Analysis of haploinsufficiency in women carrying germline mutations in the \textit{BRCA1} gene.
Different mutations, different phenotypes?

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Analysis of haploinsufficiency in women carrying germline mutations in the BRCA1 gene. Different mutations, different phenotypes?

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This thesis, submitted for the degree of Doctor of Philosophy at the Autonomous University of Madrid, has been elaborated in the Human Cancer Genetics laboratory at the Spanish National Cancer Research Center (CNIO), under the supervision of Dr. Ana Osorio and Dr. Javier Benítez Ortiz.

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This thesis is dedicated to my parents
for their love, endless support
and encouragement

♥
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BRCA1 germline mutations are associated with significantly increased lifetime risk of developing breast and ovarian cancers. However, taking into account considerable differences in disease manifestation among mutation carriers, it is probable that various BRCA1 mutations lead to formation of distinct phenotypes and haploinsufficiency effects and that both type and location of the mutation could play an important role in cancer initiation. PARP inhibitors are currently one of the most promising agents for treatment of tumors deficient in BRCA1 or BRCA2. However, cancer patients carrying germline mutation in BRCA1/2 genes show a big variability in the responses to PARP inhibitors in clinical trials. Thus, we were interested in investigating whether and how BRCA1 mutation type influences the response of cells to PARP inhibitors.

We used a panel of lymphoblastoid cell lines (LCLs) derived either from heterozygous BRCA1 mutation carriers or non-carriers in order to study haploinsufficiency effects of various mutation types (truncating vs missense). LCLs carrying truncating mutations showed significantly lower BRCA1 mRNA and protein levels and higher levels of gamma-H2AX than control cells or LCLs harboring missense mutation, indicating increased spontaneous DNA damage in these cells. However, cells carrying either of the BRCA1 mutation type showed impaired RAD51 foci formation, suggesting defective repair in mutated cells and a possible mechanism of increased susceptibility of mutation carriers to cancer.

Moreover, gene expression analysis revealed that LCLs carrying missense mutations showed more distinct expression profile than cells with truncating mutations when compared to controls, suggesting that different mutations may lead to distinct phenotypes and haploinsufficiency effects. Importantly, decreased expression of immune response-related genes in cells harboring missense mutation indicates possible mechanisms of breast cancer initiation in carriers of these particular BRCA1 mutations.

At the present study, we are describing for the first time that cells derived from carriers of missense mutations in the BRCA1 BRCT domain show higher sensitivity to PARP inhibitor olaparib than cells with truncating mutations or WTs. These results indicate that carriers of different mutation types could benefit from the treatment in a distinct way and that they could display different toxicity to the agent. Taking into account the presence of non-degraded mutated protein in cells carrying missense mutation there is a possibility that the missense mutants act in a dominant negative manner on the WT protein function. Thus, we hypothesize that there is a competition between mutated and WT BRCA1 protein in the recruitment to DNA damage sites, which could lead to an alteration the DNA repair process and decreased viability of cells carrying BRCA1 missense mutation. The mechanism of increased sensitivity of cells carrying missense mutation to olaparib could be, at least partially, also related to possible changes in olaparib-specific BRCA1 protein interactors in mutated cells. In addition, regulation of miR-222-3p and its target FOS by olaparib treatment in cells carrying missense mutation suggest a possible use of these molecules as markers of sensitivity to the treatment.

In summary, our results bring new insights on how various heterozygous mutations in BRCA1 could lead to impairment of BRCA1 function and show a strong evidence about haploinsufficiency in BRCA1 mutation carriers. Moreover, our results indicate that carriers of different types of BRCA1 mutations could benefit from the treatment in a distinct way and that could show different toxicity to the PARP inhibitor olaparib.
RESUMEN
Mutaciones germinales en BRCA1 están asociadas con un incremento significativo en el riesgo a desarrollar cáncer de mama y ovario. Sin embargo, teniendo en cuenta las considerables diferencias en la manifestación de la enfermedad en portadoras de mutación, es probable que distintas mutaciones en BRCA1 den lugar a distintos fenotipos y efectos de haploinsuficiencia, y que tanto el tipo de mutación como su localización puedan jugar un papel importante en la iniciación del cáncer. Los inhibidores de PARP son actualmente uno de los agentes más prometedores para el tratamiento de tumores deficientes en BRCA1 o BRCA2. No obstante, enfermas de cáncer portadoras de una mutación germinal en los genes BRCA1/2 muestran una gran variabilidad en la respuesta a inhibidores de PARP en ensayos clínicos. Por estos motivos, nuestro interés ha radicado en investigar si el tipo de mutación en BRCA1 influye en la respuesta de las células a inhibidores de PARP y en caso afirmativo, averiguar cuál es el mecanismo responsable.

Utilizamos un panel de células linfoblastoides (LCLs) derivadas bien de portadoras heterocigotas de mutación en BRCA1 o bien de no portadoras de mutación con el objetivo de estudiar los efectos de haploinsuficiencia que tienen distintos tipos de mutaciones (de proteína truncada vs de cambio de aminoácido). Las LCLs portadoras de mutaciones de proteína truncada mostraron niveles significativamente menores de ARNm y proteína BRCA1 y niveles superiores de gamma-H2AX que las células control o que las LCLs portadoras de mutación de cambio de aminoácido, lo que indica un incremento espontáneo en el daño del ADN en estas células. Sin embargo, las células portadoras tanto de un tipo como de otro de mutación, mostraron una disminución en la formación de focos de RAD51, lo que sugiere una reparación defectuosa en las células mutadas y un posible mecanismo de aumento de susceptibilidad al cáncer en portadoras de mutación.

Además, los análisis de expresión génica revelaron que las LCLs portadoras de mutaciones de cambio de aminoácido mostraban perfiles de expresión más diferentes que las células con mutaciones de proteína truncada al compararlas con los controles, lo que sugiere que mutaciones diferentes podrían dar lugar a distintos fenotipos y efectos de haploinsuficiencia. De manera importante, la disminución en la expresión de genes asociados a la respuesta inmune observada en las células portadoras de mutaciones de cambio de aminoácido, indica un posible mecanismo de iniciación del cáncer de mama en portadoras de estas mutaciones concretas en BRCA1.

En el presente estudio, describimos por primera vez que las células derivadas de portadoras de mutaciones de cambio de aminoácido en el dominio BRCT de BRCA1 muestran mayor sensibilidad al inhibidor de PARP olaparib que las células con mutaciones de proteína truncada o WT. Estos resultados indican que portadoras de distintos tipos de mutación podrían beneficiarse del tratamiento de distinta manera y que podrían mostrar diferente toxicidad al agente. Teniendo en cuenta la presencia de proteína mutada no degradada en las células portadoras de mutación de cambio de aminoácido, cabría la posibilidad de que los mutantes de cambio de aminoácido tengan un efecto dominante-negativo sobre la función de la proteína WT. De esta manera, planteamos la hipótesis de que existe una competición entre la proteína mutada y la WT en el reclutamiento a los sitios de daño en el ADN, lo que podría dar lugar a una alteración en el proceso de reparación del ADN y una disminución en la viabilidad de las células portadoras de mutaciones de cambio de aminoácido en BRCA1. El mecanismo de incremento en la sensibilidad a olaparib de las células portadoras de mutaciones de cambio de
aminoácido podría estar asociado, al menos en parte, a posibles cambios en las proteínas específicas para olaparib que interaccionan con BRCA1 en las células mutadas. Además, la regulación del miR-222-3p y su diana FOS por el tratamiento de olaparib en células portadoras de mutaciones de cambio de aminoácido sugiere un posible uso de estas moléculas como marcadores de sensibilidad al tratamiento.

En resumen, nuestros resultados aportan nuevos conocimientos sobre cómo distintas mutaciones heterocigotas en BRCA1 podrían dar lugar a defectos en la función de BRCA1 y muestran gran evidencia de haploinsuficiencia en portadoras de mutaciones en BRCA1. Además, nuestros resultados indican que portadoras de distintos tipos de mutaciones en BRCA1 podrían beneficiarse del tratamiento de distinta forma y podrían mostrar toxicidades diferentes al inhibidor de PARP olaparib.
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<tr>
<td>ATM</td>
<td>ataxia-telangiectasia mutated</td>
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<tr>
<td>BARD1</td>
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<td>BIC</td>
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<td>GWAS</td>
<td>genome wide association study</td>
</tr>
<tr>
<td>HEK293FT</td>
<td>Human embryonic kidney (HEK) cell line 293FT</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HR</td>
<td>homologous recombination</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity Pathway Analysis software</td>
</tr>
<tr>
<td>LCL</td>
<td>lymphoblastoid cell line</td>
</tr>
<tr>
<td>logFC</td>
<td>logarithmic fold change</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Human breast carcinoma cell line, ATCC reference name</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA; micro ribonucleic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium</td>
</tr>
<tr>
<td>NHEJ</td>
<td>non-homologous end joining</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NMD</td>
<td>nonsense-mediated mRNA decay</td>
</tr>
<tr>
<td>NUMA1</td>
<td>Nuclear Mitotic Apparatus Protein 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>OCCR</td>
<td>ovarian cancer cluster region</td>
</tr>
<tr>
<td>OLP</td>
<td>olaparib; PARP inhibitor</td>
</tr>
<tr>
<td>PALB2</td>
<td>partner and localizer of BRCA2</td>
</tr>
<tr>
<td>PAR</td>
<td>poly ADP-ribose</td>
</tr>
<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PARP1</td>
<td>poly (ADP-ribose) polymerase 1</td>
</tr>
<tr>
<td>PARP-5a</td>
<td>tankyrase 1</td>
</tr>
<tr>
<td>PARPi</td>
<td>poly (ADP-ribose) polymerase inhibitor</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PTC</td>
<td>premature termination codon</td>
</tr>
<tr>
<td>RAD51</td>
<td>recombination protein A 51</td>
</tr>
<tr>
<td>RING</td>
<td>Really Interesting New Gene finger domain</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SAINT</td>
<td>Significance Analysis of INTeractome algorithm</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sh1</td>
<td>shRNA targeting BRCA1, clone 1</td>
</tr>
<tr>
<td>sh5</td>
<td>shRNA targeting BRCA1, clone 5</td>
</tr>
<tr>
<td>shRNA</td>
<td>short-hairpin RNA</td>
</tr>
<tr>
<td>SSB</td>
<td>DNA single-strand break</td>
</tr>
<tr>
<td>TAP-MS</td>
<td>tandem affinity purification coupled with mass spectrometry</td>
</tr>
<tr>
<td>TNBC</td>
<td>triple-negative breast cancer</td>
</tr>
<tr>
<td>VLP</td>
<td>veliparib; PARP inhibitor</td>
</tr>
<tr>
<td>VUS</td>
<td>variants of unknown significance</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
INTRODUCTION
1. Breast anatomy

Human breast is a bilateral organ which has a pivotal function in synthesis, secretion, and delivery of breast milk to an infant (Hassiotou and Geddes 2013). The anatomy of the female mammary gland changes dramatically throughout the woman’s life, depending on the physiological stage of breast development. A post-pubertal mammary gland consists of glandular (secretory) and adipose (fatty) tissue which is supported by fibrous connections called suspensory (Cooper’s) ligaments (Ali and Coombes 2002) (Figure 1A). The secretory tissue is organized into 15–20 lobes, connected with the nipple by a series of branched ducts which store and transport secreted milk during lactation.

There are two major types of epithelium in the ductal network: the secretory luminal epithelium, which form the inner single layer-part of the duct, and the basal component, which is represented by the contractile myoepithelial cells at the outer layer of a duct and is in a direct contact with the adjacent fibroblast stroma (Sternlicht 2006; LaMarca and Rosen 2008) (Figure 1B). All types of epithelial cells in the mammary gland originate from multipotent mammary stem cells and are maintained due to their proliferation (LaMarca and Rosen 2008).

Figure 1. A) Anatomy of the human mammary gland (adapted from Ali and Coombes, 2002). B) Schematic view of the morphology of a developing mammary duct (adapted from Sternlicht, 2006).
2. Breast cancer

2.1. Epidemiology

Breast cancer is a complex, heterogeneous disease which is both the most frequent and the most deadly cancer in women worldwide (Ferlay and Soerjomataram, 2013). There were about 1.67 million new breast cancer cases diagnosed in 2012 worldwide, which accounts for roughly one fourth of all cancers.

Figure 2. A) Estimated age-standardized breast cancer incidence worldwide per 100,000 in 2012 (Ferlay and Soerjomataram, 2013). B) Global age-standardized rate of breast cancer mortality per 100,000 population in 2012 (Ferlay and Soerjomataram, 2013).
There is a clear variability in the breast cancer incidence (Figure 2A) and mortality (Figure 2B) rates between less and more developed regions (Ferlay and Soerjomataram, 2013). The incidence rates differ almost 4-fold depending on the world region, ranging from 27 per 100,000 in Middle Africa and Eastern Asia to 96 per 100,000 in Western Europe. The rates of breast cancer mortality were significantly reduced over the last decade, mainly due to better availability and use of medical practices such as screening programs, availability and quality of treatment. Despite this substantial progress in more developed regions, poor countries have not followed the mortality decline. Because of the more favorable survival of breast cancer in developed (high incidence) countries the mortality rates are in general smaller across the world than those for incidence. In less developed countries, breast cancer is the most common cause of cancer death. On the other hand, most female cancer patients living in the more developed regions die from lung cancer, and breast cancer is considered as a second cause of cancer death in these countries.

2.2 Risk factors

Due to its increasing incidence, there has been an enormous effort to identify factors which can modulate the chance of breast cancer development throughout the women’s lifetime. One of the highest risk factor (after a gender, i.e. being a woman) for breast cancer is age, with the highest incidence rate found among older women (Cancer Research UK, 2014). Between years 2009 and 2011, 80% of all breast cancer cases in the UK were diagnosed in women older than 50 years old, and 24% of new breast cancer patients were 75 years old or older.

Having a personal history or a close relative affected with breast cancer also considerably increase the risk of being diagnosed with the same disease (Curtis et al., 2006; Cancer Research UK, 2014). Women (and men) with a first-degree relative (mother, sister, daughter, father, or brother) previously affected with breast cancer are at increased risk of developing breast cancer as well, and the risk is further elevated if more than one first-degree relative developed breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer 2001). When compared to women with no family history, the risk of breast cancer is 1.8 times higher for women with 1 first-degree female relative diagnosed with this disease, almost 3 times higher if the woman has 2 affected relatives, and nearly 4 times higher for women with 3 or more affected relatives.
Table 1. Risk factors for breast cancer in women. Relative risk is a comparison of the absolute risk of disease among people with a particular risk factor to the risk among people without that risk factor. The relative risk which is higher than 1.0 means that the risk is higher among people with the particular risk factor than among people without the factor. (Breast Cancer Fact & Figures 2013–2014)

<table>
<thead>
<tr>
<th>Relative risk</th>
<th>Factor</th>
</tr>
</thead>
</table>
| > 4.0        | Age (65+ vs. <65 years, although risk increases across all ages until age 80)  
Biopsy-confirmed atypical hyperplasia  
Certain inherited genetic mutations for breast cancer (BRCA1 and/or BRCA2)  
Lobular carcinoma in situ  
Mammographically dense breasts  
Personal history of early onset (<40 years) breast cancer  
Two or more first-degree relatives with breast cancer diagnosed at an early age |
| 2.1–4.0      | Personal history of breast cancer (40+ years)  
High endogenous estrogen or testosterone levels (postmenopausal)  
High-dose radiation to chest  
One first-degree relative with breast cancer |
| 1.1–2.0      | Alcohol consumption  
Ashkenazi (Eastern European) Jewish heritage  
Diethylstilbestrol (DES) exposure  
Early menarche (<12 years)  
Height (tall)  
High socioeconomic status  
Late age at first full-term pregnancy (>30 years)  
Late menopause (>55 years)  
Never breastfed a child  
No full-term pregnancies  
Obesity (postmenopausal)/adult weight gain  
Personal history of endometrium, ovary, or colon cancer  
Recent and long-term use of menopausal hormone therapy containing estrogen and progestin  
Recent oral contraceptive use |

The observed difference in incidence rates worldwide is believed to be at least partially caused by the variability in reproductive patterns and other hormonal factors (Cancer Research UK, 2014). The breast cancer risk has been reported to be elevated by younger age at menarche (the first menstrual period), late menopause, lower parity (having no or fewer children), and older age at first giving birth, which are factors related to exposure to sex hormones. On the other hand, higher parity and breastfeeding at least for 6 months are associated with reduced risk of breast cancer development. In addition, current or recent use of combined oestrogen-progestogen oral contraceptives, or using combined hormonal therapy for menopausal symptoms is also classified as a breast cancer risk factor. Furthermore, recent
studies suggested that there is a correlation between certain reproductive risk factors and development of particular breast cancer subtypes (Anderson, Schwab et al. 2014).

The fact that women who migrate from low to high risk countries show an increased incidence of breast cancer suggests the existence of other, non-reproduction-related, risk factors (Ziegler, Hoover et al. 1993). Higher breast density (higher percentage of non-fat tissue) was found to be independent on sex hormones and increases risk of breast cancer up to 5 times. Other lifestyle and environmental factors which are associated with increased risk of breast cancer development are exposure to ionizing radiation, high-fat diet, increased alcohol consumption, smoking, night-shifts etc. The summary of known risk factors for breast cancer with the values of relative risk when compared to general population is presented in Table 1 (Breast Cancer Fact & Figures 2013–2014).

2.3 Breast cancer classification

Breast cancer can be divided into ductal and lobular carcinoma which is either located in situ or spreads into an adjacent tissue and gives rise to an invasive disease (Vuong, Simpson et al. 2014). Such histological classification of breast cancer was established because it has been thought for a long time that distinct histological types originate from distinct microanatomical structures of normal mammary gland (ducts versus lobes). However, Wellings and colleagues demonstrated long time ago that most of the in situ and invasive breast cancers actually arise from the terminal duct/lobular unit, independently on the histological type (Wellings and Jensen 1973; Wellings, Jensen et al. 1975). Therefore, the current division into ductal or lobular carcinoma rather describes the cytological features and immunohistochemical profiles, than the site of origin of the breast tumor (Weigelt and Reis-Filho 2009).

The traditional classification of invasive breast cancers, which helps to guide the breast cancer patient management, is performed based on the histological type and grade, the presence of lymphovascular invasion and lymph-node metastasis, and the expression of certain predictive biomarkers (Correa Geyer and Reis-Filho 2009).
2.3.1 Histological types, grade and stage

According to the newest edition of the WHO Classification of Tumours of the Breast there are more than 21 subtypes of invasive breast carcinoma (Lakhani and Ellis, 2012), the “invasive ductal carcinoma of no special type” representing the majority of breast cancer cases (40–75%). The other subtypes are classified as morphologically distinct “special” types of breast cancer.

Histological grading is a powerful prognostic factor which evaluates the proportion of tubule formation, degree of nuclear pleomorphism, and the mitotic index. The combination of such features gives rise to grades 1–3: grade 1 being the most differentiated and having a very good clinical outcome, grade 3 representing the least differentiated tumors which tend to recur and metastasize early (Elston and Ellis 1991; Rakha, Reis-Filho et al. 2010a).

In addition to tumor grading, breast cancer is also staged by the conventional TNM system which uses both clinical and pathology information, including the primary tumor size (T), the status of regional lymph nodes (N), and spread to distant metastatic sites (M) (Edge and Compton 2010).

2.3.2 Traditional predictive biomarkers and current therapeutic strategies

The expression of hormone receptors, including oestrogen (ER) and progesterone (PR) receptors, and human epidermal growth factor receptor 2 (HER2) are currently routinely used in clinical practice worldwide (Vuong, Simpson et al. 2014).

The majority of breast cancers, up to 75% of all cases, are ER-positive (ER+), and these tumors are usually well differentiated and associated with better clinical outcome (Anderson, Chatterjee et al. 2002). PR-positive (PR+) tumors account for about 60% of breast cancer. The progesterone receptor gene is known to be regulated by oestrogen and its expression is therefore considered as an indicator of an intact ER pathway (Lanari, Lamb et al. 2009). The determination of the ER/PR status has the main clinical value particularly when estimating the likelihood of the response of patient to a traditional endocrine therapy (Rakha, Reis-Filho et al. 2010a). There are several strategies how to block the oestrogen action by the pharmacologic endocrine therapy, including direct inhibition of the receptor by selective ER modulators (e.g. tamoxifen), by using selective ER down-regulators (e.g. fulvestrant), or inhibition of oestrogen
production by treatment with aromatase inhibitor antagonists (Smith and Dowsett 2003; EBCTCG 2005).

