited by the fact that it has active telomerase expression. Our attempts to introduce a ssDNA oligo with the *BRCA2*^{999del5} mutation into MCF10A cells indicated very low HDR rates. This cell line has nevertheless been used successfully in CRISPR/Cas9 HDR editing of *BRCA2* (Feng & Jasin, 2017) and could be attempted again for the *BRCA2*^{999del5} mutation with the now more streamlined protocol.

Yet another aspect of studying normal breast tissue is the question of cell type. Our results and others (Kannan et al., 2013; Kurabayashi et al., 2008; Zhou et al., 2012) indicate that the luminal epithelial cell layer of the breast, where most breast cancers originate, is of special interest with regards to telomere dysfunction and DNA damage. Studying these differences between cell types in normal breast tissue in the context of *BRCA2* heterozygosity and possible haploinsufficiency is therefore of interest. MCF10A cells express both luminal and basal/myoepithelial markers and form unique spheroid acinar structures in 3D culture that are not found in normal breast tissue (Qu et al., 2015). Another interesting model is the D492 cell line from Dr. Þórarinn Guðjónsson's lab (University of Iceland, Stem Cell Research Unit). D492 is a stem cell like breast cell line which can differentiate into both luminal and myoepithelial cells (Gudjonsson et al., 2002). Additionally, D492 forms branching structures in a 3D reconstituted basement membrane. 3D cultures better mimic *in vivo* conditions for tissue composition and cell-to-cell interactions compared to conventional 2D cell cultures.

5.3.3 Future directions - comparing BRCA2^{999del5} and BRCA2^{K3326*}

After establishing an isogenic cell line model of the *BRCA2*^{999del5} mutation, the aim was to characterize the cell lines with regards to proficiency or defects in DNA DSB repair, replication fork stalling and telomere maintenance. However, the cell lines have only recently been established and functional experiments are in early stages, thus not included in this thesis.

One important aspect of establishing a reliable functional cell line model for the *BRCA2*^{999del5} mutation is the possibility to follow up on interesting findings in a large archived sample set available from carriers of the same mutation at the University of Iceland Cancer Research Laboratory. Also of interest for the Icelandic population is the other prevalent mutation found in *BRCA2*, the *K3326*.* Contrary to the *999del5* mutation, which is a classical deleterious *BRCA* mutation in the sense of predisposing to HBOC syndrome, the *K3326** mutation is primarily linked to increased risk of small cell lung cancer, squamous cell skin carcinoma and cancers of the upper aero-digestive tract (Rafnar et al., 2018). The *999del5* mutation leads to an early truncated non-functional protein product while the *K3326** mutation is predicted to result in the loss of the last 93 amino acids of BRCA2 (Mazoyer et al., 1996). Previous functional analyses of the BRCA2^{K3326*} protein product have shown no defects in DNA DSB repair (Kuznetsov et al., 2008; Wu et al., 2005) but it might be deficient in the protection of stalled replication forks. As described in chapter 1.6.2, the difference in cancer predisposition between the two mutations is thought to stem from tissue specific dependency on separate functions of the BRCA2 protein, namely HR repair and protection of stalled replication forks (Pathania & Garber, 2018; Rafnar et al., 2018). This however remains to be functionally validated.

It would be interesting to characterize the functional implications of these two *BRCA2* mutations with regards to tissue specificity and possible haploinsufficiency. To do so, isogenic cell line models for both mutations could be generated using CRISPR/Cas9 genome editing in both normal breast epithelial (e.g. MCF10A) and normal lung epithelial (e.g. BEAS-2B) cell lines. Separation of function experiments could then elucidate different DNA repair dependencies between breast and lung tissue and shed light on the mechanism of carcinogenesis in the context of possible BRCA2 haploinsufficiency. Experiments would include well-established assays for estimating proficiency in HR repair, such as RAD51 foci staining, and DNA fiber assays with hydroxyurea treatment to detect defects in replication fork dynamics. Genomic instability and telomere dysfunction could be studied with cytogenetics and FISH-based methods. Additionally, it would be interesting to perform transcriptome analysis of the heterozygous cell lines (with RNA sequencing) to determine whether these cells upregulate alternative DNA repair mechanisms to compensate for HR deficiencies, as has been shown for BRCA deficient tumors (Kais et al., 2016; Mateos-Gomez et al., 2015) and *BRCA1* heterozygous cells (Zong et al., 2019). In light of the recent study by Tan et al. (2017) demonstrating that *BRCA2* heterozygous cells show no

signs of haploinsufficiency except upon aldehyde exposure, the discrepancy between the effects of the *999del5* and *K3326** mutations in breast and lung tissue could entirely be due to extrinsic exposures and environmental factors, which should be considered in the experimental setup.

5.4 Mutational analyses of *BRCA1*, *BRCA2* and *BRIP1* and LOH in breast and ovarian tumors

BRCA1, BRCA2 and *BRIP1* are all considered to be classical TSGs. Tumors from mutation carriers, especially ones linked to HBOC syndrome, have therefore been assumed to lose function of the wild type allele during tumorigenesis. Studies in recent years have however established that this is not always the case (Kanchi et al., 2014; Nik-Zainal et al., 2016), especially in female breast tumors from *BRCA2* mutation carriers where the wild type allele is retained in up to half of all cases (Maxwell et al., 2017).