Another commonly used indicator of poor prognosis is HER2, which is overexpressed in up to 20% of invasive breast cancers, and more than half of these tumors are hormone receptor negative (ER- and PR-) (Slamon, Clark et al. 1987; Dandachi, Dietze et al. 2002). \textit{ERBB2} gene (encoding the HER2 protein) amplification or HER2 protein overexpression is used in the clinics as a predictor of response to the humanized anti-HER2-specific monoclonal antibody, also known as trastuzumab or Herceptin (Vuong, Simpson et al. 2014). This treatment is used both in patients with HER2+ advanced disease and in the adjuvant setting for HER2+ early breast cancer. In addition, small-molecule drugs targeting the internal tyrosine kinase domain of HER2 (e.g. lapatinib) or vaccines are being developed as a new cancer therapies (Rakha, Reis-Filho et al. 2010b, 2010).

There is a distinct group of aggressive tumors representing the triple-negative breast cancer (TNBC), which displays characteristic morphology, presentation, behavior and outcome. These tumors account for about 15% of breast cancer cases and are characterized by the absence of expression of ER and PR and the lack of HER2 overexpression (Oakman, Viale et al. 2010). From the clinical point of view, this group of tumors is associated with poor prognosis and high risk of distant recurrence, and there is currently no available targeted therapy because, unlike the ER+/PR+/HER2+ tumors, these tumors do not respond to hormonal or HER2-targeted therapy (Dent, Trudeau et al. 2007; Brunello, Borgato et al. 2013). The current standard treatment option for patients with early-stage TNBC is a combination of anthracycline- and taxane-based chemotherapy regimen (Curigliano and Goldhirsch 2011).

\textbf{2.3.3 Molecular subtypes}

There has been an enormous effort in the past decade to unravel the molecular characteristics of breast cancer by applying various microarray-based high-throughput technologies in order to identify new prognostic and predictive subgroups and to individualize the therapy (van 't Veer, Dai et al. 2002; Potti, Dressman et al. 2006; Sotiriou, Wirapati et al. 2006).

The first important finding, while analyzing the diversity of primary invasive breast cancers, described by Perou et al. already in 2000, led to the development of a molecular classification of breast tumors into four distinct molecular subtypes (basal-like, \textit{ERBB2}-overexpressing,
normal breast-like, and luminal) and suggested that these subtypes might need to be treated as distinct diseases in the future (Perou, Sorlie et al. 2000). More detailed investigation by Sorlie and colleagues resulted in a further stratification of the luminal subtype into at least two separate groups, “luminal A” and “luminal B”, with distinct expression profiles and clinical outcomes for patients (Sorlie, Perou et al. 2001; Sorlie, Tibshirani et al. 2003).

The basal-like subtype is characterized by high expression of CK5 and CK17 (cytokeratine 5, cytokeratine 17), and other genes which are typical for basal/myoepithelial cells, including LAMC1 (laminin γ1), ANXA8 (annexin A8) and CDH3 (cadherin 3, type 1). In addition, basal-like tumors also often overexpress EGFR (epidermal growth factor receptor). Tumors of the ERBB2-overexpressing subtype often overexpress genes from the same chromosome region as ERBB2 (e.g. GRB7, MED24 and MED1) and show high expression of genes related to cell cycle progression. The Luminal A tumors are characterized by expression of luminal CK8 amd CK18 (cytokeratine 8, cytokeratine 18), they typically highly express transcription factors GATA3, FOXA1 and XBP1, and overexpress Cyclin D1. On the other hand, the Luminal B subtype is typical for tumors which are more proliferative and show different pattern of mutations (e.g. in TP53 and PIK3CA genes) than Luminal A tumors (Perou, Sorlie et al. 2000; Norum, Andersen et al. 2014).

Further characterization of the molecular subtypes revealed that they also display a characteristic pattern of ER, PR and HER2 expression. As shown in Table 2, the majority of luminal tumors show ER+ status, and the basal-like molecular subtype is, on the contrary, frequently associated with ER-, PR- and no overexpression of HER2 (TNBC phenotype) (Weigelt and Reis-Filho 2009). However, taking into account that only 77% of basal-like breast cancers are triple-negative and, vice versa, 71–91% of TNBC are of the basal-like phenotype, it is clear that TNBC and basal-like breast cancers overlap but they represent distinct subtypes (Oakman, Viale et al. 2010).

Both the triple-negative and basal-like breast cancer subtypes have been found to be frequently linked to the hereditary form of breast cancer caused by a germline mutation in the high-penetrance breast cancer susceptibility gene BRCA1, (Sorlie, Tibshirani et al. 2003; Penault-Llorca and Viale 2012). Interestingly, this gene has been found to be mutated also in some sporadic triple-negative breast tumors, which showed similar clinical outcomes and histological characteristics as tumors found in BRCA1 mutation carriers (Burgess and Puhalla 2014). Such shared phenotypes between sporadic and hereditary BRCA1-mutated tumors have led to a formation of a special term, “BRCAness”, which groups together sporadic cancers with
BRCA-like functional abnormalities, and which could help to identify tumors sensitive to treatments designed for familiar-BRCA tumors (Turner, Tutt et al. 2004).

Table 2. ER and HER2 status in molecular subtypes of breast cancer (Weigelt and Reis-Filho, 2009).

<table>
<thead>
<tr>
<th>Breast cancer molecular subtype</th>
<th>ER-positive median % (range)</th>
<th>HER2-positive median % (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal-like</td>
<td>16.6 (4.5–45.8)</td>
<td>13.6 (8.2–50)</td>
</tr>
<tr>
<td>HER2</td>
<td>55.7 (16.7–67.4)</td>
<td>72.7 (45.9–80)</td>
</tr>
<tr>
<td>Normal breast-like</td>
<td>83 (42.9–92.3)</td>
<td>10 (4.5–28.6)</td>
</tr>
<tr>
<td>Luminal</td>
<td>97.2 (85.4–100)</td>
<td>3.3 (1.5–27.3)</td>
</tr>
<tr>
<td>Luminal A</td>
<td>96.3 (81.8–98.8)</td>
<td>1.2 (1.2–1.2)</td>
</tr>
<tr>
<td>Luminal B</td>
<td>96.7 (87–100)</td>
<td>1.8 (1.8–1.8)</td>
</tr>
</tbody>
</table>

*ER expression evaluated by immunohistochemistry or enzyme immunoassay.
‡Only 3+ immunohistochemical expression or amplification as defined by chromogonic or fluorescence in situ hybridization was considered positive.
§Only studies where data on ER were defined by immunohistochemistry or enzyme immunoassay and HER2 by immunohistochemistry and/or in situ hybridization were included.

2.4 Hereditary breast cancer

The majority of breast cancer cases are considered as sporadic, they are characterized by a later age of onset and by lacking a particular pattern of inheritance. However, there is a substantial percentage (up to 15%) of new breast cancer patients that report a positive family history of breast cancer and are thus considered as having a “familial breast cancer” (Collaborative Group on Hormonal Factors in Breast Cancer 2001). Familial cancer is characterized by a higher number of cancer cases within a family than statistically expected, unknown inheritance model, and variable age of onset (Berliner and Fay 2007). But these features do not reliably identify women carrying a germline alteration responsible for the initiation of breast cancer since the familial clustering of breast cancer can be a consequence of several non-genetic factors: random clustering of sporadic cases in large families with many older women, similar environmental and/or lifestyle factors. In contrast to the familial breast cancer, hereditary breast cancer represents 5–10% of breast cancer cases, has a more clear pattern of inheritance, earlier age of onset (<50 years of age), and multiple primary and/or bilateral cancers.
2.4.1 Breast cancer susceptibility genes

Up to date several high-, moderate- and low-susceptibility genes, which significantly increase the risk of breast and ovarian cancer in the mutation carriers, have been identified (Figures 3A, 3B) (Foulkes 2008; Couch, Nathanson et al. 2014). As shown in the Figure 3A, mutations in the high-susceptibility genes (such as BRCA1 and BRCA2) are associated with high relative risk of breast and ovarian cancer but are very rare in population. On the contrary, low-penetrance alleles (such as variants identified in Genome Wide Association Studies (GWAS) are frequent in a population but they confer a low relative risk.

Figure 3. A) Graph of the relationship between relative risk and minor allele frequency of high-, moderate- and low-susceptibility alleles for breast cancer (adapted from Foulkes, 2008). B) High-, moderate- and low-penetrance genes predisposing mutation carriers to breast cancer (adapted from Couch et al., 2014).

2.4.1.1 High-penetrance breast cancer genes

The positional cloning experiments from early 1990s resulted in the identification of two highly penetrant breast and ovarian cancer susceptibility genes: BRCA1, located on chromosome 17, and BRCA2 on chromosome 13 (Miki, Swensen et al. 1994; Wooster,
Neuhausen et al. 1994). The estimated average cumulative risk of developing breast cancer by the age of 70 is in the range of 52–65% in BRCA1 mutation carriers and 45–49% in BRCA2 mutation carriers. Similarly, the corresponding estimates for ovarian cancer are 22–40% and 11–18%, for BRCA1 and BRCA2 mutation carriers respectively (Antoniou, Pharoah et al. 2003; Chen, Iversen et al. 2006; Chen and Parmigiani 2007; Milne, Osorio et al. 2008). However, it is important to note that the estimates of the average cumulative cancer risk in BRCA1/2-mutation carriers vary widely, depending on the target population and design of the study.

Although germline mutations in BRCA genes increase woman’s lifetime risk of developing breast and ovarian cancers, these mutations represent only about 15% of breast cancers with a hereditary component (Figure 3B) (Couch, Nathanson et al. 2014). Additional high-penetrance genes (TP53, PTEN, LKB1/STK11 and CDH1) have been described to be connected to various familial syndromes-associated increased incidence of breast cancer and are estimated to account for approximately 3% of hereditary breast cancers (Seal, Thompson et al. 2006; Rahman, Seal et al. 2007; Meindl, Hellebrand et al. 2010; Shamseldin, Elfaki et al. 2012).

2.4.1.2 Moderate- and low-susceptibility breast cancer genes

About 4% of patients with a strong family history of breast cancer could be explained by mutations in moderate-susceptibility genes (Figure 3B), which show odds ratio around 2-4 and incomplete segregation. A considerable proportion of these genes are members of the Fanconi anemia pathway (PALB2, BRIP1, RAD51C), or other DNA repair pathways (CHEK2, ATM, RAD51D, and BARD1) (Couch, Nathanson et al. 2014).

Furthermore, numerous common low-susceptibility genetic variants have been liked to breast cancer through genome-wide association (GWAS) studies (Michailidou, Hall et al. 2013). The odds ratios of these SNPs (single nucleotide polymorphisms) refer to a slight increase or reduction in risk and explain about 14% of hereditary breast cancer cases (Couch, Nathanson et al. 2014). In addition to the known variants, further 14% of SNPs, which have not been discovered yet, are predicted to contribute to hereditary breast cancer. Taking into account the small effect of these variants, the clinical utility of a single variant is limited, however, combined effects of particular variants could be useful for prevention, prediction of the cancer risk and response to treatment.

Despite the intensive efforts for identification other breast cancer susceptibility genes, our current knowledge about genes involved in predisposition of mutation carriers to breast cancer...
cancer explains only half of the cases. There are still about 50% of familial breast cancer patients which are negative for mutation in any of the previously mentioned genes. These cases are commonly called as BRCAX (or non-BRCA1/2), and their familial inheritance can be explained either by an unknown mutation in yet not discovered moderate-penetrance breast cancer gene, or by a polygenic inheritance model of several low-penetrance loci (Melchor and Benitez 2013; Couch, Nathanson et al. 2014). However, the current standard of testing for mutations in the high-susceptibility genes, in particular BRCA1 and BRCA2, is still the most powerful predictor of the likelihood of developing hereditary breast and ovarian cancers.

2.4.2 BRCA1 gene

BRCA1 gene was mapped to the human chromosome 17q21 in early 1990s based on its linkage to breast and/or ovarian cancer families (Miki, Swensen et al. 1994). The gene consists of 24 exons, covers about 110 kb of DNA and encodes for a large (1863 amino acid long) predominantly nuclear protein with a molecular mass of 220 kDa.

2.4.2.1 BRCA1 functions

There are several function-specific domains in BRCA1 protein (Figure 4A) (Roy, Chun et al. 2011). The N-terminus contains a RING domain, through which BRCA1 associates with BRCA1-associated RING domain protein 1 (BARD1). There are also two nuclear localization signal (NLS) sequences, from which only the first one is required for the transport of BRCA1 protein from cytosol into nucleus (Caestecker and Van de Walle 2013). The central region of BRCA1 is phosphorylated by CHK2 kinase at serine residue in position 988. In the C-terminus of BRCA1, there are a coiled-coil domain that binds to partner and localizer of BRCA2 (PALB2), a SQ/TQ cluster domain (SCD) with approximately 10 potential ataxia-telangiectasia mutated (ATM) phosphorylation sites, and two tandem BRCA1-C-terminal (BRCT) domains through which BRCA1 binds to ATM-phosphorylated Abraxas, CtBP-interacting protein (CtIP) and BRCA1-interacting protein C-terminal helicase 1 (BRIP1) (Roy, Chun et al. 2011).

As clear from the Figures 4A and 4B, numerous protein complexes interact with BRCA1, allowing the complexity of functions that BRCA1 is able to perform in cells. During the past two decades, BRCA1 has been found to play a critical role in various cellular processes, including DNA repair by distinct pathways, cell cycle checkpoints control, centrosome amplification,
transcriptional activation of target genes, and ubiquitin ligation (Narod and Foulkes 2004; Drost and Jonkers 2014).

Figure 4. A) BRCA1 protein domains and phosphorylation sites (based on Roy et al., 2012). B) BRCA1 functions and protein interactors (adapted from Narod and Foulkes, 2004).
2.4.2.2 *BRCA1* mutations

According to The Breast Cancer Information Core (BIC), there are more than 1800 unique sequence variants in *BRCA1*, including intronic changes, missense mutations, and small in-frame deletions and insertions (Couch, Nathanson et al. 2014); The Breast Cancer Information Core – BIC at [http://research.nhgri.nih.gov/bic/](http://research.nhgri.nih.gov/bic/).

The majority of *BRCA1* mutations are deletions, duplications, splice site, nonsense and frameshift mutations, and more than 80% of them lead to the occurrence of a premature termination codon (PTC) and truncation of the *BRCA1* coding sequence (The Breast Cancer Information Core – BIC). Most of these truncated transcripts are then subjected to degradation by a cellular surveillance mechanism called nonsense-mediated mRNA decay (NMD) pathway (Perrin-Vidoz, Sinilnikova et al. 2002).

On the other hand, missense mutations in *BRCA1* are in general much less common than other types of mutations. Pathogenic and highly penetrant missense mutations are mainly situated in the RING and BRCT domains, which play a critical role in the DNA repair activity of *BRCA1* (Roy, Chun et al. 2011); The Breast Cancer Information Core – BIC). However, there are lots of *BRCA1* variants which are spread along the coding sequence and are of unknown clinical significance (VUS). In the past years, there has been an appreciable effort of the ENIGMA consortia (Evidence-based Network for the Interpretation of Germline Mutant Alleles) to develop quantitative risk prediction methods which would help to decide whether a particular variant is pathogenic or of neutral/low effect.

Some *BRCA1* mutations are population-specific, and these founder mutations usually arise in small geographically or culturally isolated ethnic groups. The best example is a population of Ashkenazi Jewish, in which approximately 1.2% of individuals carry one of the two *BRCA1* founder mutations: c.68_69delAG (traditionally known as 185delAG), c.5266dupC (also known as 5382insC) (Roa, Boyd et al. 1996). Focusing on the Spanish population, the most common mutation is also 185delAG, and two of the three most recurrent *BRCA1* mutations are missense mutations (R71G, A1708E), which are rather rare in other populations (Milne, Osorio et al. 2008).
2.4.2.3 Heterogeneity among BRCA1-mutation carriers

BRCA1 is considered as a typical tumor suppressor gene due to its role in DNA repair and genomic maintenance and its inactivation therefore leads to an accumulation of genetic defects and increase of genomic instability. Even though carrying an inherited mutation in BRCA1 gene predisposes carriers to breast, ovarian and other cancers, there are considerable differences in the disease manifestation between mutation carriers.

Such interindividual variability could be explained by distinct environmental and genetic effects, as well as location and type of mutations in BRCA1 gene (Couch, Nathanson et al. 2014). The efforts of a Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA) have led to a discovery of numerous common genetic modifiers of breast cancer risk identified by GWAS studies. Since its establishment in 2006 and with samples from more than 40,000 BRCA1/2 mutation carriers, CIMBA provides an outstanding sample size for reliable evaluation of associations between single-nucleotide polymorphisms (SNPs) and cancer risk (Chenevix-Trench, Milne et al. 2007). Taking into account the identified modifiers of the risk, the estimated cumulative risk of developing breast cancer by the age of 80 is in the range of 81–100% (Couch, Wang et al. 2013).

Furthermore, early studies by Gayther and colleagues showed a significant correlation between the location of BRCA1 mutation and the ratio of breast/ovarian cancer incidence (Gayther, Warren et al. 1995). Nonsense and frameshift BRCA1 mutations which are located in the ovarian cancer cluster region (OCCR) were found to be significantly associated with increased risk of ovarian risk than mutations in other regions of BRCA1. In addition to the location of the BRCA1 mutation, the type of mutation seems to also play a relevant role in the biology of breast cancer. Waddell et al. have demonstrated that expression profiles of lymphoblastoid cell lines (LCLs) depend on the BRCA1 mutation type (Waddell, Ten Haaf et al. 2008). They observed differences in response to stress when comparing cells harboring missense and truncating mutations and WT controls, suggesting that the remaining WT allele in cells carrying heterozygous BRCA1 mutation might not be sufficient for normal cell function and that such haploinsufficiency thus results in an abnormal phenotype.

2.4.2.4 BRCA1 haploinsufficiency

BRCA1 behaves as a tumor suppressor, following the Knudson’s “two-hit” hypothesis, based on which, mutation in one allele of the gene is not sufficient to enable cancer
development and a second “hit” is necessary in order to impair its function (Knudson 1971). However, several studies indicate that mutation in one allele could already affect some of BRCA1 functions and that such haploinsufficiency might accelerate the loss of the second allele and lead to carcinogenesis.

Several gene expression studies published in last years support the idea about BRCA1 haploinsufficiency in mutation carriers. Comparison of BRCA1-mutation carriers-derived cells (LCLs, fibroblasts, primary breast and ovarian epithelial cells) with WT cells showed clear differences in their expression profiles when exposed to ionizing irradiation (IR) or other treatments (Kote-Jarai, Matthews et al. 2006; Bellacosa, Godwin et al. 2010; Walker, Thompson et al. 2010; Salmon, Salmon-Divon et al. 2013). Recently, using a big panel of untreated LCLs, Feilotter and colleagues for the first time distinguished BRCA1 mutation carriers from WT controls according to the gene expression patterns under basal conditions (Feilotter, Michel et al. 2014).

Importantly, BRCA1 seems not to be the only DNA repair gene showing signs of haploinsufficiency. Recent studies with lymphoblastoid cells lines (LCLs) showed that monoallelic mutations in PALB2 or MUTYH can lead to genomic instability and DNA damage response defects (Nikkila, Parplys et al. 2013; Grasso, Giacomini et al. 2014). These findings indicate the role of DNA-repair-gene haploinsufficiency in genomic instability and carcinogenesis, however, more studies will be necessary in order to fully understand the variability between mutation carriers and the initial steps of breast carcinogenesis.

3. PARP inhibitors

Poly (ADP-ribose) polymerases (PARPs) belong to a family of enzymes which catalyze a post-translational modification (poly ADP-ribosylation) of target proteins by the transfer of ADP-ribose (Ame, Spenlehauer et al. 2004). The family consists of at least 18 members which contain a conserved catalytic domain and are encoded by distinct genes. PARP enzymes have been found to play an important role in nucleic acid metabolism, modulation of chromatin structure, DNA synthesis, and DNA repair (Morales, Li et al. 2014). The first and most characterized member of the PARP family is PARP1, sharing a high similarity in its catalytic domain with PARP2. Within the past decade, inhibition of these enzymes became a hot topic when investigating new targeted therapies for hereditary breast and ovarian cancers and other malignancies.
3. 1 Synthetic lethality and other models of action of PARP inhibitors

Synthetic lethality is a concept when a combination of mutations in two genes results in cell death, but there is no negative effect on cells when only one of these two genes is compromised (Kaelin 2005). The occurrence of a synthetic lethal interaction between a tumor suppressor gene and other gene, e.g. PARP1, makes this other gene a potential therapeutic target (Figure 5).