5.4.1 Previous research on *BRCA2* LOH in female breast cancer in Iceland

Previous results from our laboratory have demonstrated this for FBC from carriers of the Icelandic $BRCA2^{999del5}$ mutation where LOH is observed in 52% of cases (Aradottir et al., 2015; Stefansson et al., 2011a). aCGH analysis of breast tumors from $BRCA2^{999del5}$ mutation carriers revealed divergent paths of tumor evolution where loss of the wild-type *BRCA2* allele (through deletions at the 13q chromosomal region containing the *BRCA2* gene) co-occurred with copy number changes linked to disease progression (Stefansson et al., 2011a). These results suggested that wild-type *BRCA2* LOH was not necessary for tumor initiation but rather a driver for advanced progression. Of clinical relevance, LOH of the *BRCA2* wild-type allele has been linked to worse breast-cancer specific survival in female $BRCA2^{999del5}$ mutation carriers, after adjusting for clinical parameters and cancer treatment (Aradottir et al., 2015). LOH is associated with tumors of the luminal subtype in $BRCA2^{999del5}$ in mutation carriers, the high-proliferating luminal B in particular, while lack of deletion at this locus is associated with triple negative tumors (Stefansson et al., 2011a). Interestingly, luminal subtype has been identified as an adverse prognostic factor in female $BRCA2^{999del5}$ mutation carriers, the complete opposite from what is seen in non-carriers (Jonasson et al., 2016). Of note, due to the sample collection period and the very recent introduction of PARP inhibitors to the breast cancer treatment regimen for *BRCA* mutation carriers, none of these patients were treated with PARP inhibitors.

A substantial portion of breast tumors from *BRCA2* mutation carriers included in the Wellcome Trust Sanger Institute's deep profiling study of 560 breast tumors (Nik-Zainal et al., 2016) and the subsequent characterization of HRDetect (Davies et al., 2017) were from Icelandic *BRCA2*^{999del5} mutation carriers (n= 13/29). These tumors were originally selected on strict criteria for the available amount and quality of tumor DNA and matching normal blood DNA, as well as tumor RNA. The high ratio of LOH among these tumors (85%, n = 11/13) does not reflect the results from a larger group of *BRCA2*^{999del5} mutation carriers described previously (Aradottir et al., 2015; Stefansson et al., 2011a) and the subset validated in this thesis.

5.4.2 General discussion on locus specific LOH analyses – methodology and limitations

Loss of heterozygosity (LOH) is the most common mechanism of wild-type allele inactivation in tumors from *BRCA* mutation carriers (Osorio et al., 2002). In two recent studies (Maxwell et al., 2017; Van Heetvelde et al., 2018) on breast and ovarian tumors from *BRCA1/2* mutation carriers, locus specific LOH most frequently occurred through deletion of the wild-type allele or copy-neutral LOH. Inactivation of the wild-type allele through somatic mutations or promoter methylation seems infrequent. As for *BRIP1*, the gene is located on chromosome 17q22, distal to *BRCA1* on 17q21, a chromosomal region frequently lost in ovarian tumors (Godwin et al., 1994). Complete loss of the wild-type allele can be observed by targeted allelotyping over genetic loci with known germline mutations while somatic mutations and epigenetic inactivation will not be detected. For tumors without apparent LOH as detected by these methods, inactivation of the wild-type allele via other mechanisms can therefore not be excluded. As most male breast tumors and ovarian tumors analyzed in this thesis exhibited loss of the wild-type allele, this is not of too much concern. Many of the female breast tumors shown to be without LOH with previous allelotyping have since been validated by aCGH analysis (Stefansson et al., 2011a), which has the limitation of not detecting copy-neutral changes, and by whole genome sequencing (Nik-Zainal et al., 2016).

Here, a subset of previously examined FBC samples were re-analyzed for methodological validation between the previously used qPCR based allelotyping and estimates of allelic ratios based on targeted Sanger sequencing. Estimates from the two methods were found to be highly correlated (**Figure 28**). All measurements based on allelic frequencies are, however, somewhat arbitrary. Very few tumors analyzed by either method

exhibit a complete loss of the wild-type allele. Most of the tumors classified with LOH in fact have a wt allele frequency ranging from 20-30%. This is to be expected since tissue samples provided for DNA extraction rarely only contain tumor cells. Tumor areas for sectioning are selected based on high tumor content but recorded estimation of the tumor percentage would be optimal, especially in the case of tumors with high levels of normal stroma, and very small tumors or tumors with high levels of tumor infiltrating lymphocytes, since high normal cell content complicates downstream analysis. Another complicating factor when interpreting sequencing results from tumor DNA is the frequent occurrence of chromosomal abnormalities, including copy-number aberrations, aneuploidy and polyploidy. Finally, some ambiguous LOH results could be explained by intra-tumoral heterogeneity. A recent study on tumor heterogeneity with regards to HR deficiency in breast tumors however indicates that such heterogeneity is infrequent (Von Wahlde et al., 2017). Assuming that the HR deficiency is a driver event for either tumor initiation or progression in these tumors, this fits with new data demonstrating almost complete homogeneity in key genetic drivers in treatment naïve epithelial tumors and their metastasis, including breast and ovarian tumors (Reiter et al., 2019).

Normal samples from mutation carriers had a measured wild-type allele frequency ranging from 45-55%. Tumors that clearly did not have LOH fell into the same range. One could argue that any wt allele frequency below that demonstrates that at least some tumor cells have lost the wild-type allele. We therefore decided to classify tumors with wt allele ratios below 40% as having LOH, applying for all three genetic loci. Ovarian tumor samples generally showed very clear results for LOH, with low wt allele percentages, while some of the breast tumors had wt allele ratios closer to the cut-off. This is most likely due to higher tumor content in ovarian tumor samples compared to breast tumor samples, in particular those from males. Ultimately, LOH status would need to be verified with more advanced sequencing methods and to determine the exact mechanism of wt allele loss.

5.4.3 The *BRCA2999del5* mutation is present in 32% of male breast cancer patients in Iceland

The first published prevalence of *BRCA2*^{999del5} mutation carriers among MBC patients in Iceland was 40% (n = 12/30) and included all cases diagnosed from 1955-1995 (Thorlacius et al., 1996, 1997). A similar proportion of 38.5% (n = 15/39) was later described for all cases diagnosed until the year 2000 (Gudmundsdottir et al., 2003). Here, we have identified the *999del5* mutation

in 32% of analyzed cases (n = 25/78) diagnosed from 1955-2018. This difference can most likely be attributed to strong penetrance of MBC in the families included in the first study having a big impact as the total number of cases was very small. The Icelandic *999del5* mutation was first identified in a family where three brothers and their first cousin had MBC, all included in the early cohorts (Thorlacius et al., 1996). With increased number of cases, the new proportion of 32% is likely a more balanced estimate but still much higher than described for other populations, with or without known founder mutations (Basham et al., 2002; Ding et al., 2011; Ottini et al., 2003; Rizzolo et al., 2016; Rubinstein, 2004; Syrjäkoski et al., 2004).

Age of diagnosis did not differ significantly between *BRCA2*^{999del5} mutation carriers and non-carriers. This is in line with other recent reports (Fostira et al., 2018; Pritzlaff et al., 2017; Silvestri et al., 2016). Carrying mutations in the *BRCA* genes has been shown to increase the risk of multiple cancer diagnoses. In the Icelandic MBC cohort, the two men who had bilateral breast cancer diagnosis were both *BRCA2* mutation carriers. 40% of *BRCA2*^{999del5} mutation carriers developed other tumors in addition to their breast tumor including 20% who were diagnosed with prostate cancer (**Table 10**). Comparing these ratios to those for non-carriers did however not reach statistical significance. Similarly, there was no statistical difference in survival between *BRCA2* mutation carriers and non-carriers (**Table 10**), even though it could be argued that there is a trend towards better survival among *BRCA2* mutation carriers. That being said, these analyses are limited due to low numbers and lack of clinical data and should be considered preliminary. The few studies that have reported on outcomes for *BRCA* mutation carriers in male breast cancer are not in complete agreement and are generally based on few cases (Gargiulo et al., 2016; Ibrahim et al., 2018; Ottini et al., 2012).

5.4.4 *BRCA2* tumor LOH in MBC and comparisons with FBC

LOH over the chromosomal area spanning the *BRCA2* gene is common in sporadic male breast tumors (Kwiatkowska et al., 2002; Prechtel et al., 1998) and the wt allele is generally assumed to be lost in mutation carriers (Gudmundsson et al., 1995; Ottini et al., 2003; Thorlacius et al., 1996). In light of accumulating data showing that the wt allele is retained in up to half of FBCs from *BRCA2* mutation carriers, a closer inspection of wt LOH in MBC is warranted. In our cohort, loss of the wt *BRCA2* allele was present in a large majority of MBC from mutation carriers (88%). This highlights differences in breast cancer tumorigenesis and/or progression between female and male *BRCA2* mutation carriers.

MBCs have been shown to differ from FBCs on a molecular level, being characterized by other recurrent somatic drivers indicating different molecular evolutionary pathways (Deb et al., 2016; Johansson et al., 2013; Piscuoglio et al., 2016). These differences can most likely be explained by dissimilarities in breast gland morphology and function between the sexes, with varied cell type composition, levels of differentiation and hormonal regulation. The tumor cell of origin may therefore be inherently different. It is possible that breast cancer susceptibility in *BRCA2* mutation carriers and dependency on wt allele LOH in the tumor is influenced by these different backgrounds.