As BRCA1 is an important player in the repair of DNA damage, BRCA1-deficient cells need to rely on other members of the DNA repair machinery. This fact makes them sensitive to the drugs which target those repair pathways, such as PARP inhibitors. PARP enzymes are involved in a DNA single-strand break (SSB) repair pathway (Helleday 2011). If inhibited, naturally occurring SSBs cannot be repaired, leading to a formation of double-strand breaks (DSBs) and replication fork collapse. Unrepaired DSBs in BRCA-deficient cells then result in cell death (Drost and Jonkers 2014). This is the basis of a synthetic lethal interaction between BRCA1/2 and PARP1 which was described for the first time by Farmer et al. and which has become a promising therapeutic strategy for breast, ovarian and other cancers (Bryant, Schultz et al. 2005; Farmer, McCabe et al. 2005).

Figure 5. Mechanism of synthetic lethality between BRCA deficiency and PARP inhibition (adapted from Banerjee et al., 2010).
Since 2005, modifications of the initial synthetic lethal principle as well as new mechanisms for the increased sensitivity of BRCA1/2-deficient cells to PARP inhibitors have been proposed. One of the alternative mechanisms is PARP trapping (Strom, Johansson et al. 2011; Murai, Huang et al. 2012). Ström and colleagues showed that treatment with PARP inhibitor traps its principal target PARP1 on the SSB, leading to a creation of PARP-SSB intermediate complex which could be more toxic than unrepaired SSB (Strom, Johansson et al. 2011). Another mechanism proposes that PARP inhibition could upregulate the error-prone NHEJ (non-homologous end joining) DNA repair pathway, which would cause genomic instability and potential lethality (Patel, Sarkaria et al. 2011). In addition, it has also been suggested that the role of PARP1 in reactivating DNA replication forks could be altered by PARP inhibitors and could lead to increased sensitivity in BRCA-mutation carriers (Hellday 2011). It is possible that a combination of previously mentioned mechanisms takes part in killing cancer cells in carriers of BRCA1/2 mutations and understanding of all consequences of PARP inhibition still remains an open question for the future.

3.2 PARP inhibitor trials in BRCA1/2-mutated breast cancers

Numerous PARP inhibitors are currently being tested in preclinical or clinical trials in patients with sporadic/hereditary breast, ovarian and other cancers. There are currently 4 distinct PARP inhibitors (including olaparib, veliparib, rucaparib and BMN 673) involved in numerous clinical trials in BRCA-associated breast cancer patients (Table 3) (Drost and Jonkers 2014). These PARP inhibitors are administered either as a monotherapy or in combination with chemotherapy, depending on a study design.

The encouraging results from phase II studies have allowed to proceed with more advanced, phase III, trials using either of the four PARP inhibitors in BRCA1/2-mutated breast and ovarian cancers (Burgess and Puhalla 2014; Liu, Konstantinopoulos et al. 2014). There are currently 7 phase III clinical trials (ClinicalTrials.gov identifiers: NCT01945775, NCT02163694, NCT01905592, NCT01847274, NCT01844986, NCT01874353, NCT01968213) recruiting carriers of BRCA mutation suffering from breast or ovarian cancer (www.ClinicalTrials.gov).
Table 3. Current clinical trials involving PARP inhibitors in BRCA-associated breast cancer (based on Drost and Jonkers, 2014). Search criteria in ClinicalTrials.gov: PARP inhibitors, breast cancer, BRCA.

<table>
<thead>
<tr>
<th>PARP inhibitor</th>
<th>Company</th>
<th>Types of cancer</th>
<th>Combination</th>
<th>Phase clinical trial</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olaparib (KU-0059436, AZD2811)</td>
<td>Astrazeneca</td>
<td>Breast, ovarian, breast, ovarian, prostate, pancreatic breast, ovarian, cervical, endometrial, peritoneal, fallopian tube</td>
<td>No</td>
<td>II</td>
<td>Completed[10]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No</td>
<td>II</td>
<td>Active, not recruiting[21]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carboplatin</td>
<td>I</td>
<td>Recruiting</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carboplatin</td>
<td>I</td>
<td>Recruiting</td>
</tr>
<tr>
<td>Veliparib (ABT-888)</td>
<td>Abbott</td>
<td>Solid breast, ovarian, peritoneal, fallopian tube</td>
<td>Temozolomide</td>
<td>I</td>
<td>Completed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Temozolomide</td>
<td>II</td>
<td>Active, not recruiting[72]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cyclophosphamide</td>
<td>II</td>
<td>Active, not recruiting[72]</td>
</tr>
<tr>
<td>Rucaparib (CO-338, AG-014699, PF-0136738)</td>
<td>Clovis Oncology</td>
<td>Solid breast, ovarian</td>
<td>No</td>
<td>II</td>
<td>Recruiting</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No</td>
<td>II</td>
<td>Recruiting</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cisplatin</td>
<td>II</td>
<td>Recruiting</td>
</tr>
<tr>
<td>BMN 673</td>
<td>BioMarin Pharmaceutical</td>
<td>Solid breast, ovarian</td>
<td>No</td>
<td>I</td>
<td>Recruiting</td>
</tr>
</tbody>
</table>

3.3 Sensitivity and resistance to PARP inhibitors

Although more than 60% of the patients with germline mutation in BRCA1 and BRCA2 genes showed clinical benefit after treatment with PARP inhibitor olaparib, a substantial fraction of patients was resistant to the agent (Fong, Boss et al. 2009).

Several mechanisms of acquired resistance to PARP inhibitors have been hypothesized and are expected to be multifactorial in etiology. One of the expected cause of resistance to PARP inhibition is the occurrence of secondary reversion mutations in BRCA1/2 genes (Barber, Sandhu et al. 2013). Another way of loosing sensitivity to the treatment with PARP inhibitors is by maintaining a certain basal activity of mutated BRCA1. Drost et al. showed that C61G mutation in RING domain does not disrupt all the BRCA1 functions and that this basal activity is sufficient to reduce sensitivity to PARP inhibitors (Drost, Bouwman et al. 2011). There are also BRCA-independent mechanisms of acquired resistance to PARP inhibitors. For example, resistance of a mouse model for BRCA1-associated breast cancer to olaparib has been described to be driven by upregulation of P-glycoprotein efflux pumps (Rottenberg, Jaspers et al. 2008). In addition, a loss of 53BP1 has been found to partially restore the homologous recombination (HR) pathway in BRCA1-deficient cells (Jaspers, Kersbergen et al. 2013). It has also been recently proposed that overexpression of PARP1 could be responsible for a fraction of PARP inhibitor-resistant cases (Gilabert, Launay et al. 2014).
Taking into consideration the phenotypic variability between *BRCA1* mutation carriers and the described changes in response to PARP inhibitors in carriers of various *BRCA1* mutations (as illustrated in Figure 6), it would be interesting to investigate the differences in therapy response among carriers of various *BRCA1* founder mutations. Clearly, further investigation is necessary for the best utilization of these potent compounds in the clinics.

Figure 6. Effect of *BRCA1* mutation status on the response to PARP inhibiting therapy (adapted from Caestecker et al., 2013). DSB HR = double strand break homologous recombination; PARPi = poly (ADP-ribose) polymerase inhibiting therapy.
OBJECTIVES
Considering the variability in disease manifestation among BRCA1 mutation carriers, we hypothesized that different types of mutations may give rise to various phenotypes. Therefore, the primary objective of this work was to investigate whether different germline BRCA1 mutations (missense versus truncating) lead to distinct haploinsufficiency effects.

Moreover, cancer patients carrying germline mutation in BRCA1 gene show a wide range of responses to PARP inhibitors. Thus, we were interested in investigating whether and how BRCA1 mutation type influences the response of cells to PARP inhibitors.

Taking into account the general objectives, the specific goals of this thesis were:

1. Investigate BRCA1 haploinsufficiency in carriers of missense or truncating mutations in BRCA1 gene by checking DNA repair capacity, gene and miRNA expression profiles.

2. Check the response of cells harboring missense or truncating BRCA1 mutations to various PARP inhibitors and investigate the factors which could potentially influence the response of cells to PARP inhibitors.

3. Using functional studies and gene and miRNA expression profiling, explore the mechanism of variability in response to PARP inhibitor olaparib depending on BRCA1 mutation status.
Cell lines and cell culture

1.1 Patient-derived cell lines

Lymphoblastoid cell lines (LCLs) were established by EBV-transformation of peripheral blood mononuclear cells (PBMC). Briefly, the PBMC were isolated from blood samples with Ficol (Sigma; #F2637), washed 1x with 1xPBS (Lonza; #BE17-516F), resuspended in freezing media (complete growth medium with 10% DMSO; see detailed information about growth conditions in the “Cell culture and treatments” section) and shipped frozen to our collaborators from the Cancer Epigenetics and Biology Program at the Bellvitge Biomedical Research Institute (IDIBELL) in Barcelona, where the cell immortalization was carried out. 5 million PBMCs were incubated (3 hours, 37ºC) with 1ml of supernatant of 0.45 μm filtered culture of the B95.8 strain EBV producer cell line. 3ml of a complete growth medium was added and cells were treated with Phytohemaglutinin-L (final concentration 10 μg/ml). Cells were checked daily and media was changed when necessary during the next 2–3 months, until the immortalization was achieved.

Blood samples from 15 women carrying heterozygous mutations in BRCA1 (BRCA1+/−) and 6 non-carrier relatives (BRCA1+/+) were collected to establish the final panel of LCLs. Mutational analysis had been previously performed by Sanger sequencing (primers available upon request). None of the individuals included in the study had personal antecedents of cancer. Detailed information about the individuals included in the study is shown in Table 4.

1.2 Other cell lines

Breast cancer cell line MDA-MB-231, derived from a sporadic breast cancer tumor, was obtained from the Cancer Epigenetics Group at the Bellvitge Institute for Biomedical Research (Barcelona, Spain). MDA-MB-231 was transduced by BRCA1-specific short-hairpin RNAs (shRNAs) or control shRNA (shScramble) and these newly generated cell lines were then used to confirm a synthetic lethal interaction between BRCA1 and PARP1.

HEK293FT cells were provided by the lab of Dr. Alvaro Monteiro during my 4-month short stay at the Moffitt Cancer Center (USA, FL, Tampa).
### Table 4. List of lymphoblastoid cell lines (LCLs)

<table>
<thead>
<tr>
<th>LCL ID</th>
<th>BRCA1 mutation</th>
<th>Mutation type</th>
<th>Exon</th>
<th>NMD</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>065179-L</td>
<td>WT</td>
<td>WT</td>
<td>-</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td>095797-L</td>
<td>WT</td>
<td>WT</td>
<td>-</td>
<td>-</td>
<td>27</td>
</tr>
<tr>
<td>105889-L</td>
<td>WT</td>
<td>WT</td>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>11566-L</td>
<td>WT</td>
<td>WT</td>
<td>-</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>115534-L</td>
<td>WT</td>
<td>WT</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>115954-L</td>
<td>WT</td>
<td>WT</td>
<td>-</td>
<td>-</td>
<td>35</td>
</tr>
<tr>
<td>0651159-L</td>
<td>c.5123C&gt;A; p.Ala1708Glu</td>
<td>missense</td>
<td>18</td>
<td>-</td>
<td>37</td>
</tr>
<tr>
<td>105890-L</td>
<td>c.5123C&gt;A; p.Ala1708Glu</td>
<td>missense</td>
<td>18</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>1051202-L</td>
<td>c.5123C&gt;A; p.Ala1708Glu</td>
<td>missense</td>
<td>18</td>
<td>-</td>
<td>53</td>
</tr>
<tr>
<td>11565-L</td>
<td>c.5117G&gt;A; p.Gly1706Glu</td>
<td>missense</td>
<td>18</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td>11567-L</td>
<td>c.5117G&gt;A; p.Gly1706Glu</td>
<td>missense</td>
<td>18</td>
<td>-</td>
<td>34</td>
</tr>
<tr>
<td>0751291-L</td>
<td>c.3239T&gt;A; p.Leu1080X</td>
<td>nonsense</td>
<td>11</td>
<td>+</td>
<td>34</td>
</tr>
<tr>
<td>0651167-L</td>
<td>c.3331_3334delCAAG; p.Gln1111fs</td>
<td>frameshift</td>
<td>11</td>
<td>+</td>
<td>33</td>
</tr>
<tr>
<td>095491-L</td>
<td>c.815_824dup10; p.Thr276fs</td>
<td>frameshift</td>
<td>11</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>095546-L</td>
<td>c.212+1G&gt;A; p.?</td>
<td>splice</td>
<td>5</td>
<td>Unknown</td>
<td>42</td>
</tr>
<tr>
<td>095798-L</td>
<td>c.2410C&gt;T; p.Gln804X</td>
<td>nonsense</td>
<td>11</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>10544-L</td>
<td>c.4309delT; p.Ser1437fs</td>
<td>frameshift</td>
<td>13</td>
<td>+</td>
<td>22</td>
</tr>
<tr>
<td>1051177-L</td>
<td>c.68_69delAG; p.Glu23fs</td>
<td>frameshift</td>
<td>2</td>
<td>re-initiation*</td>
<td>27</td>
</tr>
<tr>
<td>115376-L</td>
<td>c.212+1G&gt;A; p.?</td>
<td>splice</td>
<td>5</td>
<td>unknown</td>
<td>39</td>
</tr>
<tr>
<td>115384-L</td>
<td>c.212+1G&gt;A; p.?</td>
<td>splice</td>
<td>5</td>
<td>unknown</td>
<td>75</td>
</tr>
<tr>
<td>1151004-L</td>
<td>c.981_982delAT; p.Cys328X</td>
<td>frameshift</td>
<td>11</td>
<td>+</td>
<td>25</td>
</tr>
</tbody>
</table>

1-6 indicate which LCLs were established from relatives (sisters or mother/daughter)

2 Mutation nomenclature listed uses GenBank reference sequences NM_007294.3 with numbering starting at the A of the first ATG, following the journal guidelines ([www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen)); p.?, unknown protein nomenclature (variant causing skipping of exon 5 of BRCA1)

3 NMD not activated by the variant (-), degradation of mutated transcript by NMD (+), not known functional interpretation (unknown), variant leading to translation re-initiation (re-initiation)

4 NMD status determined by prediction according to the BIC database (BRCA1 master list from 19 July 2013) or experimentally investigated (Buisson, Anczukow et al. 2006) (*)

5 Age of women at the time of blood extraction to establish LCL

### 1.3 Lentiviral constructs

BRCA1 gene down-regulation was performed in MDA-MB-231 cell line using shRNAmir target gene set obtained from Open Biosystems (13 human GIPZ lentiviral shRNAmir individual clones; #RHS4531). The design of shRNAmir is based on the primary microRNA-30 transcript and allows processing via the endogenous RNAi pathway resulting in more specific and guaranteed gene silencing.
Six different shRNA constructs were transduced (details in Supplementary Table S1), and two of them, which provided the best knockdown efficiency, were selected to silence BRCA1 in the breast cancer cell line. A non-targeting shRNA vector (pGIPZ-shRNAmir-Scramble) was used as a negative control.

### 1.4 Generation of stable cell lines

Schematic overview of the generation of stable cell line is shown in Figure 7. Each human GIPZ lentiviral shRNAmir vector was co-transfected with lentiviral packaging plasmids (helper plasmids VSVG and psPAX2; kindly provided by Dr. F. Real from CNIO) into 293FT cells (Invitrogen) using transfection reagent Fugene 6 (Promega; #E2691) following the manufacturer’s instructions. The virus-containing supernatants were collected 36 hours post-transfection and filtered. MDA-MB-231 cells were then infected with lentiviral supernatant supplemented with 1μg/ml polybrene (Santa Cruz; #sc-134220). The stably-transduced cells were selected by adding medium containing 1 mg/ml puromycin (Sigma; #P9620). Downregulation of BRCA1 expression was confirmed by Western blotting and quantitative RT-PCR.

![Figure 7](image)

**Figure 7.** Schematic overview of shRNA-mediated BRCA1 knockdown in MDA-MB-231 breast cancer cell line.
1.5 Cell culture and treatments

LCLs were cultured in RPMI-1640 media (Sigma-Aldrich; #R8758) supplemented with non-heat inactivated 20% fetal bovine serum (Sigma-Aldrich; #F7524), 1% penicillin-streptomycin (Gibco; #15070-063) and 0.5% Fungizone (Gibco; #15290-018). Cell culture was carried out in 25 cm$^2$ flasks (Corning; #3056) and LCLs were maintained in exponential growth by daily dilution to 1 x 10$^6$ cells per ml of full media. Early passages (passage 1–10) of LCLs were used for all experiments. MDA-MB-231 and HEK293FT cells were grown in RPMI-1640 or DMEM media, respectively, and both media were complemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.5% Fungizone. All cells were maintained in an atmosphere of 5% CO$_2$ at 37°C.

Where noted, DNA damage was induced by exposure to 10 Gy ionizing radiation (MDS Nordion Gammacell 1000). Following treatment, cells were allowed to recover for 4 hours under normal growth conditions prior to fixation for immunofluorescence experiments. In experiments with PARP inhibitors, LCLs were incubated with either olaparib = OLP (Axon Medchem; #1464) or veliparib = VLP (Axon Medchem; #1593) for indicated period of time at 37°C in 5% CO$_2$ atmosphere and treatment with DMSO (Sigma; #D8418) dissolvent was used as a control.

2 RNA expression analysis

2.1 RNA extraction and quantification

RNA from an early passage of LCLs or MDA-MB-231 cell line (parental cell line or stably expressing shRNA) was extracted using TRIzol Reagent (Invitrogen; #15596-026) according to the manufacturer’s instructions. In the case of LCLs, ten million cells from each cell line, either non-treated or treated with DMSO or olaparib, were used for RNA isolation. The equal amount of cells utilized for RNA extraction was used in order to prevent a selective loss of certain miRNAs from total RNA (Kim, Yeo et al. 2012). RNA quality and quantity was determined using LabChip technology on an Agilent 2100 BioAnalyser (Agilent Technologies). All samples had the value of RNA integrity number (RIN) in the range 7.5 to 10.0.
2.2 Real-time PCR analysis

For detection of BRCA1 gene expression levels, 500 ng of total RNA was reverse-transcribed by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; #4368814) according to the manufacturer’s instructions. Using labeled probes (Roche Universal ProbeLibrary; #04683633001) and TaqMan Universal PCR Mix (Applied Biosystems; #4304437) the cDNAs were analyzed by quantitative RT-PCR assay in an ABI Prism Sequence Detection System 7900HT (Applied Biosystems). Following oligonucleotides were used in the quantitative RT-PCR: F-primer 5’-TTAAAGAAAGAAAAATGCTGA-3’ and R-primer 5’-GTTGGTTTCTTCCATTGACC-3’ for detection of BRCA1 expression; F-primer 5’-CTGGAGGACGACAAGGAAA-3’ and R-primer 5’-TGTGTGCTACCGATACCGTA-3’ to detect PARP1 gene expression. Expression levels of HPRT1 and MRLP19 were used as internal controls and enabled normalization of samples. Three independent experiments were performed and the relative expression was calculated using a comparative Ct method.

3 Protein-based assays

3.1 Protein extract preparation

In order to detect levels of certain proteins in cells by Western blotting, whole cell lysates or proteins from a nuclear fraction were prepared. Exponentially growing cells were harvested by centrifugation and washed 2x with ice-cold PBS (Lonza; #BE17-516F).

For detection of BRCA1, a protein subcellular fractionation was performed. Briefly, washed cellular pellet was resuspended in RSB buffer (Tris 10 mM pH 7.5, NaCl 10 mM, MgCl₂ 3 mM, protease inhibitor) and incubated 20 min on ice. Nuclei were sedimented by centrifugation and the supernatant (cytoplasmic fraction) was transferred into a new tube and stored at -20°C. In order to remove any contaminating cytoplasm the pellet (nuclei) was washed 1x with full RSB buffer and 2x with RSB buffer which did not contain protease inhibitors. Nuclear pellet was then resuspended in NB buffer (Tris 10 mM pH 7.5, NaCl 0.4 mM, EDTA 1 mM, protease inhibitor) by pipetting several times and incubated 15 min at 4°C while shaking. Samples were centrifuged and the concentration of the nuclear fraction of proteins (supernatant) was determined using DC Protein Assay Kit II (Bio-Rad; #500-0112) with BSA as a standard.
For PARP1 and PAR detection, whole cell lysates were prepared. Shortly, cellular pellets were washed twice with ice cold PBS and lysed in RIPA buffer (Sigma-Aldrich; #R0278) containing protease inhibitor (Roche; #11697498001) for 1 hour on ice. Lysates were centrifuged at 16,000 g for 20 min at 4°C and supernatants (containing proteins from whole cell lysate) were transferred into new tubes for further quantification of proteins with Bio-Rad protein assay kit as in the case of nuclear proteins.