MBC is a very rare disease, even in *BRCA2* mutation carriers. While female *BRCA2* mutation carriers face a life-time risk of up to 70% of developing breast cancer (Kuchenbaecker et al., 2017), estimations for male mutation carriers are in the single digits (Evans et al., 2010; Tai et al., 2007). What separates the male mutation carriers who develop breast cancer from the others? Known risk factors for male breast cancer such as hormonal imbalance could be one explanation, along with other environmental factors. Data on this is not readily available for our cohort. Another possibility is the presence of other genetic variants having a modifying effect on the breast cancer risk. This is underscored by the fact that MBC seems to be more prevalent in some *BRCA2* mutation carrier families than others (Thorlacius et al., 1996). We are currently waiting for genealogical data for the whole MBC cohort where some of the cases, not limited to $BRCA2^{999del5}$ mutation carriers, are known to aggregate in families.

The largest pathological assessment on MBCs from *BRCA2* mutation carriers to date showed that these tumors represent a subgroup with aggressive biology within MBCs and present at higher stage, higher histological grade and are more frequently ER/PR positive compared to FBC from mutation carriers (Silvestri et al., 2016). The fast proliferating luminal B subtype has been linked to mutations in DNA repair genes in MBC (Piscuoglio et al., 2016). Interestingly, breast tumors with *BRCA2* wt LOH from Icelandic female $BRCA2^{999del5}$ mutation carriers are associated with the luminal B subtype and worse prognosis, as previously mentioned. We do not have tumor subtype information for the male breast cancer cohort at the time of writing but it will be interesting to see whether similar trends apply.

One male breast tumor had a measured mutant allele frequency of 30% and was classified as not having LOH. This patient was a confirmed *BRCA2* mutation carrier based on analysis of his normal tissue. The same result was obtained after multiple PCR amplifications and sequencing runs. To rule out

any kind of sample mix-up in the tumor sample, new sections have been requested and will be analyzed from scratch. If confirmed, this result could be explained by LOH of the mutant allele instead of the wt allele, which has been previously shown to occur stochastically in breast tumors from *BRCA* mutation carriers (King et al., 2007).

Based on the analysis presented in this thesis, we do not have the means to determine whether *BRCA2* LOH is a causal event for MBC in mutation carriers or a stochastic byproduct of genomic instability. The frequent LOH observed however suggests that complete loss of *BRCA2* is important for tumor formation in these men. Three male *BRCA2* mutation carriers had breast tumors without LOH. These tumors could have arisen in a more sporadic manner due to other risk factors such as heightened estrogen levels. Another possible influencing factor, related to tissue specific haploinsufficiency, is prior treatment with DNA-damaging chemotherapy (discussed further in chapter **5.4.9** below). Two out of the three MBC cases with no LOH had a previous cancer diagnosis and possibly received some form of chemotherapy.

5.4.5 Mutations in *BRCA1* and *BRIP1* do not contribute to MBC in Iceland

Neither germline *BRIP1* nor *BRCA1* mutations seem to contribute to male breast cancer in the Icelandic population. The $BRCA1^{G5193A}$ mutation was not detected in any patient. This is not surprising since mutations in *BRCA1* are generally less frequent in MBC than *BRCA2* mutations (Pritzlaff et al., 2017) and the prevalence of *BRCA1* germline mutations in the Icelandic population is very low (Bergthorsson et al., 1998). The two men carrying the $BRIP1^{2040\_2041insTT}$ mutation were also carriers of the $BRCA2^{999del5}$ mutation and had LOH of *BRCA2* while retaining the wild-type *BRIP1* allele. This indicates that loss of the wild-type *BRCA2* allele is a tumorigenic driving force in these tumors. Previous studies have not reported germline mutations in *BRIP1* to be associated with increased risk of MBC (Silvestri et al., 2011). In the largest multi-gene panel testing of MBCs to date, only one patient of the 708 patients analyzed carried a mutation in *BRIP1* but very interestingly was also a *BRCA2* mutation carrier (Pritzlaff et al., 2017). See further discussion on dual *BRCA2/BRIP1* mutation carriers in chapter **5.4.11** below.

5.4.6 Future perspectives of the male breast cancer project

In this study of MBC we have so far screened the samples for three of the most prominent founder mutations in HR genes in the Icelandic population

linked to HBOC syndrome; *BRCA1*^{G5193A}, *BRCA2*^{999del5} and *BRIP1*$^{2040\_2041insTT}$. Apart from rarer mutations in these genes, there are other known HR-associated gene mutations in the population, for example in *PALB2* and *CHEK2* (information from Landspitali Genetic Counseling Unit). Germline mutations in both of these genes have been associated with increased risk of MBC (Pritzlaff et al., 2017). The future aim for this project is to perform exome sequencing on the MBC cohort, both tumor samples and normal tissue. We will then be able to detect other germline mutations in HR genes as well as somatic mutations and importantly, mutational signatures indicating HR deficiency. As shown here, 32% of the cases already have a confirmed germline mutation in *BRCA2* and most exhibit loss of the wt allele in the tumor. High scores of HR deficiency are expected in these tumors but of course remain to be verified. Promoter methylation of selected HR genes will also be analyzed with pyrosequencing. It will be interesting to see the proportion of male breast tumors that are deficient in HR as it is possible that MBC is largely a disease driven by defects in HR and many patients would benefit from targeted treatment with PARP inhibitors.

Pathological assessment has previously been described for all MBC cases diagnosed in Iceland between 1955-2000 (Gudmundsdottir et al., 2003; Jonasson et al., 1996) and is currently being updated for the whole cohort. Data on various clinical parameters and cancer treatment is also being gathered. TMAs have been created and will be stained by IHC for all clinically relevant markers such as ER, PR, HER2, Ki-67, P53 and AR with the possibility of follow-up staining to verify loss of expression of proteins shown to be mutated in genetic analysis or affected by promoter methylation. This is dependent on the availability of good antibodies, routine IHC staining for BRCA2 is for example not considered feasible due to lack of reliable antibodies. Finally, recent papers have described RAD51 foci staining on archived paraffin embedded tissue samples being an option for estimating HR efficiency in tumors (Castroviejo-Bermejo et al., 2018; Cruz et al., 2018). Such staining would be a great alternative to assess HR deficiency in tumors that are not available for exome sequencing, as well as being a functional validation of the genomic analyses.

The MBC cohort is small so we do not necessarily expect to be able to detect differences in response to cancer treatment and/or survival between patients with tumors deficient in HR and not. The preliminary survival analysis presented here (**Figure 32**), comparing *BRCA2* mutation carriers to non-carriers, showed non-significant differences in survival. In addition to the low number of cases, an obvious flaw to these survival estimates is the lack of

correction for important clinical factors such as tumor staging, histological subtype and treatment. The “non-carrier” group moreover almost certainly includes patients with other mutations in HR genes, either in the germline or somatic in the tumor, likely skewing the results. Another complicating factor is the length of the study period, with diagnosis and treatment evolving drastically over the 63-year period. This will all be addressed upon completion of data gathering.

To conclude, the results presented here on MBC are the initial steps in a thorough analysis of the genetic landscape in MBC at a population level coupled with detailed clinical data. To our knowledge, a study of this kind has not been performed at a population level before.

5.4.7 Prevalence of *BRCA1*, *BRCA2* and *BRIP1* mutation carriers in ovarian, fallopian tube and peritoneal cancer in Iceland

We screened 288 cases of ovarian, fallopian tube and peritoneal cancer diagnosed over a 15-year period in Iceland (1999-2013) for mutations in *BRCA1*, *BRCA2* and *BRIP1.* Of analyzed tumors, 1.8% carried the $BRCA1^{G5193A}$ mutation, 7.3% carried the $BRCA2^{999del5}$ mutation and 4.5% carried the $BRIP1^{2040\_2041insTT}$ mutation. These results are similar to an earlier screen of ovarian cancers diagnosed in Iceland between 1991-2000 where 1.2% of patients were carriers of the $BRCA1^{G5193A}$ mutation and 6% carried the $BRCA2^{999del5}$ mutation (Rafnar et al., 2004). The $BRIP1^{2040\_2041insTT}$ mutation has previously been shown to confer high risk of ovarian cancer in the population (Rafnar et al., 2011). Germline mutations in *BRCA1/2* are generally estimated to be responsible for 5-15% of ovarian cancers which resonates with our findings (Daly et al., 2010; Ramus & Gayther, 2009). Founder effects in *BRCA* mutations in Iceland however result in different frequencies than observed elsewhere, where mutations in *BRCA1* are typically more common (Ramus & Gayther, 2009). In a recently published population-based study on ovarian cancer genetics in Denmark, germline mutations in 12 HR genes (including *BRCA1/2* and *BRIP1*) were collectively detected in 9.8% of tumors (Hjortkjær et al., 2019). In our study, three founder mutations are collectively present in 12.8% of cases (n = 37/288, two women carried both the $BRCA2^{999del5}$ and the $BRIP1^{2040\_2041insTT}$ mutations, thus only counted once). Screening for other rarer mutations in the *BRCA* and *BRIP1* genes as well as other HR-related genes is likely to raise this number.

Analysis of age at diagnosis of ovarian cancer between *BRCA1/BRCA2/BRIP1* mutation carriers and non-carriers revealed no

significant differences apart from *BRIP1* mutation carriers being diagnosed at a later age. In the last cohort studied in Iceland, *BRCA1/2* mutation carriers were not diagnosed significantly younger than non-carriers although a trend was seen (Rafnar et al., 2004). Such associations have frequently been reported by others, often in larger cohorts (Kanchi et al., 2014; Ramus & Gayther, 2009). Mutation carriers had significantly higher incidence of other cancer diagnoses, breast cancer in particular (**Table 12**). The higher breast cancer incidence can most likely be attributed to *BRCA1/2* mutation carriers but not *BRIP1* mutation carriers, this observation is however based on very few cases.