3.2 SDS-PAGE and Western blotting

For the detection of BRCA1 protein, 75 or 100 ug of nuclear proteins from LCLs or MDA-MB-231 cells, respectively, was loaded per well of NuPAGE 3–8% Tris-Acetate gel (Invitrogen). To detect other proteins, 75 ug of whole cell lysate per well was loaded into NuPAGE 4–12% Bis-Tris gel (Invitrogen). Separated proteins were transferred to a nitrocellulose membrane (Whatman) using the NuPage transfer system (Invitrogen) and the membrane was then blocked in Tris buffered saline containing 0.05% Tween-20 (TBS-T) and 5% milk. Blots were probed with following primary antibodies: mouse anti-BRCA1 (Calbiochem; #OP92), rabbit anti-PARP1 (Cell Signaling; #9542), rabbit anti-PAR (Calbiochem; #528815), rabbit anti-RAD51 (Santa Cruz; #sc-8349), rabbit anti-GAL4-DBD (Santa Cruz; #sc-577). Mouse anti-β-actin (Sigma; #A5441) was used as a loading control for whole cell lysates, and mouse anti-HSC70/HSP70 antibody (Enzo; #ADI-SPA-820-D) or rabbit anti-nucleolin (Abcam; #ab22758) served as loading controls for nuclear proteins. The secondary antibodies were HRP-conjugated (Dako) and the immunoblots were developed using the ECL system (GE Healthcare). Western blot X-ray films (Kodak) were scanned and analyzed using ImageJ software.

3.3 Mass spectrometry (performed during my short stay at the MOFFITT Cancer Center)

DMSO- or OLP (16μM)-treated HEK293FT cells overexpressing pNTAP-BRCA1-BRCT construct (previously described by Woods, Mesquita et al. 2012) were collected, lysed and proteins were extracted. A previously described protocol was used (Woods, Mesquita et al. 2012) to perform the mass spectrometry experiment. Briefly, protein fractions created by SDS-PAGE were excised, destained, reduced, alkylated and digested with trypsin (Promega). Peptides were eluted from the gel and concentrated using vacuum centrifugation. A nanoflow liquid chromatograph (U3000, Dionex, Sunnyvale, CA) coupled to an electrospray ion trap mass
spectrometer (LTQ or LTQ-Orbitrap, Thermo, San Jose, CA) was used for tandem mass spectrometry peptide sequencing. Raw files were searched against SwissProt human database (release year 2014) using a search engine Mascot (Matrix Science) (Perkins, Pappin et al. 1999). Assignments were manually verified by inspection of the tandem mass spectra and coalesced into Scaffold reports (v.2.0, available at www.proteomesoftware.com) for statistical analysis and data presentation. The Scaffold software was used to validate MS-MS based peptide and protein identifications. After applying the SAINT (Significance Analysis of INTeractome) algorithm and setting a probability threshold of 0.8–1, a final list of 121 protein interactors was selected.

4 Functional and cell-based assays

4.1 DNA repair evaluation by confocal microscopy

Where noted, DNA damage was induced by exposure to ionizing radiation (MDS Nordion Gammarcell 1000). Control and 10-Gy irradiated LCLs were cultured 4 hours before fixation with 4% paraformaldehyde (Aname; #15710). Two hours before fixation, cells were counted and seeded into poly-L-lysine-coated (Sigma-Aldrich; #P4832) µCLEAR bottom 96-well plate (Greiner Bio-One; #655087) at a density 75000 cells/100ul full media/well. LCLs were then allowed to attach to the surface of the wells for additional 2 hours, fixed for 15 min at room temperature (RT), permeabilized in 0.5% Triton X-100 in PBS for 20 min at 4°C. Blocking step with heat-inactivated 20% human serum for 15 min in RT was followed by staining with primary and secondary antibodies and 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI; #D9542) to visualize nuclei. To detect gamma-H2AX we used mouse monoclonal anti-phospho-histone H2AX antibody (Millipore; #05-636), for detection of RAD51 a rabbit polyclonal anti-RAD51 antibody was utilized (Santa Cruz Biotechnology; #sc-8349). Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies from Molecular Probes (Invitrogen; #A-11034 and #A-11031, respectively) were used, and fluorescent images were automatically acquired from each well of the 96-well plate using an Opera High-Content Screening System (Perkin Elmer). Pictures were taken at no saturating conditions using a 40x magnification lens and the intensity of gamma-H2AX nuclear signal and the number of RAD51 foci in nuclei were calculated.
4.2 MTT colorimetric assay

The effect of PARP inhibition on cell viability was assessed using the MTT colorimetric assay. Briefly, 200 ul of cellular suspension in full media was plated to each well of 96-well plate at the density of 10^4 cells/well. Cells were then treated with DMSO (control) or PARP inhibitors olaparib (concentrations 2, 4, 8 μM) or veliparib (10, 25, 50, 100 μM) for 72 hours under standard growth conditions. As LCLs tend to form large aggregates of cells, cells were resuspended by pipetting up and down once a day using multichannel pipette in order to ensure equal distribution of the treatment to all cells. Adherent breast cancer cell line MDA-MB-231 did not have to be resuspended by pipetting. 3 days after the beginning of treatment, 50 ul of MTT dissolved in PBS (stock 5mg/ml MTT) was added onto plate and incubated under normal growth conditions for 4 hours. Afterwards, 50 ul of SDS/HCl solution (500ml SDS 10% + 150µL HCl 37%) was transferred into each well and plates were kept in a cell culture incubator for additional 16 hours. Absorbance at 544 nm was read on a spectrophotometer (Victor 3 Plate Reader; PerkinElmer), the data were then normalized to a mean absorbance detected in wells containing media without cells, and the results were expressed as a percentage (%) of the control (DMSO-treated cells).

4.3 Colony formation assay

MDA-MB-231 cells expressing either shScramble or shBRCA1-1 or shBRCA1-5 clone were treated with DMSO or with indicated concentration of PARP inhibitor OLP or VLP for 24 hours. Afterwards, the cells were seeded at a density of 300 cells/well in 6-well plates and incubated under normal growth conditions for 7 days. Medium was removed and cells were washed twice with PBS, fixed with ice-cold methanol (Sigma; #32213) for 10 min, and stained with 0.5% crystal violet solution (made in 25% methanol) for 10–20 min. Crystal violet (Sigma; #C3886) was pour out, plates were rinsed several times with distilled water and allowed to dry over night at room temperature. Number of colonies containing more than 50 cells was counted.

4.4 Basal PARP enzymatic activity

The PARP enzymatic activity was determined using the HT Chemiluminescent PARP/Apoptosis Assay kit (Trevigen; #4685-096-K) according to the manufacturer's
instructions with several modifications. Eight million exponentially growing cells were washed once with ice-cold PBS and lyzed in 100 ul of Trevigen Cell Extraction Buffer (1x l-PAR Assay buffer; 0.4 mol/L NaCl, 0.9 % Triton X-100, Complete protease inhibitor cocktail [Roche; #11697498001]) on ice for 30 min and periodically vortexed. After centrifugation of the cellular lysates at 14,000 rpm at 4°C for 10 min, protein concentration was quantified using DC Protein Assay Kit II (Bio-Rad; #500-0112) using BSA as a standard. A basal PARP enzymatic activity was determined using the HT Chemiluminescent PARP/Apoptosis Assay kit according to the manufacturer’s instructions with several modifications. The cellular lysate (10 μg/well) was added in duplicates to the wells of 96-well plate, which contained PARP buffer and PARP cocktail, and incubated at room temperature for 1 hour. Activated DNA was added only to the standards but not to extracts, which allowed to measure a basal enzymatic activity. The wells were washed three times with PBS and 0.1% Triton X-100 (PBS-T) and three times with PBS and then incubated with streptavidin horseradish peroxidase, diluted in strep diluent buffer 1:1000, for 1 hour. The wells were then washed as previously described (3x with PBS-T + 3x with PBS). Chemiluminescent detection was performed according to the manufacturer’s instructions (EnVision Multilabel Plate Reader; PerkinElmer). The background luminescence was subtracted from the readings of the samples and standards served for calculation of PARP activity from a standard curve.

4.5 Cellular growth rate calculation

Differences in the cellular growth rate were determined using the above mentioned MTT colorimetric assay with some modifications. From each LCL 10⁴ cells/well/200ul full media were plated in hexaplicates into two separate 96-well plates. The first plate was subjected to an MTT addition at time 0 hours whereas the MTT solution was added into the second plate 72 hours later, i.e. the same duration as for determination of sensitivity to PARP inhibitors was used to determine growth rate of LCLs. Plates were incubated with the MTT for 4 hours which was followed by incubation with SDS/HCl solution for additional 16 hours. Absorbance at 544 nm was measured on a spectrophotometer and the data were normalized to a mean absorbance detected in wells containing media without cells. Growth rate was calculated as a ratio between the mean absorbance from 72 hour timepoint and the mean absorbance at timepoint 0 hours.
4.6 **BRCA1 transcriptional activation assay** (performed during my short stay at the MOFFITT Cancer Center)

The ability of BRCA1 BRCT domain (WT or mutated) in the presence or absence of PARP inhibitor OLP was determined using a highly reproducible assay, transcription activation assay. Construct containing WT BRCA1 (amino acids 1396–1863) was used as a positive control and deleterious variant M1775R as a negative control (as previously described by Carvalho, Pino et al. 2009). HEK293FT cells were seeded in 24-well tissue culture plates in a concentration $5 \times 10^4$ cells/well. 24 hours later, cells were transfected with vectors containing studied BRCA1 variants in triplicates with Fugene 6 (Promega; #E2691) according to the manufacturer's instructions. To mimick the heterozygous status, co-transfection of WT BRCA1 and pFLAG-CMV3 vector containing the BRCA1 A1708E variant (exons 16–24) and lacking the GAL4 DNA binding domain was performed. The vectors containing the variants were co-transfected with pG5Luc, which contains a firefly luciferase reporter gene driven by GAL4-responsive binding sites, and phGR-TK, which contains a *Renilla* luciferase gene driven by a constitutive TK basal promoter used as an internal control. 24 hours post-transfection, growth medium was replaced by a medium containing either DMSO (control) or PARP inhibitor OLP of a final concentration 16μM. Cells were harvested 24 hours after the treatment, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega; #E1910) following the manufacturer's instructions. Activity was plotted as a percentage of the WT BRCA1 activity of DMSO-treated cells.

5 **Gene and miRNA expression profiling**

5.1 **mRNA expression profiling**

Whole-genome transcriptional profiling was performed using Agilent Human Gene Expression G3 v2 8x60K Microarrays (Agilent microarray design ID 039494, P/N G4851B). Briefly, LCLs were either non-treated or treated with DMSO (control for olaparib-treated cells) or PARP inhibitor olaparib (8 μM) for 72 hours and total RNA was then extracted. LCLs 100 ng of total RNA was labeled using the Low Input Quick Amp Labeling Kit (Agilent Technologies) following manufacturer's instructions and the One-Color Microarray-Based Gene Expression Analysis approach. Labeled samples were purified with silica-based RNeasy spin columns (Qiagen) and 600 ng of labeled extract of total volume 50 μl was hybridized at 65°C for 17
hours. Images were scanned on a G2505C DNA microarray scanner (Agilent) and data were extracted with Feature Extraction software (Agilent; version 10.7). Background correction (performed via the normexp method) and between arrays normalization (utilizing the quantiles method) was performed using Babelomics 4.3 web tool, which is available at http://babelomics.bioinfo.cipf.es. Microarray dataset from non-treated samples have been deposited in the Gene Expression Omnibus (GEO) public repository with GSE60396 GEO accession number.

5.2 miRNA expression profiling

miRNA expression profiling was performed using the 7th generation miRCURY LNA™ microRNA Array kit (Exiqon). Briefly, 300 ng of total RNA, isolated from from non-treated or DMSO/8 µM olaparib-treated (for 72 hours) LCLs, was first incubated with CIP and subsequently labeled with Hy3 fluorescent dye using the miRCURY LNA™ microRNA Hi-Power Labeling Kit (Exiqon). Labeled RNA was then hybridized onto miRNA microarray slides for 16 hours at 56°C using Agilent SureHyb hybridization chambers and a hybridization oven with rotation. After washing and drying steps, the arrays were scanned and the images were analyzed using Feature Extraction software (Agilent; version 10.7), using a modified Exiqon protocol. The microarray background correction was performed using normexp method and normalization between arrays was carried out via quantiles method in R statistical software (version 2.13.0). Microarray dataset from non-treated samples is publicly available at the GEO public repository (GEO accession number GSE60444).

6 Bioinformatic and statistical analysis

6.1 Microarray data analysis

Replicate probes from the normalized data were merged by their mean profile and mRNAs and miRNAs with low expression variation (VAR<1xE-9 for mRNA profiling; VAR<0.002 for miRNA data) across samples were excluded. These data pre-processing steps were performed using the preP web server application available in the Asterias web server (http://asterias.bioinfo.cnio.es/). To identify differentially expressed genes and miRNAs between control and mutated LCLs we used POMELO II web tool, which is accessible in the
Asterias web server. The estimated significance level (unadjusted P value) was corrected for multiple hypotheses testing using Benjamini and Hochberg false discovery rate (FDR) adjustment. mRNAs with FDR<0.15 were considered as significantly differentially expressed, whereas FDR<0.05 was used as a threshold to select differentially expressed miRNAs. For the unsupervised hierarchical clustering analysis, GENE-E software version 3.0.204 was used (Broad Institute; http://www.broadinstitute.org/cancer/software/GENE-E/index.html) performing one minus Pearson correlation with Average-Linkage clustering method.

6.2 Pathway analysis using IPA

To annotate DEG and BRCA1 BRCT protein interactors into biological networks and for evaluation of their functional significance the Ingenuity Pathway Analysis software package was used (IPA; http://www.ingenuity.com). IPA uses the Ingenuity Pathway Knowledge Base, which is derived from known functions and interactions of genes published in literature, allowing the identification of biological networks, global functions and functional pathways of a particular set of data.

To determine the top canonical pathways, biological functions, and molecule networks associated with analyzed datasets (DEG derived from microarray analysis, protein interactors obtained using the mass spectrometry approach), a core analysis was performed from which the most dramatically affected functions/pathways were extracted (p-value < 0.05). The p-value, calculated with the right-tailed Fisher’s exact test, estimates the probability that the association between the dataset and canonical pathways/biological functions/molecule networks might be due to a random chance.

6.3 Integration of miRNA and mRNA gene expression signatures

As single miRNA can target many mRNAs and, on the contrary, a single mRNA can be targeted by multiple miRNAs, we used IPA to identify only those miRNA-mRNA pairs which has been previously experimentally validated (using TarBase, miRecords and Ingenuity Expert Findings databases). The miRNA and mRNA microarray expression profiles from DMSO/OLP-treated LCLs were used, in particular the 215 miRNAs, which passed the normalization and pre-processing step, and the 312 genes, which were differentially expresses between DMSO- and OLP-treated WT cells. IPA microRNA Target Filter was then utilized to select only miRNA-mRNA
pairs showing anti-correlated expression pattern and to prioritize experimentally validated mRNA targets.

6.4 Statistical analysis

To test the statistical difference between groups of LCLs (when analyzing quantitative RT-PCR, Western blotting, immunofluorescence experiment, PARP enzymatic activity assay, cellular growth rate), independent samples Student’s t-test was used for normally distributed variables and Mann-Whitney U test for non-normal data distribution. For analysis of the differences in sensitivity of LCLs to OLP and VLP, the cellular growth rate was used as a covariate in a linear regression model. To check association between two variables, Pearson or Spearman correlation coefficients were calculated for normally or not normally distributed data, respectively. P-value lower than 0.05 was considered as statistically significant. Calculations were done using SPSS Statistics software (version 17.0; IBM) and dot plots were created with GraphPad Prism (version 5.03; GraphPad Software; www.graphpad.com).
Taking into account the variability in breast and ovarian cancer incidence and age of onset in carriers of BRCA1 mutations, the first objective of this thesis was therefore to explore BRCA1 haploinsufficiency in cell lines harboring either missense or truncating mutation in this gene by checking BRCA1 expression, DNA repair capacity, and comparing gene or miRNA expression profiles.

1.1 BRCA1 expression is reduced in carriers of heterozygous truncating mutation in BRCA1

To determine whether there were any differences in the level of BRCA1 expression among mutation carriers, we compared both BRCA1 mRNA and protein levels in BRCA1 +/- and BRCA1 +/+ cells. For this purpose, we established a panel of lymphoblastoid cell lines (LCLs) from 15 healthy women carrying heterozygous missense or truncating mutation in BRCA1 and 6 healthy ancestry-matched controls negative for BRCA1 mutation (Table 4).

The BRCA1 mRNA level was determined by real-time RT-PCR using BRCA1-specific primers that did not distinguish between wildtype and mutant allele, and the expression curves were normalized to the expression of housekeeping genes MRLP19 and HPRT1. The average BRCA1 expression in cells harboring heterozygous mutation was 88.6% of that of control cells derived from non-carriers (Figure 8A; P = 0.208). However, when we stratified the mutations according to their type (missense versus truncating) we observed a significantly lower BRCA1 expression in cells with truncating mutation in comparison to WT cells (Figure 8B; P = 0.028). These results are in agreement with the commonly accepted fact that the presence of a premature stop codon in mRNA often leads to an activation of a nonsense-mediated mRNA decay (NMD) pathway (Conti and Izaurralde 2005). On the other hand, cells harboring missense mutation showed comparable level of expression of BRCA1 as control cells (Figure 8B; P = 0.593) and significantly higher BRCA1 expression than cells with truncating mutation (Figure 8B; P = 0.010), suggesting that the mutated transcripts do not undergo degradation by the cellular RNA decay machinery.
Figure 8. LCLs carrying a monoallelic truncating mutation in *BRCA1* show decreased *BRCA1* mRNA and protein expression. A) Relative *BRCA1* expression in 6 WT LCLs and 15 LCLs carrying heterozygous mutation. Mutation carriers show 88.6% of the normal *BRCA1* expression level in WTs (two-tailed Student’s t-test; *P* = 0.208 with a mean of 1.202 ± 0.08 SEM for WT cells and 1.065 ± 0.06 SEM for mutated cells). Means are shown by lines and error bars represent SEM. B) Relative *BRCA1* mRNA level in 6 WT LCLs, 5 LCLs carrying missense mutation, and 10 LCLs with truncating mutation in *BRCA1* (two-tailed Student’s t-test; *P* = 0.028 for comparison between WT and truncating LCLs, and *P* = 0.010 when comparing LCLs with missense and truncating mutation; mean of 1.202 ± 0.08 SEM for WT, 1.263 ± 0.08 SEM for missense LCLs, and 0.967 ± 0.06 SEM for truncating LCLs). C) *BRCA1* protein expression in LCLs
derived from non-carriers (6 LCLs) and BRCA1 mutation-carriers (15 LCLs) (Mann-Whitney U test; P = 0.119 with a mean of 2.197 ± 0.55 SEM for WT cells and 1.460 ± 0.30 SEM for mutated cells). Intensity of protein bands was quantified by Image-J and normalized to WT sample 10S889-L. D) BRCA1 protein level in control LCLs and cells harboring missense or truncating heterozygous mutation (two-tailed Student’s t-test; P = 0.047 with a mean of 2.197 ± 0.55 SEM for WT and 1.065 ± 0.24 SEM for truncating LCLs). E) Western blot analysis of BRCA1 expression in a panel of LCLs. Full-length BRCA1 was detected using a monoclonal anti-BRCA1 antibody (Calbiochem, #OP92) and HSC70/HSP70 served as a loading control. First sample in each blot is identical (WT cell line 10S889-L) and served for between blots-normalization when analyzing protein band densities by ImageJ. * indicates LCLs which could not be included in the final panel of analyzed cell lines.

In order to check the BRCA1 protein level and the stability of mutated protein, we performed a Western blotting of the nuclear protein fraction of LCLs (Western blot scan shown in Figure 8E). Using an anti-BRCA1 antibody with an epitop against N-terminus of the protein we observed that LCLs harboring monoallelic BRCA1 mutation show 66.5% of the WT protein level (Figure 8C; P = 0.199). As in the case of mRNA, the division of LCLs based on their mutation type allowed us to detect a significantly less full-size BRCA1 (~250 kDa) in cells with truncating mutation than in control cells (Figure 8D; P = 0.047). Interestingly, we observed comparable full-size BRCA1 protein level in cells harboring monoallelic missense mutation when compared to control cells (Figure 8D; P = 0.952), suggesting that, although altered, the proteins are stable.

It has been reported in previous studies that BRCA1 is present in cells as heterogeneous species, depending on its phosphorylation status, and various sets of Ser residues are being phosphorylated throughout the cell cycle and in response to DNA damage (Thomas, Smith et al. 1997; Okada and Ouchi 2003). We were able to distinguish a hypo- and hyper-phosphorylated form of BRCA1 in our Western blots. As in the case of a total BRCA1 protein level, there were no statistically significant differences in the level of hypo- or hyper-phosphorylated protein between mutated and control cells (Figure 9A; P = 0.057, and Figure 9B; P = 0.399, respectively). However, the stratification of samples according to the BRCA1 mutation type once again showed that cells with truncating mutation expressed significantly less hypo-phosphorylated form of BRCA1 than control cells (Figure 7C; P = 0.029). In addition, the level of hypo-phosphorylated BRCA1 correlated with the BRCA1 gene expression (Figure 10).
Interestingly, an evaluation of the ratio of hyper-P/hypo-P BRCA1 revealed that control cells contained a higher level of hypo-P BRCA1 than the phosphorylated form (hyper/hypo-P ratio of 0.938) whilst cells harboring either of the BRCA1 mutation type contained almost 2.2-times more phosphorylated protein form than hypo-P BRCA1 (Figure 9D; P = 0.005). Even though the cells with missense mutations showed elevated hyper-P/hypo-P BRCA1 ratio, the variability among these samples did not allow to reach significance when compared with control cells (Figure 9E; P = 0.168). However, we again observed a significant difference between cells with truncating mutation and control cells (Figure 9E; P = 0.001).

Finally, we checked the stability of potentially presented truncated proteins which should avoid the NMD by triggering translation re-initiation, which has been described for the c.68_69delAG mutation (Buisson, Anczukow et al. 2006), but we were unable to detect any truncated BRCA1 protein in this particular LCL.

Figure 9. Differences in the proportion of hypo-/hyper-phosphorylated form of BRCA1 protein between WT and mutated LCLs. A) Relative protein level of hypo-P BRCA1 in 6 WT LCLs and 15 LCLs with heterozygous mutation. Mutated LCLs show 70% of the protein level of WT (Mann-Whitney U test; P = 0.057 with a mean of 1.838 ± 0.50 SEM for WT cells and 1.288 ± 0.37 SEM for mutated cells). Means are demonstrated by lines and error bars represent SEM values. Band intensities were measure using...
Image-J and normalized to expression of WT sample 10S889-L. B) Relative expression of hyper-P BRCA1 in WT and mutated LCLs. Mutated LCLs show 126% of the protein level detected in WTs (two-tailed Student’s t-test; $P = 0.399$ with a mean of $1.627 \pm 0.39$ SEM for WT cells and $2.054 \pm 0.27$ SEM for mutated cells). C) Comparison of the levels of hypo-P BRCA1 between 6 WT, 5 missense, and 10 truncating LCLs (two-tailed Student’s t-test; $P = 0.029$ with a mean of $1.838 \pm 0.50$ SEM for WT and $0.855 \pm 0.11$ SEM for truncating LCLs). D) Normalized ratio of hyper-P and hypo-P form of BRCA1 in LCLs derived from non-carriers (6) and BRCA1 mutation-carriers (15) (Mann-Whitney U test; $P = 0.005$ with a mean of $0.938 \pm 0.16$ SEM for WT cells and $2.227 \pm 0.33$ SEM for mutated cells). E) Hyper-P/hypo-P normalized BRCA1 ratio in 6 WT LCLs, 5 LCLs carrying missense mutation and 10 LCLs with truncating mutation in BRCA1 (Mann-Whitney U test; $P = 0.001$ with a mean of $0.938 \pm 0.16$ SEM for WT and $2.300 \pm 0.32$ SEM for truncating LCLs).

![Figure 10](image)

Figure 10. Correlation between mRNA and hypo-P BRCA1 protein levels. Significant positive Spearman correlation between gene and protein expression in panel of LCLs. WTs are represented by light grey dots (○), LCLs with missense mutation are shown as grey squares (□), and cell lines carrying truncating mutation are displayed as black triangles (∆).

1.2 LCLs derived from BRCA1 mutation carriers show elevated levels of gamma-H2AX and impaired RAD51 foci formation

BRCA1 plays an important role in multiple repair pathways, including single-strand annealing and the repair of DNA double-strand breaks (DSBs) by homologous recombination (HR) or non-homologous end joining (NHEJ) (Roy, Chun et al. 2011). As we detected differences
in *BRCA1* mRNA and protein levels, depending on the mutation status of LCLs, we hypothesized that mutated cells could be haploinsufficient for the DNA repair under basal conditions. To test this hypothesis we performed a high-throughput screening of the intensity of gamma-H2AX signal (commonly used as a marker of DNA damage, especially DSBs) and the number of RAD51 foci (protein assisting in the repair of DSBs) in nuclei of either non-irradiated or 10 Gy-irradiated cells.

Monitoring gamma-H2AX signal by high content confocal microscope revealed that, even under basal conditions, LCLs harboring mutated *BRCA1* accumulated significantly more DNA damage than control cells (*Figure 11A, Figure 11C;* P=0.027) and significantly lower number of RAD51 foci per nucleus (*Figure 11B, Figure 11C;* P=0.020).

As expected, 10 Gy irradiation increased the intensity of the gamma-H2AX nuclear signal in both control and mutated cells, but the level of DNA damage was significantly elevated in LCLs with *BRCA1* mutations (*Figure 11A;* P<0.001). No significant difference in the number of RAD51 foci after 10 Gy irradiation was observed between control and mutated cells (*Figure 11B;* P=0.253).

In order to confirm our observation, we analyzed a double amount of nuclei (240) in a replicated experiment and we detected even more significant evidence of increased DNA damage and decreased number of RAD51 foci in non-irradiated mutated LCLs (data not shown; P<0.001 for both gamma-H2AX signal intensity and number of RAD51 foci). However, we were not able to replicate the significant differences in DNA damage and repair after IR.

Surprisingly, stratification of LCLs according to their *BRCA1* mutation status revealed that cells harboring truncating mutation present significantly increased level of DNA damage under basal conditions when compared to either WT cells or cells with missense mutation (*Figure 11C, Supplementary Figure S1A;* P<0.001). The number of RAD51 foci per nuclei in non-irradiated cells was, however, significantly decreased in cells harboring either of the *BRCA1* mutation type (*Figure 11C, Supplementary Figure S1B;* P<0.001).

In order to test if the differences in number of RAD51 foci between groups of non-treated LCLs resulted from changes in RAD51 protein levels, a Western blot analysis of whole cell lysates was performed. As shown in *Figures 12A and 12B*, there were no statistically significant differences in RAD51 protein levels between groups of LCLs, indicating that the changes in RAD51 foci formation under basal conditions are not associated with a corresponding change in the cellular level of RAD51 protein, and that *BRCA1* haploinsufficiency in mutated LCLs leads
to an impairment of RAD51 redistribution to nuclear foci rather than to a failure to express normal levels of the protein.

Figure 11. Effect of the BRCA1 germline mutation and IR on the level of DNA damage and repair. A) High-throughput microscopy quantification of gamma-H2AX signal intensity in nuclei of WT LCLs or cells with mutated BRCA1 which were either non-treated or irradiated with 10 Gy. The data represent a signal intensity detected in 120 individual nuclei of each LCL in the group and the grey line indicates mean intensity of the gamma-H2AX signal. The differences between groups were evaluated using Mann-Whitney U test (* represents P<0.05 and *** signifies P<0.001). B) Average number of RAD51 foci per
nucleus in control or mutated cells either non-irradiated or exposed to 10 Gy. The data represent a mean from 120 analyzed nuclei of each LCL in the particular group +/- SEM. The P-value was calculated using Mann-Whitney U test (* represents P<0.05). C) Representative immunofluorescence images of non-irradiated cell lines harboring WT BRCA1 (10S889-L) or heterozygous missense (11S56-L) or truncating (09S798-L) mutation. Each column consists of the same field of view using DAPI to visualize nuclei, mouse monoclonal anti-phospho-histone H2AX antibody to detect gamma-H2AX, and rabbit polyclonal anti-RAD51 antibody to detect RAD51 foci.

Figure 12. BRCA1 is not required for maintaining normal RAD51 protein levels in LCLs. A) Western blot analysis of RAD51 expression in a panel of LCLs (WT, MIS = missense, TRUN = truncating). RAD51 was detected using a rabbit polyclonal anti-RAD51 antibody (Santa Cruz; #sc-8349) and β-actin served as a loading control. First sample in both blots is identical (WT cell line 06S179-L) and served for between blots-normalization when analyzing protein band densities by ImageJ. * indicates LCLs which could not be included in the final panel of analyzed cell lines. B) RAD51 protein level in control LCLs and cells harboring missense (MIS) or truncating (TRUN) heterozygous mutation (two-tailed Student’s t-test; no significant differences between groups; mean of 1.390 ± 0.24 SEM for WT, 1.408 ± 0.12 SEM for MIS, and 1.509 ± 0.12 SEM for TRUN). Intensity of protein bands was quantified by Image-J and normalized to WT sample 06S179-L.
1.3 Gene expression profiles vary depending on the BRCA1 mutation status

Taking into account the dramatic elevation of lifetime risk of breast cancer development in BRCA1 mutation carriers, we aimed to investigate whether such increased risk could be connected with changes in gene expression profiles in cells harboring either missense or truncating monoallelic mutations.

Unsupervised hierarchical clustering did not provide a clear splitting of the samples according to their mutation type, reflecting their similarity in gene expression profiles (Figure 13). However, we observed a tendency of separate clustering between cells harboring missense mutation (highlighted in red) and the rest.

![Unsupervised hierarchical clustering](image)

**Figure 13.** Unsupervised hierarchical clustering of LCLs harboring WT BRCA1 or heterozygous missense or truncating mutation. Dendrograms derived from unsupervised hierarchical clustering based on expression of 42807 transcripts that remained after normalization and pre-processing. Color labels define particular BRCA1 mutation status: WTs in blue, missense highlighted in red, and truncating represented by black color. The cluster tree shows relations of particular samples to each other.

We then compared expression profiles of controls with those of individuals with missense or truncating mutations, aiming to identify differentially expressed genes (FDR < 5% or logFC > 2 in absolute value). By comparing the set of 42807 transcripts which passed the normalization and pre-processing data transformation steps, we were able to identify a set of genes whose
expression was significantly up- or down-regulated between studied groups of samples (Figure 14A). Interestingly, the highest number of differentially expressed genes was observed when comparing control samples with cells harboring missense mutation (Figures 14A and 14B), indicating that their expression profiles are the most distinct from all other comparisons which is in agreement with the trend observed in unsupervised hierarchical clustering (Figure 13). The annotated genes which differentiated between specific groups are listed in Table 5 and their differential expression is shown in a heatmap in Figure 14C.

Figure 14. Differentially expressed transcripts/genes between groups of LCLs depending on their BRCA1 mutation status. A) T-test with 20022 permutations was used in order to obtain genes that
differed between LCLs. The graph shows a comparison of the number of differentially expressed transcripts with logFC > |2| between WT, missense (MIS), truncating (TRUN) and mutated (MUT = MIS + TRUN) LCLs. B) Venn diagram illustrating the number of genes being differentially expressed (logFC > |2|) between: WT vs MIS, WT vs TRUN and MIS vs TRUN. For each comparison, the overlap of genes is shown. The numbers highlighted in red are up- or down-regulated genes that are specific for particular comparison. C) Gene expression patterns of 20 genes differentiating LCLs depending on their BRCA1 mutation status. LCLs and their BRCA1 mutation status are indicated at the top of the figure. The mean values of normalized and pre-processed probe intensities are visualized in the heat map. Probe intensities above and below the global value are denoted by shades of red and blue, respectively, and those at the global mean level are shown in white. Up- or down-regulated genes in a group of LCLs (in bold) from particular comparison are marked with a red and blue arrow, respectively.

Table 5. Description of the 20 genes that differentiate groups of LCLs depending on the type of BRCA1 mutation.

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<th>Down-regulated in</th>
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</table>

NOTE: logFC, logarithmic fold change. Only differentially expressed genes with logFC > |2| are shown in the table.

*a genes involved in immune response (according to the Reactome_Immune_system gene set) are marked with #
1.4 Haploinsufficiency in \textit{BRCA1} leads to a defective expression of genes involved in immune response and cancer

In order to get more insight into the consequences and biological relevance of differential gene expression between groups of LCLs, we used Ingenuity Pathway Analysis (IPA) software and investigated molecular/cell function alterations and interaction networks of differentially expressed genes.

Genes, which were differentially expressed in cells with heterozygous missense mutation when compared to WTs, regulate 26 molecular and cellular functions (Supplementary Table S2). Among the top five altered biological functions, most of them are known to be relevant for cancer development (Figure 15A). Then we subjected the list of 13 differentially expressed genes to IPA gene network analysis in order to generate interaction network and calculate a probability score. 10 out of 13 input genes were included into a network consisting of 34 molecules (Figure 15B) and displaying a probability score of 25 (score = 25 represents $p$-value $= 10^{-25}$). In addition, BRCA1 was artificially added in order to visualize its possible interactions with the network, and was found to be both directly (through TNF) and indirectly (via NF\textsubscript{k}B complex) associated with the pathway.

Interestingly, the members of this pathway were found to be significantly associated with many diseases and functions, mainly immune and inflammatory responses, cellular development, cell signaling, cellular proliferation, cell death and survival etc. Visualization of the involvement of the network members in cell death (6 out of the set of 13 differentially expressed genes involved; $p = 2.32 \times 10^{-5}$) and inflammatory response (2/13 differentially expressed genes implicated; $p = 1.32 \times 10^{-6}$) pathways is shown in Supplementary Fig. S2.

We performed a similar analysis for the two differentially expressed genes in cells harboring heterozygous truncating mutation and found that they are involved in regulation of 8 molecular and cellular pathways (Supplementary Table S3), including cellular movement, development, proliferation, interaction, cell death etc. The IPA gene network analysis did not provide any signs of interaction between \textit{MMP7} and \textit{LY6D}, the two differentially expressed genes in cells carrying truncating mutation in \textit{BRCA1} gene, in a common network.
Figure 15. Downstream effect analysis and network of differentially expressed genes between WTs and cells with BRCA1 missense mutation. A) Top 5 molecular and cellular functions significantly associated with genes differentially expressed in cells harboring heterozygous missense mutation in BRCA1. The grey line represents a significance threshold (P-value 0.05). B) IPA-based network of differentially expressed genes (underlined) between WTs and cells with BRCA1 missense mutation. 12 differentially expressed genes were input into the analysis, 10 of them (underlined) are represented in the 34 molecule output pathway. The legend specifies the molecule type, type of interaction and up-/down-regulation of differentially expressed genes.
1.5 No alteration of miRNA expression found in cells carrying monoallelic BRCA1 mutation

Changes in expression of microRNAs (miRNAs) have been found to be associated with breast cancer development, metastasis, prognosis and response to treatment (Mulrane, McGee et al. 2013). In addition to gene expression profiling, we also aimed to investigate whether cells derived from BRCA1 mutation carriers show deregulated miRNA expression.

miRNA expression profiles showed a high similarity between WT LCLs and cells harboring heterozygous mutation in BRCA1. There were no significantly differentially expressed miRNAs between groups of LCLs with FDR<0.05 or logFC > 2 in absolute value. The list of differentially expressed miRNAs passing the threshold of unadjusted P-value <0.05 is shown in Supplementary Fig. S3.
The second objective of this work was to investigate whether there are any differences in response of LCLs to various PARP inhibitors, depending on their BRCA1 mutation status. In addition, we aimed to look at factors which could potentially influence the response of cells to PARP inhibitors, including PARP1 expression, its enzymatic activity and cellular growth rate.

2.1 **BRCA1 knockdown sensitizes the breast cancer cell line MDA-MB-231 to PARP inhibition, confirming a synthetic lethal interaction between BRCA1 and PARP1**

In order to confirm the synthetic lethal interaction between BRCA1 and PARP1, BRCA1 was silenced using a set of six short hairpin RNAs (shRNAs) in the BRCA1-proficient breast cancer cell line MDA-MB-231 and the ability to form colonies and changes in proliferation in response to PARP inhibitors olaparib (OLP) or veliparib (VLP) was evaluated in these cells. Western blotting analysis of nuclear proteins revealed a substantial variability in the silencing ability between particular shRNAs (Figure 16A). Only shBRCA1-1 (sh1) and shBRCA1-5 (sh5), which decreased the level of full-length BRCA1 protein the most, were used for further experiments and their effect was compared to the effect of control non-targeting shRNA (shScramble). The quantitative RT-PCR confirmed a lower BRCA1 mRNA level in cells expressing sh1 and sh5 when compared to shScramble (Figure 16B), confirming the silencing ability of these shRNAs at both mRNA and protein level.

BRCA1 knockdown itself dramatically decreased the number of colonies that the cells were able to form 7 days after plating, to 10% (sh1) or 69% (sh5) of the colony quantity found in control cells. As expected, both OLP and VLP further altered the ability of sh1 and sh5 cells to form colonies (Figure 17A). Consistently with these results, treatment with PARP inhibitors also markedly decreased proliferation of cells with silenced BRCA1 when compared to a mild effect on the proliferation of control cells (Figure 17B). In addition, the concentration range of PARP inhibitors used in these experiments showed that OLP acts synthetically lethal with BRCA1 at a lower concentration than VLP. Such confirmation of a synthetic lethal interaction between BRCA1 and PARP1 provided a confidence to use these two agents in further experiments with our panel of LCLs.
Figure 16. BRCA1 silencing ability of a set of human GIPZ lentiviral shRNAmir. A) Western blot analysis of BRCA1 protein expression in MDA-MB-231 cells, parental or transduced with 6 BRCA1-specific shRNA clones (sh1–6) or control shRNA (shScramble). Nucleolin was used as a loading control for nuclear proteins. B) Relative BRCA1 mRNA expression in cells overexpressing non-targeting shRNA or BRCA1-specific sh1 or sh5.

Figure 17. Effect of BRCA1 depletion on sensitivity of MDA-MB-231 cells to PARP inhibitors. A) Colony formation assay of control or BRCA1-depleted cells pre-treated either with DMSO (control) or with
indicated concentrations of olaparib (OLP) or veliparib (VLP). Values represent a mean and standard deviation of 6 replicates. Concentrations of PARP inhibitors are indicated in μM. Compact group of more than 50 cells was considered as a colony. B) MTT assay displaying proliferation changes of control or BRCA1-depleted cells after treatment with OLP or VLP for 72 hours. A representative example of one of two independent experiments is shown.

2.2 Carriers of heterozygous truncating mutation in BRCA1 show reduced expression of PARP1

As we aimed to investigate the response of LCLs to PARP inhibitors, we first checked the gene and protein expression of PARP1 and its enzymatic activity, factors which could potentially influence the response of cells to PARP inhibitors.

The PARP1 gene expression was determined by quantitative RT-PCR and the expression curves were normalized to the expression of housekeeping genes MRLP19 and HPRT1. Interestingly, we observed a significant decrease in PARP1 expression in cells harboring truncating mutation in BRCA1 (Figure 18A; P = 0.028), the same cells which showed lower expression of the BRCA1 gene (Figures 8B and 8D). Western blotting analysis of whole cell lysates revealed a significant positive correlation between PARP1 mRNA and protein expression (Figure 18B; P = 0.027). Similarly as in the case of PARP1 gene expression, cells harboring truncating mutations in BRCA1 showed decreased levels of PARP1 protein, however the variability between cell lines did not allow to reach statistical significance (Figure 18C; P = 0.073). Interestingly, unlike cells carrying truncating mutation, cells harboring missense mutation in BRCA1 showed comparable PARP1 protein level as WTs.