5.4.8 High wt LOH frequency in *BRCA1/BRCA2/BRIP1* mutation carriers reflects the HR deficient nature of many ovarian tumors

We previously identified a number of *BRCA1/BRCA2/BRIP1* mutation carriers with ovarian cancer in a pilot study for this larger project and included them in the LOH analyses to increase number of cases (**Table 11**). Locus specific LOH was observed in all tumors from *BRCA1*^{G5193A} mutation carriers and 92% of tumors from *BRCA2*^{999del5} mutation carriers. Our results are in agreement with previously published data showing that wt allele LOH is present in majority of ovarian tumors from *BRCA1* and *BRCA2* mutation carriers. Kanchi et al. (2014) reported LOH in 100% and 76%, respectively, of high-grade serous ovarian tumors from *BRCA1* and *BRCA2* mutation carriers included in the TCGA and the Women's Health Initiative Exome Sequencing Project (WHISP). Maxwell et al. (2017) then reported locus specific LOH in 93% of cases from *BRCA1* mutation carriers and 84% of cases from *BRCA2* mutation carriers in ovarian cancer cases from the University of Pennsylvania and the TCGA.

Ovarian tumors from carriers of the *BRIP1*$^{2040\_2041insTT}$ mutation have LOH in 75% of cases in our cohort. These results are in concordance with previously published results on this same mutation where full or partial LOH was seen in 8 out of 10 tumors examined using the same targeted Sanger sequencing method (Rafnar et al., 2011). The authors concluded that BRIP1 behaved as a classical tumor suppressor in ovarian cancer. The cases examined in that study probably overlap with some of the 13 cases analyzed here, taking the study periods into consideration. Five of the *BRIP1* ovarian tumors described here were diagnosed after Rafnar et al. published their study and do therefore not overlap.

In summary, LOH in ovarian tumors from *BRCA1/BRCA2/BRIP1* mutation carriers is frequent and underscores the strong link to HR deficiency in

ovarian cancer. Large scale studies on ovarian cancer have revealed that up to half of all cases have underlying germline or somatic changes in HR-related genes (Cancer Genome Atlas Research Network, 2011; Cunningham et al., 2015; Kanchi et al., 2014).

5.4.9 The effects of germline mutations in *BRCA1/BRCA2/BRIP1* and LOH on survival in ovarian cancer

The majority of ovarian cancer patients receive platinum-based chemotherapy as first-line therapy. *BRCA* mutation carriers have been shown to respond better to platinum-based chemotherapy than non-carriers (Cass et al., 2003) and carrying *BRCA* mutations is associated with overall better survival in ovarian cancer patients (Bolton et al., 2012). This is however dependent on the loss of the wt allele, since these treatments do not affect heterozygous cells by principle (Bryant et al., 2005; Farmer et al., 2005). In fact, *BRCA* mutation carriers without locus specific LOH in their ovarian tumor have significantly lower overall survival compared to tumors with LOH when treated with adjuvant platinum-based chemotherapy, more similar to sporadic cases, and this likely extends to PARP inhibitors (Maxwell et al., 2017). It is therefore important to know tumor LOH status in mutation carriers since absence of LOH can cause primary resistance to these treatments.

In a pilot study we performed earlier on a small cohort with overrepresentation of *BRCA* mutation carriers, $BRCA2^{999del5}$ mutation carries were shown to have significantly better 5-year survival than non-carriers after correcting for age and year at diagnosis, histological stage and residual postoperative tumor, while $BRCA1^{G5193A}$ mutation carriers did not differ from non-carriers (data not shown, Sigurður Ingi Magnússon's B.Sc project, University of Iceland, 2018). The survival analysis performed here for the 1999-2013 cohort did however not reveal any differences in survival between carriers of *BRCA1/BRCA2/BRIP1* mutations compared to non-carriers, neither pooled nor separated by gene (**Figure 34A&Figure 35**). Apart from low sample numbers in some groups, this discrepancy can likely be explained by the lack of important clinical factors which should be incorporated in the survival models, including histological stage and grade, ovarian/fallopian tube/peritoneal cancer histological subtypes and treatment information. Previous or later cancer diagnoses can have a confounding impact on the result of survival analyses. Preferably, patients with previous or later non-ovarian cancer diagnosis should be excluded. Subsetting the group to only include primary tumor diagnoses or using ovarian cancer specific survival as an end-point did not change the results. We feel further analysis

of survival in relation to mutational status or tumor LOH is not warranted at this time, comparisons between tumors with and without LOH are as well not likely to be meaningful due to low numbers in the latter group (**Figure 34B**). Additionally, in a disease like ovarian cancer where relapse rate is high, progression free survival time is a more accurate and established end-point estimate for studying response to treatment.

As previously discussed, most *BRCA1/BRCA2/BRIP1* mutation carriers in our cohort had tumor locus-specific LOH in ovarian tumors. An interesting observation is that all three *BRCA2* mutation carriers that had absence of LOH in their ovarian tumor had a previous breast cancer diagnosis and therefore may have received DNA damaging chemotherapy. Normal cells from *BRCA* mutation carriers are not considered to be more sensitive to standard chemotherapeutic agents. However, there might be selective pressure towards retention of BRCA function in precancerous cells during these treatments.