In order to check whether the decrease in PARP1 mRNA level is related to an alteration of BRCA1 expression, we measured its expression in MDA-MB-231 cells where the BRCA1 gene was knocked down by sh1. BRCA1 silencing led to a decrease of PARP1 mRNA levels (of about 18% when compared to control cells) (Figure 18D), indicating that BRCA1 could be involved in regulation of PARP1 gene expression.
Figure 18. PARP1 expression in LCLs and MDA-MB-231 cells with depleted BRCA1. A) Relative PARP1 mRNA level in 6 WT LCLs, 5 LCLs carrying missense mutation, and 10 LCLs with truncating mutation in BRCA1 (two-tailed Student’s t-test; P = 0.028 for comparison between WT and truncating LCLs; mean of 1.270 ± 0.05 SEM for WT, 1.151 ± 0.05 SEM for missense LCLs, and 1.081 ± 0.05 SEM for truncating LCLs). B) Significant positive Pearson correlation between PARP1 mRNA and protein levels in a panel of LCLs. C) PARP1 protein level in control LCLs and cells harboring missense or truncating heterozygous mutation in BRCA1 gene (two-tailed Student’s t-test; P = 0.073 for comparison between WT and truncating LCLs; mean of 1.569 ± 0.18 SEM for WT, 1.482 ± 0.26 SEM for missense LCLs, and 1.129 ± 0.14 SEM for truncating LCLs). D) Relative PARP1 mRNA expression in cells overexpressing non-targeting shRNA or BRCA1-specific sh1. E) Western blot analysis of PARP1 expression in a panel of LCLs. Full-length PARP1 (116 kDa) and the large fragment of cleaved PARP1 (89 kDa) were detected using anti-PARP1 antibody (Cell Signaling; #9542) and β-actin served as a loading control. First sample in each blot is identical (WT cell line 095179-L) and served for between blots-normalization when analyzing protein band densities by ImageJ. * indicates LCLs which could not be included in the final panel of analyzed cell lines.
Since it has been previously demonstrated that PARP1 expression and protein level only partially correlate with its enzymatic activity (Zaremba, Ketzer et al. 2009; Zaremba, Thomas et al. 2011), we decided to check PARP enzymatic activity in our panel of LCLs using two distinct methods. Firstly, we measured the level of PAR (poly ADP-ribose), which is a post-translational modification that the family of PARP proteins transfers to itself and to other target proteins (Ame, Spenlehauer et al. 2004). Using an anti-PAR antibody and Western blot technique, we did not find any statistically significant differences in the cellular level of PAR polymers between groups of LCLs (Figure 19A), confirming the lack of correlation between PARP1 expression and enzymatic activity. However, there was markedly higher variability in PAR levels in BRCA1 mutated cells than in WTs. In order to measure the enzymatic activity more precisely, we used a modified in vitro PARP activity assay which allowed us to determine a basal PARP activity in cell lysates (i.e. non-induced naturally activated PARP present in LCLs). Using this assay, we did not find any statistically significant differences in basal PARP activity between groups of LCLs depending on their BRCA1 mutation status (Figure 19B). Nevertheless, results from both experiments indicate that cells harboring heterozygous missense mutation in BRCA1 show slightly elevated PARP activity than cells with truncating mutation (Figures 19A and 19B).

**Figure 19.** PARP enzymatic activity in LCLs. A) Relative PAR polymer levels in whole cell lysates isolated from 6 WT LCLs, 5 LCLs carrying missense mutation, and 10 LCLs with truncating mutation in BRCA1. PAR was detected by Western blot using anti-PAR antibody. Median of the data is represented by the line inside the box plots (median of 1.74 ± 0.15 SEM for WT, 1.76 ± 0.30 SEM for missense LCLs, and 1.49 ± 0.22 SEM for truncating LCLs). B) PARP activity in the cellular extracts of LCLs measured as the amount of ribosylation on histone-coated plates. Line inside of the box plot indicate median of the data (median of 32.48 ± 2.95 SEM for WT, 35.33 ± 2.15 SEM for missense LCLs, and 33.42 ± 1.67 SEM for truncating LCLs). Graph shows representative results from one out of three independent experiments.
LCLs harboring heterozygous missense mutations in BRCA1 BRCT domain are more sensitive to PARP inhibition than WTs or cells with truncating mutations

Taking into account the different levels of both BRCA1 and PARP1 expression between groups of LCLs, we were wondering whether cells carrying various types of BRCA1 mutation display different sensitivity to PARP inhibitors OLP and VLP. Indeed, our results with PARP inhibitor OLP showed significantly increased sensitivity of cells derived from mutation carriers harboring missense mutation in BRCA1 (Figure 20A). Interestingly, LCLs with truncating mutations showed comparable low sensitivity levels to OLP as WT cells. Moreover, the PARP1 enzymatic activity, measured by an in vitro assay, negatively correlated with viability of the cells after treatment with low concentration of OLP (Figure 20C), being in line with previously published results (Gottipati, Vischioni et al. 2010). No association between sensitivity of cells to OLP and BRCA1 or PARP1 expression was found, indicating that expression levels of these two proteins do not play a role in regulation of response to this agent.

Figure 20. Effect of BRCA1 germline mutation on sensitivity of LCLs to PARP inhibitors. A) MTT assay displaying proliferation changes of LCLs treated with indicated concentrations of PARP inhibitor OLP for
72 hours, relative to control cells (treated with DMSO). 6 WT LCLs, 5 LCLs carrying missense mutation, and 10 LCLs with truncating mutation in BRCA1 were used in this experiment. Values represent a mean and standard deviation of viabilities of all LCLs belonging to one group, obtained in 3 independent experiments. P-values were calculated using a linear regression model taking into account the cellular growth rate as a covariate. B) MTT assay of LCLs treated with indicated concentrations of VLP for 72 hours, relative to control DMSO-treated cells. 6 WT LCLs, 5 LCLs carrying missense mutation, and 10 LCLs with truncating mutation in BRCA1 were used in this experiment. Values represent a mean and standard deviation of viabilities of all LCLs belonging to one group, obtained in 3 independent experiments. P-values were calculated using a linear regression model taking into account the cellular growth rate as a covariate. C) Significant negative Pearson correlation between PARP enzymatic activity and viability of LCLs treated with PARP inhibitor OLP 4μM.

Experiments with VLP, the second PARP inhibitor, revealed that higher concentration of the reagent had to be used in order to reach 50% inhibition of viability. As in the case of OLP treatment, WT cells were the most resistant to VLP treatment (Figure 20B). However, cells harboring either of the BRCA1 mutation type, and not only missense mutation, showed significantly increased sensitivity to higher concentrations of VLP.

It is a commonly accepted fact that rapidly dividing cells are more sensitive to DNA damaging agents, such as chemotherapeutic drugs (Stark, Zhang et al. 2010). Therefore, we raised a question of whether cellular growth rate could act as a confounding variable, influencing sensitivity of LCLs to PARP inhibitors. As shown in Figure 21A, there were no significant differences in growth rate between groups of LCLs depending on their BRCA1 mutation status. However, we indeed found a significant negative correlation between cellular growth rate and viability of cells after treatment with the highest concentration of OLP or VLP (Figures 21B, 21C). Because of this relationship, growth rate was taken into account as a cofounding variable while performing the statistical analysis of cellular response to OLP and VLP (P-values mentioned in Figures 20A and 20B). However, the significant differences in response of mutated cells to OLP or VLP were still present after incorporating the cellular growth rate as a covariate, indicating a growth rate-independent variability in cellular sensitivity to OLP and VLP.
Figure 21. Cellular growth rate in LCLs and its effect on sensitivity of cells to PARP inhibitors. **A)** Growth rate of 6 WT LCLs, 5 LCLs carrying missense mutation, and 10 LCLs with truncating mutation in *BRCA1* (mean of 3.988 ± 0.32 SEM for WT, 4.896 ± 1.10 SEM for missense LCLs, and 4.260 ± 0.40 SEM for truncating LCLs). Cellular proliferation was measured using modified MTT assay, comparing absorbances (544nm) taken at timepoints 0 hours and 72 hours. **B)** Significant negative Spearman correlation between cellular growth rate and viability of LCLs treated with PARP inhibitor OLP 8μM. **C)** Significant negative Spearman correlation between cellular growth rate and viability of LCLs treated with PARP inhibitor VLP 100μM.
The final part of the thesis was focused on an exploration of the mechanism underlying the increased sensitivity of cells with *BRCA1* missense mutation to PARP inhibitor OLP. We were particularly interested whether the missense mutant could act in a dominant negative manner in heterozygous cells. To gain better understanding of the molecular mechanisms behind OLP action, we performed studies focusing on the BRCT domain of BRCA1 protein (transcriptional activation assay, analysis of BRCT-protein interactions by tandem affinity purification coupled to mass spectrometry), where both of the studied missense mutations are located. Moreover, profiling of miRNA and gene expression in OLP-treated cells was performed in order to select candidate genes involved in the response of cells to this treatment. Finally, an integration of the data from gene and miRNA expression was carried out.

### 3.1 Transcriptional activation function of the BRCA1 BRCT domain is not involved in the response of cells to OLP

BRCA1 has been found to be involved in several crucial cellular processes, including regulation of transcription of other genes through its BRCT domains (Monteiro, August et al. 1996). As the two deleterious missense mutations present in our panel of LCLs are located in the BRCA1 BRCT domain, we hypothesized that the increased sensitivity of these cells to OLP is caused, at least partially, by alteration of this function. Using a well described cell-based transcriptional assay (Monteiro, August et al. 1996) we analyzed the transcriptional activity of constructs containing WT BRCA1 (positive control), missense variants M1775R (negative control), G1706E, A1708E, and combination of WT+A1708E in the HEK293FT cellular model (Figures 22A and 22B). The co-transfection of WT and A1708E constructs mimicked the heterozygous status present in our LCLs and allowed us to test a possible dominant-negative effect of the mutated variant on the WT protein function.

Western blot analysis confirmed an overexpression of all transfected BRCA1 constructs in both DMSO- and OLP-treated HEK293FT cells (Figure 22C). As expected, WT BRCA1 was able to activate transcription of the reporter gene (Figure 22D). The negative control, as well as both tested missense variants (G1706E, A1708E), displayed an altered ability to transcribe the luciferase gene, which is in agreement with previously published studies (Lee, Green et al.
Interestingly, a co-transfection of constructs containing WT and A1708E variants of BRCA1 led to an transcriptional activation which was comparable to the one induced by WT construct alone, indicating that the A1708E variant does not act in a dominant-negative manner to a WT BRCA1 transcriptional activation function. It was also interesting to note that the treatment with PARP inhibitor OLP slightly decreased the ability of WT BRCA1 to activate the transcription of the target gene (82% of the WT).

Figure 22. Functional analysis of missense variants in BRCA1. A) Diagram of construct visualizing BRCA1 missense variants tested in this experiment. Grey boxes indicate the DNA binding domain (DBD) of GAL4, and the N- and C-terminal BRCT domains. B) Diagram of the five GAL4 DNA binding sites located upstream of the luciferase reporter gene. Adapted from Carvalho et al., 2009. C) Transfection efficiency was confirmed by Western blot using anti-GAL4-DBD antibody. High levels of a specific antibody and an overabundance of the overexpressed protein caused intense localized signals and, consequently, white bands at exposed film. D) Quantitative transcriptional assay in HEK293FT cells treated with DMSO (control) or OLP for 24 hours. Results are displayed as a percentage of DMSO-treated cells overexpressing WT construct, the error bars represent standard deviation from triplicates. The graph shows results from one out of three independent experiments.
3.2 OLP induces changes in BRCA1 BRCT protein interactors and phosphorylation of threonine residue at position 1834

To further investigate whether OLP treatment influences functions mediated by the BRCA1 BRCT domain, we decided to analyze BRCT-mediated protein interactions, examined whether they are modulated by PARP inhibition and checked which molecular functions are the protein interactors involved in. For this purpose, we used HEK293FT cells overexpressing the BRCA1 BRCT domain, which was either WT or carrying the G1706E variant, and treated them either with DMSO (control) or OLP (16μM). We then analyzed BRCT-bound protein complexes by tandem affinity purification coupled with mass spectrometry (TAP-MS) (detailed protocol in the Materials and Methods chapter).

As shown in Figure 23A of a Coomassie stain, we were unable to overexpress the mutated BRCT at levels sufficient for further mass spectrometry analysis, even after several rounds of transfection optimizations. Thus, we were only able to obtain information about WT BRCT protein interactions before and after treatment with OLP, which, however, still provide a valuable insight into the mechanism of OLP action. After applying the SAINT (Significance Analysis of INteractome) algorithm to our data and setting a probability threshold of 0.8–1, we ended up with a list of 121 protein interactors (Figure 23B) (full list shown in Supplementary Table S4). Most of the proteins (108/121 = 89%) were found to interact with BRCT in both control and OLP-treated cells. However, 11 interactors were found to be specific only for OLP-treated cells, several of them being involved in cellular proliferation, transcription or cellular signaling (Table 6).

![Figure 23A and B](image-url)

**Figure 23.** BRCA1 BRCT protein interactors in cells treated with DMSO or OLP. A) Coomassie staining of purified TAP-tagged BRCA1 BRCT constructs from DMSO- or OLP-treated HEK293FT cells. Arrows indicate the TAP-tagged bait protein. B) Venn diagram showing the number of proteins interacting with
WT BRCT after treatment with DMSO (control) or 16μM OLP. Only protein interactors which passed the SAINT cutoff of 0.8 and above were included.

Table 6. BRCA1 BRCT interacting proteins specific for OLP-treated cells. (Source: www.genecards.org)

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<th>Gene name</th>
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<th>Fold change (OLP/DMSO)</th>
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<td>STRN</td>
<td>86</td>
<td>5</td>
<td>positive regulation of estrogen receptor signaling</td>
</tr>
<tr>
<td>STRN3</td>
<td>Striatin-3</td>
<td>STRN3</td>
<td>87</td>
<td>4</td>
<td>neg. regulation of estrogen receptor signaling; regulation of transcription</td>
</tr>
</tbody>
</table>

To get further insights into the functional roles of proteins interacting with the BRCA1 BRCT domain, we performed an enrichment analysis of the set of 121 proteins by using Ingenuity Pathway Analysis (IPA), taking into account the value of fold change spectral counts. As expected, among the top canonical pathways related to this set of proteins was “Role of BRCA1 in DNA Damage Response”, which was found to be downregulated (as indicated by the negative z-score value of -1.6) and which indicates an alteration of this pathway by OLP treatment (Figure 24A). The interactors were also found to be significantly involved in several molecular and cellular functions, such as “DNA Replication, Recombination, and Repair”
(Figure 24B), and they were connected into a highly scored network within members of this function (score 61) (Figure 24C).
Figure 24. Functional analysis of the set of 121 BRCA1 BRCT-interacting proteins. A) Top five canonical pathways across the entire dataset. The significance was calculated with a Fisher’s exact test right tailed. The blue bar indicates predicted pathway inhibition after treatment with OLP. The pathways shown in grey color mean that no prediction can currently be made. The orange points connected with a thin line represent a ratio of genes from our dataset to all genes involved in this canonical pathway. B) Top 5 molecular and cellular functions being related to the set of protein interactors. The orange line represents a significance threshold (P-value 0.05). C) IPA-based network of proteins involved in DNA Replication, Recombination, and Repair, Gene expression and Cell morphology functions with a score of 61. 30 BRCA1 BRCT interactors were included in the 35 molecule output pathway. The legend specifies the molecule type, type of interaction, and up- (green) or down- (red) regulation of the protein binding after treatment with OLP.

We were also able to detect a new BRCA1 phosphorylation site at position T1834 (located at the end of the C-terminal BRCT domain) in the OLP-treated cells. According to the PhosphoSitePlus database (www.phosphosite.org), there are 105 BRCA1 phosphorylation sites reported up to date and they are implicated in various cellular processes (Figure 25). However, the phosphorylation site at threonine 1834 has not been previously reported and could therefore represent an OLP-specific response.

Figure 25. BRCA1 phosphorylation sites. A screenshot from the PhosphoSitePlus database showing the known phosphorylation sites of BRCA1 and their involvement in various cellular processes.
3.3 Gene expression profiling revealed downregulation of breast cancer-related genes as a response to OLP treatment

After focusing our attention on the BRCA1 BRCT domain, we aimed to get a more global picture of the cellular response to PARP inhibitor OLP, and thus we searched for genes and miRNAs which are differentially expressed as a consequence of OLP treatment. We used DMSO-treated cells as controls and compared their gene and miRNA expression profiles to those of OLP-treated LCLs.

We first checked the number of differentially expressed genes (FDR < 0.15 or logFC > 2 in absolute value) between DMSO- and OLP-treated WT cells and then we compared those numbers between WT cells, cells with missense mutations and cells carrying truncating mutations. Such comparison gave us an idea about inducement of an expression phenotype by OLP, taking into account the BRCA1 mutation status.

Interestingly, when we used the most stringent threshold (FDR < 0.15) to identify OLP-induced differential gene expression, we did not find any differentially expressed genes (DEg) in WT cells, however we detected 3842 DEg in mutated cells (missense and truncating together) (Figure 26A). After stratification according to the BRCA1 mutation type, only cells harboring truncating mutation had changed expression after treatment with OLP (2144 DEg). A closer look at the normalized pre-processed data revealed that most of these differentially expressed genes were of a low logarithmic fold change (Figure 26B), suggesting that the OLP-induced changes were small, but very consistent.

As we wanted to check the response of WT cells to OLP, i.e. the standard response of cells to OLP, we decided to use the second stringent threshold (logFC > |2|). We were able to identify 312 genes which were differentially expressed after treatment of WTs with OLP (Figures 26B and 26C). IPA-based functional analysis of these genes revealed their involvement in numerous cancer-related molecular and cellular functions, including regulation of cell morphology and movement or cell death and survival (Figure 26D). Importantly, most of the DEg were related to the disease “Cancer” (p-value of 4.7x10^{-6}), and the direction of their expression indicated a significant downregulation of “Cancer”-related genes upon treatment with OLP (as predicted from a negative z-score value of -2.019).
Figure 26. OLP-induced differential expression of genes in groups of LCLs and their implication in molecular functions. A) A diagram showing the numbers of OLP-induced differentially expressed genes with FDR < 0.15 in WT, missense (MIS), truncating (TRUN), and mutated (MUT = MIS + TRUN) LCLs. B) A graph displaying the numbers of differentially expressed genes (logFC > |2|) after treatment with OLP in WT, MIS, TRUN, and MUT LCLs. C) Venn diagram illustrating the number of genes being differentially expressed after treatment with OLP (logFC > |2|) in WT cells, or LCLs harboring MIS or TRUN mutation. For each comparison, the overlap of genes is shown. The numbers highlighted in blue are up- or down-regulated genes in WT cells. The underlined number represents OLP-induced DEg specific only for WT and TRUN, but not for missense. D) Top 5 molecular and cellular functions being associated with genes differentially expressed after OLP treatment of WT cells. The orange line represents a significance threshold (P-value 0.05).

Using the MALACARDS database (http://www.malacards.org/) of genes involved in human diseases, we searched whether any of the 289 reported breast cancer-associated genes were differentially expressed in our WT samples after treatment with OLP. Interestingly, 4 commonly upregulated breast cancer genes (ERBB2, FOS, HSPB2, AGR3) were found to be downregulated by OLP, indicating that this treatment could have additional positive effects than just a simple synthetic lethality.
3.4 miRNA expression is changed by OLP treatment and regulates expression of breast cancer-related genes ERBB2 and FOS

The final high throughput method we used in order to better understand the response of cells to OLP was miRNA expression profiling and its integration with gene expression. As in the case of gene expression, we first checked the numbers of differentially expressed miRNA (DEm). Interestingly, the miRNome was strongly affected by OLP treatment in all groups of cells (Figures 27A and 27B), however the absence of any DEm when applying the significance threshold of logFC > |2| suggests that the expression of these miRNAs is consistent between cell lines but does not change dramatically after treatment with OLP.

As obvious from the Figure 27B, most of the OLP-induced DEm were common for all groups of LCLs (154 miRNAs). To check the standard changes in miRNA expression induced by PARP inhibitor OLP, we focused on WT cells and with the help of IPA we performed the integration study between the 187 DEm and the 321 DEg. After several filtering steps (selection of miRNAs recognized by IPA, miRNAs with experimentally validated targets, miRNA-mRNA pairs showing anti-correlated expression pattern), we selected 7 DEm targeting 9 DEg (Table 7). Interestingly, the list of miRNA targeted genes included FOS and ERBB2, the breast cancer related genes, whose expression was downregulated in WT cells by OLP treatment. The OLP-induced upregulation of miR-222-3p and miR-548ap-5p and subsequent downregulation of FOS or ERBB2 gene expression, respectively, indicate the complexity of action of PARP inhibitor OLP.
Table 7. Selection of 7 miRNAs targeting 9 genes which are differentially expressed in OLP-treated WT LCLs. Involvement of the genes in IPA canonical pathways is specified.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>p-value</th>
<th>logFC</th>
<th>Gene</th>
<th>p-value</th>
<th>logFC</th>
<th>IPA Pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-155-5p</td>
<td>1.76E-02</td>
<td>0.364</td>
<td>CTLA4</td>
<td>6.05E-02</td>
<td>-2.388</td>
<td>CD28 Signaling in T Helper Cells, CTLA4 Signaling in Cytotoxic T Lymphocytes, T Cell Receptor Signaling</td>
</tr>
<tr>
<td></td>
<td>1.76E-02</td>
<td>0.364</td>
<td>MEIS1</td>
<td>9.19E-02</td>
<td>-2.111</td>
<td>Transcriptional Regulatory Network in Embryonic Stem Cells</td>
</tr>
<tr>
<td>hsa-miR-155-5p</td>
<td>1.76E-02</td>
<td>0.364</td>
<td>TNFRSF10A</td>
<td>1.25E-01</td>
<td>-2.036</td>
<td>Death Receptor Signaling, p53 Signaling, Retinoic Acid Mediated Apoptosis Signaling, Tec Kinase Signaling</td>
</tr>
<tr>
<td>hsa-miR-17-5p</td>
<td>6.53E-03</td>
<td>0.557</td>
<td>TP63</td>
<td>1.74E-01</td>
<td>-2.057</td>
<td>Calcium Signaling, p53 Signaling</td>
</tr>
<tr>
<td>hsa-miR-181a-5p</td>
<td>7.77E-02</td>
<td>0.156</td>
<td>CDX2</td>
<td>2.75E-02</td>
<td>-2.605</td>
<td>Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency, Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency, Transcriptional Regulatory Network in Embryonic Stem Cells</td>
</tr>
<tr>
<td>hsa-miR-21-5p</td>
<td>8.75E-03</td>
<td>-0.362</td>
<td>SOX5</td>
<td>2.19E-02</td>
<td>2.376</td>
<td>Wnt/B-catenin Signaling</td>
</tr>
<tr>
<td>hsa-miR-222-3p</td>
<td>1.10E-02</td>
<td>0.369</td>
<td>FOS</td>
<td>2.24E-03</td>
<td>-3.031</td>
<td>14-3-3-mediated Signaling, Acute Phase Response Signaling, April Mediated Signaling, Aryl Hydrocarbon Receptor Signaling, B Cell Activating Factor Signaling, CCR5 Signaling in Macrophages, CD27 Signaling in Lymphocytes, CD28 Signaling in T Helper Cells, CD40 Signaling, CDC42 Signaling, Ceramide Signaling, Chemokine Signaling, Cholecystokinin/Gastrin-mediated Signaling, Colorectal Cancer Metastasis Signaling, Corticotropin Releasing Hormone Signaling, CCR4 Signaling, EGF Signaling, Endothelin-1 Signaling, ErbB Signaling, ERK/MAPK Signaling, ERK5 Signaling, Erythropoietin Signaling, Estrogen-Dependent Breast Cancer Signaling, GDNF Family Ligand-Receptor Interactions, Glucocorticoid Receptor Signaling, GPRH Signaling, Growth Hormone Signaling, HSF Signaling, HMGB1 Signaling, JNK Signaling, IL-1 Signaling, IL-10 Signaling, IL-12 Signaling and Production in Macrophages, IL-17A Signaling in Fibroblasts, IL-17A Signaling in Gastric Cells, IL-2 Signaling, IL-3 Signaling, IL-6 Signaling, IL-8 Signaling, ILF Signaling, JAKOS Signaling, JAK/Stat Signaling, LPS-stimulated MAPK Signaling, MIF Regulation of Inflammatory Response, Molecular Mechanism of Cancer, Neuropathic Pain Signaling in Dorsal Horn Neurons, Neuropeptide Y Signaling, NF2-mediated Oxidative Stress Response, P2Y Purinergic Receptor Signaling Pathway, PGG2 Signaling, PKA Signaling in B Lymphocytes, PDC Signaling in T Lymphocytes, PPAR Signaling, Production of Nitric Oxide and Reactive Oxygen Species in Macrophages, Proteasome Signaling, RAC1 Signaling in Osteoclasts, RAR Activation, Regulation of IL-2 Expression in Activated and Immature T Lymphocytes, RasAxon Signaling, Renal Cell Carcinoma Signaling, Renin-Angiotensin Signaling, Role of Macrophages, Fibroblasts, and Endothelial Cells in Rheumatoid Arthritis, Role of NF-κB in Regulation of the Immune Response, Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis, Signaling by P2X Purinergic Receptors, Systemic Lupus Erythematosus Signaling, T Cell Receptor Signaling, Tec Kinase Signaling, TGF-β Signaling, Thrombopoietin Signaling, Tight Junction Signaling, THP-1 Signaling, TNF-α Signaling, Toll-like Receptor Signaling, UVA-Induced MAPK Signaling, UBV-Induced MAPK Signaling, UVB-Induced MAPK Signaling, VEGF Family Ligand-Receptor Interactions</td>
</tr>
<tr>
<td>hsa-miR-29a-3p</td>
<td>2.23E-03</td>
<td>0.907</td>
<td>COL1A1</td>
<td>7.10E-02</td>
<td>-2.748</td>
<td>Atherosclerosis Signaling, Dendritic Cell Maturation, Hepatic Fibrosis, / Hepatic Stellate Cell Activation, IL-6 Signaling, Intrinsic Protein Kinase Activation Pathway, Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis</td>
</tr>
<tr>
<td>hsa-miR-548ap-5p</td>
<td>4.37E-03</td>
<td>0.290</td>
<td>ERBB2</td>
<td>6.38E-03</td>
<td>-2.517</td>
<td>Agin Interactions at Neuronal Synapse Function, Axon Guidance Signaling, Bladder Cancer Signaling, Endometrial Cancer Signaling, ErbB Signaling, ErbB2/ErbB3 Signaling, HER-2 Signaling in Breast Cancer, Her-2 Signaling, Non-Small Cell Lung Cancer Signaling, Pancreatic Adenocarcinoma Signaling</td>
</tr>
</tbody>
</table>
Taking into account the apparent importance of this cascade in response of cells to OLP, we checked the expression of these two miRNAs and their two gene targets in LCLs harboring missense or truncating mutation in BRCA1 gene. Surprisingly, both miR-222-3p and miR-548ap-5p were found to be significantly upregulated in both WTs and cells with truncating mutation, however, they were significantly downregulated in cells carrying missense mutation (Table 8). In addition, FOS expression was significantly downregulated in WTs and cells with truncating mutation (Table 8; Figure 26C – underlined number), but not in cells harboring missense mutation. These results indicate that OLP-induced regulation of FOS expression could be at least partially responsible for the observed differences in sensitivity of cells to PARP inhibitor OLP (Figure 20A), and confirm distinct effects of the various types of BRCA1 mutation. Importantly, these results also indicate that breast cancer patients carrying BRCA1 mutation are likely to show different toxicity to PARP inhibitor OLP, depending on the mutation type, and possibly also variability in the response to these agents.

Figure 27. OLP-induced differential expression of miRNAs in groups of LCLs depending on their BRCA1 mutation status. A) A diagram showing number of OLP-induced differentially expressed miRNA with FDR < 0.05 in WT, missense (MIS), truncating (TRUN), and mutated (MUT = MIS + TRUN) LCLs. B) Venn diagram illustrating the number of miRNAs being differentially expressed after treatment with OLP (FDR < 0.05) in WT cells, or LCLs harboring MIS or TRUN mutation. For each comparison, the overlap of miRNAs is shown. The numbers highlighted in blue are up- or down-regulated miRNAs in WT cells.
Table 8. OLP-induced differential expression of miR-222-3p and miR-548ap-5p and their target genes *FOS* and *ERBB2*, respectively, in panel of LCLs. FDR < 0.15 and logFC > |2| were considered as significance thresholds for miRNAs and genes, respectively.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>FDR &lt; 0.15</th>
<th>logFC</th>
<th>Gene</th>
<th>logFC &gt;</th>
<th>2</th>
<th></th>
<th>LCL group</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-222-3p</td>
<td>0.01390</td>
<td>0.4</td>
<td><em>FOS</em></td>
<td>-3.0</td>
<td></td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.02368</td>
<td>-0.4</td>
<td></td>
<td>no significant change</td>
<td>MIS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.00002</td>
<td>0.5</td>
<td></td>
<td>-2.1</td>
<td></td>
<td>TRUN</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-548ap-5p</td>
<td>0.00629</td>
<td>0.3</td>
<td><em>ERBB2</em></td>
<td>-2.5</td>
<td></td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.02368</td>
<td>-0.2</td>
<td></td>
<td>no significant change</td>
<td>MIS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.00002</td>
<td>0.3</td>
<td></td>
<td>no significant change</td>
<td>TR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Impaired DNA repair capacity and gene expression indicate haploinsufficiency in healthy heterozygous \textit{BRCA1} mutation carriers

\textit{BRCA1} plays a critical role in various cellular processes, including DNA repair, cell cycle regulation, transcriptional activation, and ubiquitin ligation (Zhang and Powell 2005). Carrying an inherited mutation in this gene significantly increases individual’s lifetime risk to develop breast, ovarian and other cancers (Antoniou, Pharoah et al. 2003; Chen, Iversen et al. 2006; Milne, Osorio et al. 2008). \textit{BRCA1} is considered as a tumor suppressor gene, i.e. both alleles of the gene have to be affected to impair its function and a single “hit” is not sufficient to enable cancer development (Knudson 1971). However, it has been suggested in several studies that a mutation in one allele of \textit{BRCA1} could already affect some of its functions and such haploinsufficiency might accelerate the loss of the second allele and lead to carcinogenesis (Gayther, Warren et al. 1995; Thompson, Easton et al. 2002; Waddell, Ten Haaf et al. 2008; Drost and Jonkers 2014). Moreover, taking into account the variability in disease manifestation among mutation carriers it is possible that different \textit{BRCA1} mutations have diverse phenotypic and haploinsufficiency effects and that both type and location of mutation may influence cancer initiation and response to treatment.

In the first part of the thesis, we aimed to gain a better understanding of the molecular and biological mechanisms which predispose \textit{BRCA1} mutation carriers to hereditary breast cancer by investigating the mechanism of haploinsufficiency in LCLs carrying different types of germline truncating and missense mutations. Using this cellular model, we analyzed \textit{BRCA1} expression, DNA repair capacity, and gene and miRNA expression profiles.

1.1 Cells derived from \textit{BRCA1} mutation carriers show variable \textit{BRCA1} expression, depending on the mutation type

First, we aimed to determine whether cells derived from carriers of various types of \textit{BRCA1} mutations (missense versus truncating) show altered expression at mRNA and protein levels. As expected, we found significantly decreased \textit{BRCA1} mRNA expression in cells harboring truncating mutation, indicating that the truncated transcripts were most probably subjected to a degradation by the nonsense-mediated mRNA decay (NMD) pathway (Conti and Izaurralde 2005). Our findings are also in agreement with the study published by Perrin-Vidou \textit{et al.}, demonstrating that most of the \textit{BRCA1} transcripts containing a premature termination
codon (PTC) are degraded by NMD which leads to up to 5-fold reduction in mRNA abundance (Perrin-Vidoz, Sinilnikova et al. 2002). Consistently with the probable degradation of PTC-containing transcripts, we were unable to detect any truncated protein in our optimized Western blotting experiments. It has been proven that some mutations fail to trigger NMD because of translation re-initiation, which is the case of the BRCA1 mutation c.68_69delAG (Buisson, Anczukow et al. 2006). However, we didn’t detect such truncated protein suggesting that it was probably degraded after its synthesis. These findings are in agreement with previous reports (Buisson, Anczukow et al. 2006; Anczukow, Ware et al. 2008) and indicate that endogenously expressed truncated BRCA1 are highly unstable and is possible to detect them by Western blotting only when overexpressed.

On the contrary to truncating mutations, the BRCA1 mRNA level in cells with heterozygous missense mutation was comparable to WT LCLs, indicating that the mutated mRNA transcripts are stable, which is in agreement with previously published results (Lovelock, Healey et al. 2006; Bouwman, van der Gulden et al. 2013). In addition, the average level of the total full-size BRCA1 protein was also similar to the level found in controls. Such finding was surprising because previous studies indicated that particularly these two mutants (p.Ala1708Glu and p.Gly1706Glu) have a severe folding defect which leads to destabilization of the protein structure (Lovelock, Healey et al. 2006; Lee, Green et al. 2010; Rowling, Cook et al. 2010). Further analysis of our results revealed that two of the three LCLs harboring the p.Ala1708Glu mutation showed lower level of total BRCA1 protein than those carrying p.Gly1706Glu. The discrepancy in the stability of these two BRCA1 variants could be explained by a previously reported partial skipping of mutated exon 18 in cells with p.Ala1708Glu mutation which might give rise to even less stable protein (Millevoi, Bernat et al. 2010).

Importantly, the presence of non-degraded mutated protein brings the possibility of a dominant-negative effect of the altered product on WT protein functions. It has been described already more than 10 years ago by Fan and colleagues that non-degraded truncated or mutated full-length BRCA1 proteins can abrogate some functions of the remaining WT BRCA1 allele (Fan, Yuan et al. 2001). Consistently with these results, it has also been found that the presence of BRCA1-Δ(1808–5556) truncated protein in mouse epithelial ovarian cancer cell line, which contains two endogenous BRCA1 WT alleles, leads to an increase of tumorigenicity in vivo and chemosensitivity (Sylvain, Lafarge et al. 2002). A more recent paper from Coene and colleagues shows the competition of the recombinant C-terminal BRCA1 fragment for binding to F-actin (Coene, Gadelha et al. 2011), further supporting the possibility of a dominant-negative function of BRCA1 mutated protein. Results from these studies together
with our findings suggest that the dominant-negative phenomenon could play a considerable role in hereditary breast and ovarian cancers, especially in carriers of \textit{BRCA1} missense mutations which maintain the mutated proteins in cells.

1.2 Heterozygous mutations in \textit{BRCA1} gene lead to haploinsufficiency in the repair of spontaneously occurring DNA damage

The observed differences in \textit{BRCA1} mRNA and protein expression between LCLs also encouraged speculations about haploinsufficiency and manifestation of distinct phenotypes depending on the \textit{BRCA1} status. Taking into account the role of BRCA1 in several DNA repair pathways, we aimed to evaluate the level of spontaneously occurring or IR-induced DNA damage (measured by detection of gamma-H2AX nuclear signal) and the efficiency of DSB repair (evaluated by counting number of RAD51 nuclear foci) in different LCLs. We were able to demonstrate that, at the basal conditions, LCLs established from mutation carriers showed increased level of DNA damage, measured by the intensity of gamma-H2AX nuclear signal. Interestingly, the same cells also showed increased proportion of hyper-phosphorylated BRCA1. As various sets of Ser residues are being phosphorylated throughout cell cycle and in response to DNA damage (Thomas, Smith et al. 1997; Okada and Ouchi 2003), and we didn’t find any significant differences in the cellular growth rate between control and mutated LCLs, it is likely that the increased ratio of hyper-P BRCA1 in mutated cells was a consequence of a higher requirement of the active (phosphorylated) protein due to the elevated level of DNA damage.

It’s worth to note that cells harboring \textit{BRCA1} truncating mutation showed the highest level of DNA damage at basal conditions and also displayed the highest ratio of hyper-P/hypo-P BRCA1. These results highlight the differences between distinct types of \textit{BRCA1} mutations and suggest that increased DNA damage (in particular dangerous DSBs) might be implicated in the initial steps of breast cancer development in carriers of truncating mutations.

Interestingly, we also observed a significantly lower amount of RAD51 foci in non-irradiated mutated LCLs when compared to control cells grown under basal conditions, indicating haploinsufficiency of mutated cells in DNA repair. Although we did not find such altered RAD51 foci formation in irradiated cells, more RAD51 foci would be expected to be formed in mutated cells taking into account the elevated level of DNA damage caused by irradiation with respect to controls. Thus, it makes us to interpret the equal level of RAD51 foci
as an actual defect of DNA repair in mutated cells. Moreover, RAD51 protein levels in the western blot were similar among LCLs suggesting that BRCA1 haploinsufficiency results in impairment of RAD51 foci formation but not of the maintenance of normal protein levels, being in line with a prior study (Bhattacharyya, Ear et al. 2000).

These results indicate that the presence of one mutated allele in the BRCA1 gene may already lead to DNA repair defects and that these cells are therefore haploinsufficient for repair of such damage which could be implicated in initial steps of neoplastic transformation and increased susceptibility to cancer. Our observation that heterozygous mutations in BRCA1 gene could lead to haploinsufficiency in the repair of spontaneously occurring DNA damage are also in line with other previously published studies. For example, Konishi et al. used MCF10A non-tumorigenic human breast epithelial cells MCF10A and introduced a common BRCA1 c.68_69delAG mutation into one BRCA1 allele and described impaired HR-mediated DSB repair (Konishi, Mohseni et al. 2011). Our results are also in agreement with the observation of a spontaneous hyper-recombination phenotype and reduced efficiency of HR repair of DSBs in MCF7 cells carrying only a single BRCA1 allele (Cousineau and Belmaaza 2007). However, most of other studies found impaired DNA repair only after irradiation of BRCA1 heterozygous cells.

The elevated level of spontaneously occurring DSBs together with impairment of their repair could increase the toxicity related to a DNA-damaging treatment in BRCA1 mutation carriers. In this regard, it has been suggested that BRCA1/2 mutation carriers could be at higher risk to develop specific side effects related to chemotherapy (Huszno, Budryk et al. 2013). Therefore, a further investigation on therapy-induced toxicity in carriers of various BRCA1 mutations would be recommended in the future.

1.3 Gene expression profiles vary depending on the BRCA1 mutation status, indicating differences in haploinsufficiency and phenotypic effects

In order to identify genes which would discriminate the LCLs according to their BRCA1 mutation status we performed a gene and miRNA expression analysis in non-treated cells. Unsupervised hierarchical cluster of expression differences between control cells and LCLs from BRCA1 mutation carriers suggested that monoallelic BRCA1 mutations do not lead to dramatic changes in gene expression profile (Fig. 4). These results are consistent with a study published by Kote-Jarai and colleagues, where they analyzed expression profiles of normal skin fibroblasts isolated from carriers of BRCA1 or BRCA2 mutation (Kote-Jarai, Matthews et al.
2006). In this study they were able to predict \textit{BRCA1/2} carrier genotypes only after induction of DNA damage after irradiation, but not under basal conditions. Similarly, Salmon et al. reported the inability to distinguish non-carriers of \textit{BRCA1} mutation from mutation carriers based on expression profiling of non-treated LCLs (Salmon, Salmon-Divon et al. 2013).

However, we noticed that cells harboring missense mutation tended to cluster away from the rest. Consistently with this finding, we observed the highest number of differentially expressed genes when comparing LCLs with missense mutation and controls. The fact that we found less differentially expressed genes when analyzing WT vs all mutated LCLs than when comparing WT cells with any of the mutation type alone suggests differences in effects of the two types of mutations. Similarly to our results, it has also been shown by Waddell et al. that missense pathogenic mutations in \textit{BRCA1} show distinct expression profile which is more similar to BRCAX individuals than BRCA1/2 samples (Waddell, Ten Haaf et al. 2008). Taking into account the comparable BRCA1 protein level between WTs and cells with missense mutation and their distinct expression profiles, it is possible that the presence of non-degraded mutated protein could either have a dominant negative effect on the WT protein functions or could lead to occurrence of novel protein interactions and activation of alternative pathways.