5.4.10 Future directions of the ovarian cancer project

No large-scale studies combining genetic, pathological and clinical data on cancer treatment have been performed for ovarian cancer in the Icelandic population. We have recently participated in a large collaborative effort aiming to study this important subject, starting with cases diagnosed over a 15-year period ending in 2013 enabling us to obtain complete 5-year survival data for the whole study group. Here, I have described mutational analysis for the three most prominent founder mutations related to HBOC syndrome in the population and linked it to the limited available clinical data and prognosis. Gathering of data is in its final stages at the time of writing and these preliminary analyses will therefore soon be updated.

Apart from characterizing ovarian cancer in Iceland, another important aim of this research project is to study the prevalence of promoter methylation of selected DNA repair genes with pyrosequencing in our cohort, most notably *BRCA1* and *RAD51C*, and determine whether epigenetic silencing has the same effects on prognosis as carrying deleterious mutations in these genes. Previous studies are not in agreement on whether this is the case for ovarian cancer (Bernards et al., 2018; Cancer Genome Atlas Research Network, 2011; Ruscito et al., 2014; Stefansson et al., 2012) but epigenetic silencing of *BRCA1* and *RAD51C* by promoter methylation has been shown to cause HR deficiency signatures in breast tumors (Polak et al., 2017), suggesting these tumors should respond well to platinum chemotherapy or PARP inhibitors. Promoter methylation of *BRCA1* has been studied on a breast cancer cohort

in Iceland and linked to improved response to platinum-based therapy ((Stefansson et al., 2011b); Olafur A. Stefansson, in press). Results from our ovarian cancer pilot study were inconclusive due to low sample numbers (Sigurður Ingi Magnússon, B.Sc thesis, University of Iceland, 2018).

Pathological assessment is being performed for all the tumors included in the study and TMAs will be stained with IHC for important clinical markers such as p53 and CA125 as well as BRCA1 to confirm loss of expression. As previously described for the male breast cancer cases, it would be interesting to perform RAD51 foci staining to detect tumors that are HR deficient, both to verify sequencing results and identify tumors with other germline or somatic mutations in HR genes than we have screened for. Apart from defects in HR repair, a subset of ovarian tumors have been shown to be deficient in MMR repair (Murphy & Wentzensen, 2011). Seven cases included in our study presented with SEOC which is in some instances caused by Lynch Syndrome (Takeda et al., 2018). Of the genes associated with Lynch syndrome, germline mutations in in *PMS2* and *MSH6* are most prevalent in the Icelandic population and confer risk to colorectal, endometrial and ovarian cancers among a few others (Haraldsdottir et al., 2017). Promoter methylation of *MLH1* has also been shown to be present in a subset of Lynch-syndrome associated tumors, including ovarian cancer (Losi et al., 2018). IHC staining for these proteins will be performed and used to guide subsequent analysis by sequencing.

5.4.11 Double mutation carriers of *BRCA2* and *BRIP1*

BRCA1-interacting protein C-terminal helicase 1 (BRIP1) is a tumor suppressor protein involved in DNA DSB repair through its interaction with BRCA1 (Cantor et al., 2001). BRIP1 is also involved in the repair of DNA ICLs through the FA pathway where biallelic mutations in *BRIP1* lead to the Fanconi anemia complementation group J (FANCJ) (Levran et al., 2005). BRIP1 seems to be involved in many important functions for maintaining genomic stability, for example in alleviating replication stress through resolving G-quadruplexes, but many of these roles are still not well understood (Brosh & Cantor, 2014). Like many other proteins involved in the same pathways, deleterious mutations in *BRIP1* have been linked to increased risk of breast and ovarian cancer.

We have not analyzed female breast tumor samples for the $BRIP1^{2040\_2041insTT}$ mutation in this study but previously published data indicates that this mutation does not increase breast cancer risk in the Icelandic population (Rafnar et al., 2011). The effects of truncating *BRIP1*

mutations on breast cancer risk are similarly debated elsewhere (Easton et al., 2016). This does not mean that such cases do not exist but whether biallelic inactivation of *BRIP1* is associated with high HR deficiency scores in breast cancer remains controversial, so is the significance of some other HR-related genes such as *ATM* and *CHEK2* (Polak et al., 2017). Even if the *BRIP1*$^{2040\_2041insTT}$ mutation is not associated with increased breast cancer risk at a population level, it would be interesting to examine breast tumors from female mutation carriers with regards to LOH and HR deficiency. The same goes for the *BRIP1* LOH ovarian cancer cases.

One of the two *BRCA2*^{999del5} breast tumors in the Sanger study (Davies et al., 2017; Nik-Zainal et al., 2016) that did not have LOH of *BRCA2*, was interestingly from a woman also carrying the *BRIP1*$^{2040\_2041insTT}$ germline mutation. The tumor had LOH of the wild-type *BRIP1* allele and a high HRDetect score, suggesting that loss of *BRIP1* was the driving event for this tumor and the reason for its HR deficiency, even though this could not be ascertained based on one sample (Davies et al., 2017). Another *BRCA2*^{999del5} breast tumor included in the same study was shown to also carry the *BRIP1*$^{2040\_2041insTT}$ mutation. Neither of these cases had been genotyped for *BRIP1* mutations beforehand. Inversely, this breast tumor had LOH of *BRCA2* but retention of the *BRIP1* wild-type allele (Davies et al., 2017). This patient was however later diagnosed with peritoneal cancer, (case 199 included in the 1999-2013 ovarian cancer cohort). As described above, that tumor had retained both *BRIP1* and *BRCA2* wild-type alleles. These estimates are based on targeted sequencing over the locus and inactivation of either gene by other mechanisms cannot be excluded. It would be very interesting to validate this finding with more extensive sequencing methods and establish whether the peritoneal tumor shows signs of HR deficiency. Although not demonstrated by this case, the tissue specificity between *BRCA2*, which if mutated is more associated with breast cancer, and *BRIP1* which predominantly increases risk for ovarian cancer, is intriguing.

An interesting angle would be to examine families where both *BRCA2*^{999del5} and *BRIP1*$^{2040\_2041insTT}$ are present. Is there higher cancer penetrance in individuals with dual germline mutations and is there a trend in which wild-type allele is lost in different tumor types? We currently have very limited information on the families of the 5 individuals carrying both mutations described here (2 MBC cases, 1 FBC, 1 FBC/PTC and 1 OC case) but they are likely from at least 3 different immediate families (data not shown). Of these five individuals, two have been diagnosed with more than one cancer; the breast/peritoneal tumor case 199 described above as well as a man with

two distinct breast cancer diagnoses (case 16). In addition, ovarian cancer case 356 had two DCIS diagnoses. While the male breast tumors seem to be *BRCA2* associated, the other female breast tumors and ovarian/peritoneal tumors display a variety of LOH combinations. More cases would be needed to identify any patterns in cancer occurrence or driver events. It should be noted that multiple cancer diagnoses are also frequently observed in mutation carriers of *BRCA2* alone. Furthermore, double mutations in the *BRCA* genes are extremely rare, even in populations with multiple known founder mutations like Ashkenazi Jews. The few double carriers studied do not seem to have higher risk of developing breast and ovarian cancers than carriers of one mutation in either gene (Lavie et al., 2011). Double mutation carriers of the $BRCA2^{999del5}$ and the $BRCA1^{G5193A}$ have not been identified in the Icelandic population to our knowledge but compound mutation carriers of the *999del5* and *K3326** mutations in *BRCA2* have been described (Rafnar et al., 2018).

The combination of carrying two mutations in HR-involved genes is interesting in terms of cancer predisposition on a functional level. Could there be slight haploinsufficiency in both genes contributing to cancer risk or is BRCA2 deficiency the main culprit? Functional studies on BRIP1 are scarce. Using the CRISPR/Cas9 genome editing technology, heterozygous cell line models could be established with the $BRCA2^{999del5}$ and $BRIP1^{2040_2041insTT}$mutations, separate and together, to compare the effects on DNA repair efficiency and genomic stability in general (similar to what was described earlier for comparison of the *999del5* and $K3326^{*}$ mutations in *BRCA2).* Here, the choice of cell lines is equally important if tissue specific effects are to be addressed. Even then, it is possible that functional work in cell lines could not distinguish between these without the context of tissue structure, microenvironment and hormonal regulation over a lifetime.

6 Conclusions

The work described in this thesis covers various aspects of possible BRCA2 haploinsufficiency. Expanding on previously reported data showing that retention of the wild-type *BRCA2* allele is common in female breast cancers from *BRCA2*^{999del5} mutation carriers, I show that wild-type locus specific LOH is nearly always present in male breast cancer and ovarian cancer cases from carriers of the same mutation. This highlights different tumor development and/or progression pathways between these tissues and indicates tissue-specific BRCA2 haploinsufficiency in the female breast.

Focusing on a possible haploinsufficiency effect on telomeres in female *BRCA2*^{999del5} mutation carriers, no differences in telomere length or levels of telomere dysfunction were detected between mutation carriers and non-carriers. However, shorter TL in both blood and normal breast tissue were associated with earlier breast cancer occurrence in *BRCA2*^{999del5} mutation carriers but not in non-carriers. This suggest that short TL, often indicative of dysfunctional telomere maintenance, may be a modifying factor of breast cancer risk in *BRCA2* mutation carriers. Consistent with previously reported data, a clear difference in telomere length was seen in different cell types within normal breast tissue. The shortest telomere length and highest levels of DNA damage were seen in luminal epithelial cells which is highly relevant as these are the cells from which most breast cancers originate.

To further elucidate BRCA2 haploinsufficiency on a functional level, I have recently established an isogenic cell line model mimicking the *BRCA2*^{999del5} mutation. Functional characterization of such models in different cell lines could shed light on the mechanism of possible BRCA2 haploinsufficiency in tumorigenesis as well as tissue-specificity.

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Original publications

Paper I