Interestingly, there was an overrepresentation of immune response-related genes (\textit{IFNG}, \textit{IRF5}, \textit{ICOS}, \textit{ITGB5}) that were downregulated in LCLs harboring missense mutation, suggesting that this pathway might be altered specifically in carriers of these particular mutations (Table 2). Moreover, looking at the Catalogue of somatic mutations in cancer (COSMIC), we observed that \textit{IFNG}, \textit{IRF5}, \textit{ICOS} have been previously found to be mutated in ductal breast carcinoma and three out of the four downregulated genes (\textit{IRF5}, \textit{ICOS}, \textit{ITGB5}) were described to be mutated in serous ovarian carcinoma. Importantly, Feilotter et al. have recently published a study showing alteration of gene expression due to \textit{BRCA1} haploinsufficiency and, consistently with our results, they also detected alterations in the interferon-regulated transcriptional pathway in LCLs harboring monoallelic \textit{BRCA1} mutations (Feilotter, Michel et al. 2014). It has also been shown that the tumor suppressor \textit{IRF5} is down regulated in patients with ductal carcinoma in situ and invasive ductal carcinoma and that loss of this gene correlates with increased invasiveness (Bi, Hameed et al. 2011). In addition, the involvement of the differentially expressed genes in cancer-relevant functions (such as cell death, cellular movement and proliferation) indicate that these molecular functions and the immune response pathway may be deregulated under conditions of \textit{BRCA1} haploinsufficiency and that such deregulation could contribute to a neoplastic transformation.
Another interesting gene than we found to be downregulated in carriers of BRCA1 missense mutations was PPARG (peroxisome proliferator-activator receptor gamma) that has been previously shown to be connected to breast carcinogenesis. This nuclear receptor acts as a tumor suppressor and the loss of its expression has been shown to contribute to creation of protumorigenic environment in breast tissue (Skelhorne-Gross, Reid et al. 2012; Apostoli, Skelhorne-Gross et al. 2014). Therefore, the downregulation of this tumor suppressor could, together with impaired functions of BRCA1, could contribute to the increased susceptibility of carriers of specific BRCA1 missense mutations (p.Ala1708Glu, p.Gly1706Glu) to breast cancer.

Our results from gene expression profiling suggest that mutation in one of the two alleles of the BRCA1 gene may lead to insufficient maintenance of gene expression. Moreover, we have found that cells harboring missense mutations show more altered pathways than those with truncating mutations when compared with controls, which suggest that different mutations could give rise to differences in haploinsufficiency and phenotypic effects. These findings could contribute to a better understanding of the initial steps of hereditary breast cancer and could also have implications for the surveillance and treatment of cancer patients.

2 Cells carrying germline missense mutation in BRCA1 show increased sensitivity to the PARP inhibitor OLP

Various PARP inhibitors are currently being tested in numerous preclinical or clinical trials in patients with sporadic/hereditary breast, ovarian and other cancers. The promising results from phase II studies in BRCA1/2-mutated breast and ovarian cancers allowed to proceed to a more advanced phase III (Burgess and Puhalla 2014; Liu, Konstantinopoulos et al. 2014). Despite the success, a substantial fraction of patients displayed resistance to the treatment (Fong, Boss et al. 2009), which could be at least partially explained by secondary mutations in BRCA1/2 genes, remaining a basal activity of mutated BRCA1 protein, upregulation of P-glycoprotein efflux pumps, loss of 53BP1, or overexpression of PARP1 (Rottenberg, Jaspers et al. 2008; Drost, Bouwman et al. 2011; Barber, Sandhu et al. 2013; Jaspers, Kersbergen et al. 2013; Gilabert, Launay et al. 2014).

Taking into consideration the phenotypic variability between BRCA1 mutation carriers, the second objective of this thesis was to investigate whether and how BRCA1 mutation type influences the sensitivity of cells to PARP inhibitors. In addition, the factors which could
influence the response of cells to PARP inhibitors olaparib (OLP) and veliparib (VLP), such as PARP1 expression and enzymatic activity or cellular growth rate, were examined.

2.1 Carriers of heterozygous truncating mutation in BRCA1 show reduced expression of PARP1

First, we wanted to validate the previously described synthetic lethal interaction between BRCA1 and PARP1 (Bryant, Schultz et al. 2005; Farmer, McCabe et al. 2005) by testing OLP and VLP in BRCA1-proficient and -deficient breast cancer cells. As expected, BRCA1 knockdown in BRCA1-proficient breast cancer cell line MDA-MB-231 led to a decrease in cellular viability after treatment with these PARP inhibitors. In addition to our proliferation experiment, we also observed a reduced ability to form colonies in PARP inhibitor-treated BRCA1-depleted cells, which further confirms the synthetic lethal interaction between BRCA1 and PARP1. The observed variability in the ability to create colonies which was detected between sh1 and sh5 was not related to their BRCA1-knockdown efficiency, as both of them silenced the gene in a similar level. Instead, it was probably related to its random integration in the MDA-MB-231’s genome, which could have led to an occurrence of additional mutation, and thus to a different response to the treatment.

We then examined several factors which could potentially influence the response of cells to PARP inhibitors. Unexpectedly, we found that PARP1 expression was downregulated in cells harboring truncating mutation in BRCA1. As these cells also express lower levels of BRCA1, it is possible that BRCA1 helps to maintain normal PARP1 expression. Such connection between BRCA1 and base excision repair pathway members is not new because it has been previously reported that BRCA1 stimulates the base excision repair pathway by increasing the activity of OGG1, NTH1, and REF1/APE1 (Saha, Rih et al. 2010). The role of BRCA1 deficiency in this phenotype was further confirmed by a decreased PARP1 expression in MDA-MB-231-sh1 cells, even though only a weak PARP1 expression downregulation was observed in these cells. Such variation could be explained by differences between these two cellular models or by just a partial control of PARP1 expression by BRCA1.

As various studies indicate that PARP1 expression correlates with its enzymatic activity only partially (Zaremba, Ketzer et al. 2009; Zaremba, Thomas et al. 2011), we decided to measure the activity of this enzyme by two methods. Neither detection of PAR polymers by Western blot (a less accurate measurement) nor in vitro PARP activity assay revealed any
significant differences between groups of LCLs, confirming the independency of PARP activity on the level of protein expression. Nevertheless, an elevated PARP activity in cells harboring missense mutation in comparison to cells with truncating mutation, found in both assays, indicates differences between these mutation types and could modulate their response to the drug.

2.2 The type of BRCA1 germline mutation influences the sensitivity of cells to OLP

We then evaluated the differences in the sensitivity to OLP according to the BRCA1 mutation type. Consistently with the Knudson’s model of “two-hit” hypothesis for tumor suppressors, treatment with OLP led to a comparable sensitivity of cells harboring heterozygous truncating mutations as in WT cells. Taking into account that we detected lower levels of BRCA1 and PARP1 in cells with truncating mutations, our results indicate that the expression levels of these two proteins are not associated with the response of these cells to OLP. However, it was interesting to find out that cells with heterozygous missense mutation in BRCA1 were significantly more sensitive to the treatment then both WTs and cells with truncating mutation. These findings again highlight the different behavior of missense and truncating mutants and indicate it might be beneficial to consider them separately in the clinics.

By analyzing the factors which could influence the response of cells to OLP, we found that the higher is the PARP enzymatic activity, the lower is the viability of cells after the treatment. Such negative correlation indicates that the sensitivity to OLP is, at least partially, dependent on the ability of PARP to play its enzymatic role. Previous research by Gottipati et al. shows that hyperactivation of PARP1 is associated with an increased sensitivity of cells to PARP inhibitors, and they relate the increased sensitivity to these agents to a homologous recombination-defective phenotype (Gottipati, Vischioni et al. 2010). Our results from immunofluorescence experiment indicate that cells harboring either of the BRCA1 mutation types show alteration in DNA repair, as shown by a significantly decreased level or RAD51 foci. However, our experimental desing allows only indirect evaluation of homologous recombination efficiency, which brings the possibility that a more stringent assay (e.g. the Traffic Light Reporter System described by (Certo, Ryu et al. 2011) could reveal additional differences between missense and truncating mutations. As expected, the cellular growth rate positively correlated with the viability of OLP-treated cells, which is in line with other studies showing that rapidly dividing cells are more sensitive to DNA damaging agents (Stark, Zhang et
However, the significant differences in response of mutated cells to OLP or VLP were maintained after incorporating the growth rate as a covariate, indicating that the difference in sensitivity to the agent is independent on the growth rate.

Taking into account the expected presence of mutated protein in cells carrying missense mutation and the absence of truncated protein in cells with truncating mutation, there is a possibility that the missense mutants act in a dominant negative manner on the WT protein function. Considering our results, we propose a model which involves a dominant negative effect of the BRCA1 BRCT missense mutant and a previously described two-phase BRCA1 recruitment to the DNA DSBs (Baer 2013; Li and Yu 2013). Based on the research carried on by Li et al., WT BRCA1 heterodimerizes with BARD1 through its RING domain and is recruited into DSBs by interaction of BARD1 with PAR polymers within an minute post damage (early recruitment) (Figure 28). On the other hand, during the late recruitment, BRCA1 is directed into damaged chromatin within minutes after damage. The later phase requires functional BRCA1 BRCT domains and is dependent on a direct interaction with p-Abraxas, a member of a BRCA1-A complex subunit.

OLP treatment inhibits the early recruitment of BRCA1 into DSBs by inhibition of PAR polymeration, which makes the cells more dependent on the late recruitment phase. Cells harboring heterozygous truncating mutation in BRCA1 gene usually lack the mutated protein due to the degradation of a mutated transcript by NMD mechanism. But the remaining WT protein can function in the DSB repair through late recruitment phase. On the contrary, cells carrying heterozygous missense mutation in BRCA1 contain both the functional (WT) and defective (mutant) proteins which could compete to undergo the late recruitment path to repair DNA DSBs. In addition, the slight elevation of PARP activity in these cells makes them even more dependent on the late phase after treatment with OLP. We therefore hypothesize that the competition of mutated and WT protein could lead to altered DNA repair process and decreased viability of cells carrying BRCA1 missense mutation.
Figure 28. Two-phase recruitment of BRCA1 to distinct subcompartments of a DSB. During the early recruitment phase, BRCA1 interacts with BARD1 through their RING domain and a recruitment of the heterodimer into DSB is mediated by BARD1 BRCT recognition of PAR. On the other hand, the late phase is dependent on BRCA1 BRCT domains and their interaction with phosphorylated Abraxas. Adapted from Baer et al., based on the results from Li et al.

Importantly, the OLP concentration range, which was used in our experiments, was comparable to the peak plasma levels found in patients treated with these agents in clinical trials (Fong, Boss et al. 2009). On the other hand, the VLP concentrations used in our experiments were few fold higher than the maximal plasma levels in treated patients or animals (Donawho, Luo et al. 2007; Kummar, Kinders et al. 2009). Higher VLP dosage was also used in our breast cancer cellular model with silenced BRCA1 in order to achieve synthetic lethality between BRCA1 and PARP1, and such high concentrations were also necessary in other in vitro studies (Horton, Jenkins et al. 2009; Stordal, Timms et al. 2013). Such difference between PARP inhibitors could be related to a lower potency of VLP to trap PARP1 at the DNA damage site (Murai, Huang et al. 2012), an alternative mechanism of function of PARP inhibitors, and is a reason behind choosing OLP for exploring the mechanism of action of PARP inhibitors in mutation carriers.
3 OLP changes BRCA1 BRCT protein interactors and leads to downregulation of breast cancer-related genes ERBB2 and FOS

In the last part of the thesis, we aimed to explore the mechanism of increased sensitivity of cells carrying missense mutation to PARP inhibitor OLP. We used transcriptional assay to assess how BRCA1 missense variants and OLP treatment influence the ability of cells to activate transcription of target genes. In addition, utilizing the mass spectrometry technique allowed us to determine the OLP-specific BRCT-protein interactors. Finally, we explored which genes or miRNAs are involved in the response of LCLs to this PARP inhibitor and performed integration of the high-throughput data.

3.1 Transcriptional activation function of the BRCA1 BRCT domain does not seem to be involved in the response of cells to OLP

There are several structural and functional assays, including protease sensitivity, peptide binding activity and specificity, and transcriptional activity assays, which have been developed to test specific functions of BRCA1 and to determine the effect of BRCA1 variants (Lee, Green et al. 2010). The missense mutations represented in our panel of LCLs are located specifically in the BRCA1 BRCT domain, which is known to be involved in the maintenance of genomic stability and transcriptional activation of target genes (Roy, Chun et al. 2011). Therefore, we used a well described transcriptional activation assay to check how the missense variants and OLP treatment influence this BRCT-related function. As expected, WT protein strongly induced transcription of the target gene, whereas both missense variants disrupted the transcriptional activation function of BRCA1. These findings are in agreement with previous studies and indicate the pathogenicity of these missense mutations (Phelan, Dapic et al. 2005; Carvalho, Pino et al. 2009; Lee, Green et al. 2010). In order to mimic the heterozygous status present in mutated LCLs, we also overexpressed both WT and A1708E missense variant which allowed us to explore a potential dominant negative effect of the mutated protein. However, we observed that a simultaneous expression of both WT and mutant variant did not affect the phenotype and the transcriptional activation was comparable to the response of WT alone, suggesting that the variant does not influence the WT transcriptional activation function in a dominant negative manner. Interestingly, OLP treatment led to a slight decrease of the transcriptional activation, however only less than 50% of the WT activity is generally considered as deleterious (Carvalho, Pino et al. 2009). Hence this function of the BRCA1 BRCT domain seems not to be
dramatically affected by OLP treatment and our results indicate that the increased sensitivity of cells carrying heterozygous missense mutation to OLP is not related to an alteration of the BRCA1 transcriptional activation function.

3.2 OLP downregulates the interaction of BRCA1-A complex with BRCA1 and induces phosphorylation of threonine residue at position 1834

The ability to activate transcription of target genes is not the only function mediated by the BRCT domain of BRCA1. The number of BRCT protein interactors allows BRCA1 to play a role also in such complex cellular processes like cell cycle checkpoint regulation and maintenance of genomic stability (Roy, Chun et al. 2011). Thus, in order to further puzzle out whether and how OLP treatment affects functions mediated by the BRCA1 BRCT domain, we looked at the BRCA1 BRCT protein interactors in OLP-treated cells. From the 121 BRCT interactors, identified using a proteomics approach, most of them were found in both control and OLP-treated cells, indicating that the treatment did not lead to dramatic changes of protein complexes. The 11 identified OLP treatment-specific BRCA1 BRCT interactors were found to be involved in various biological processes, such as cell cycle and transcription regulation (www.genecards.org). The most interesting protein from the selection was NUMA1 because it has been previously described as an acceptor of PAR polymers from tankyrase 1 (PARP-5a). Such PARylation of NUMA1 is required for a proper assembly of mitotic spindle pole, chromatin remodeling, and RAD51-dependent homologous recombination repair (Chang, Dynek et al. 2005; Chang, Coughlin et al. 2009; Vidi, Liu et al. 2014). Moreover, it has also been recently suggested that NUMA1 forms a complex with BRCA1, PARP-5a, and ATM, and that creation of this protein complex is required for the PARylation of NUMA1 and its proper function in mitosis (Palazzo, Della Monica et al. 2014). However, as PARP inhibitor OLP does not inhibit enzymatic activity of PARP-5a (Wahlberg, Karlberg et al. 2012), it is likely that OLP-treatment and the subsequent increased binding of NUMA1 to BRCA1 does not affect function of NUMA1.

Unexpectedly, OLP treatment induced phosphorylation of BRCA1 on the threonine residue at position 1834 which is located at the very end of the C-terminal BRCT domain. Phosphorylation of more than 100 distinct sites have been identified in BRCA1 up to date (www.phosphosite.org), however their involvement in various cellular processes is elucidated only for some of them. For example, it has been known already for a long time that ATM-
dependent phosphorylation of S1423 and S1524 is critical for BRCA1-mediated DNA damage response (Cortez, Wang et al. 1999) and activation of UV-induced apoptosis (Martin and Ouchi 2005). On the other hand, a promotion of error-free HR and suppression of the error-prone NHEJ repair pathways is independent on the phosphorylation on serines 1423 and 1524, and is related to a Chk2-mediated phosphorylation at S998 (Zhang, Willers et al. 2004). As the OLP-induced phosphorylation of T1834 has not been previously described, we can only hypothesize that it might serve as an activator of downstream pathways related to a response to this treatment.

In order to shed light on the molecular processes regulated by the set of BRCA1 BRCT interactors and to determine how OLP could influence these processes, we performed an enrichment analysis with IPA. The investigation revealed that BRCA1 interacts with proteins involved in canonical pathways such as DNA damage response, hereditary breast cancer and oestrogen signaling, or assembly of RNA polymerase II Complex. Such results are in line with previously known roles of BRCA1 BRCT in the maintenance of genome integrity and regulation of cell cycle checkpoint and gene expression (Roy, Chun et al. 2011). Consistently with these results, the BRCT interactors also created a network of interconnected proteins which were mainly involved in DNA replication, recombination, repair, gene expression functions.

Interestingly, the canonical pathway “Role of BRCA1 in DNA Damage Response” seemed to be downregulated upon OLP treatment. In particular, most of the members of the BRCA1-A complex (RAP80, BRCC3, Abraxas), showed decreased binding to BRCA1 BRCT in response to OLP treatment. As this complex plays a crucial role in DNA damage response by recognition of ubiquitinated H2AX and recruiting BRCA1 into the DNA damage site (Wang, Matsuoka et al. 2007; Wang, Hurov et al. 2009), our results indicate that OLP treatment might partially inhibit this BRCA1-mediated function. Coming back to our model of dominant-negative effect of missense variants, it seems that OLP treatment does not only inhibit the PAR-dependent early phase but partially also the BRCA1-A complex-dependent late phase of BRCA1 recruitment into DSBs in normal cells. This could make the cells dependent on the remaining function of the late BRCA1 recruitment phase in case of DSBs occurrence. The potential competition of the WT and defective BRCA1 protein in carriers of BRCA1 missense mutation could thus lead to intolerable effects and consequently to a decreased viability of OLP- treated mutant cells.

The inability to perform an identical analysis of the protein interactors of mutated BRCA1 only allowed us to speculate that cells carrying missense mutation in this domain could display a different set of protein interactors. The mutation-mediated change of interacting partners or
their abundance could then alter the response of mutated cells to OLP treatment and might also disrupt their ability to phosphorylate the OLP-specific T1834 site. Clearly, further methodological optimization will be necessary to express the mutated BRCA1 BRCT in a sufficient level and to be able to address our speculations.

3.3 PARP inhibitor OLP changes gene and miRNA expression profiles and downregulates expression of breast cancer-related genes ERBB2 and FOS

The transcriptional activation assay and mass spectrometry approach gave us an idea of which BRCA1-BRCT-related functions are changed in response to PARP inhibition by OLP. In order to get more complex understanding of the cellular response to OLP and the effect of BRCA1 mutation type in sensitivity of cells to this agent we studied genes and miRNA expression in our panel of treated LCLs.

It was surprising that when we used the most stringent threshold of significance (FDR < 0.15) we found more than two thousand DEg between DMSO- and OLP-treated LCLs harboring truncating mutation in BRCA1, but no genes showed differential expression in WTs or cells with missense mutation. Looking at the data for each particular truncating cell line revealed that OLP induced only small but very consistent changes in gene expression. On the contrary, the OLP-mediated changes of gene expression were much higher and not so consistent among WT cells, as indicated by the high number of genes showing more than 2-fold logarithmic change. The inability to detect any DEg in WT or missense LCLs when applying FDR < 0.15 was probably related to a lower number of cell lines in these two groups. In addition, a clear outlying pattern of one WT DMSO-treated cell line (11S66-L) probably contributed to an increased heterogeneity between LCLs and did not allow any gene to reach the stringent significance threshold.

By investigating the OLP-induced DEg in WT cells (using the less stringent threshold, logFC > |2|) we found 312 DEg and got better idea about the standard response of cells to this agent. The functional analysis of these 312 genes with IPA revealed a significant downregulation of cancer-related genes in OLP-treated cells, which could indicate that the global effect of this agent is not damaging in non-mutated cells. Interestingly, four well described breast cancer-related genes (ERBB2, FOS, HSPB2, AGR3) were found to be significantly downregulated by the treatment. The amplification and/or overexpression of ERBB2 have been related to breast, ovarian, and other cancers (Hynes and Lane 2005). Breast
cancer patients expressing high levels of this protein are nowadays treated with Herceptin, a humanized monoclonal antibody against the extracellular domain of ERBB2, which can delay mortality from 9 months to 3 years (Slamon, Leyland-Jones et al. 2001). The second downregulated gene in response to OLP was FOS which encodes a leucin zipper protein and forms part of the transcription factor complex AP-1 (Jochum, Passegue et al. 2001). There are many evidences of the connection of FOS with breast cancer, e.g. it has been described to support growth of malignant breast cancer and has been involved in mammary cell proliferation and transformation (Lu, Shen et al. 2005; Motrich, Castro et al. 2013). The HSPB2 gene encodes for a protein which belongs to the superfamily of small heat-shock proteins, which are normally induced under environmental stress (Georgopoulos and Welch 1993). Up to date, there are numerous studies linking this protein to breast cancer, for example HSPB is known to serve as a risk factor of malignant progression in benign proliferating breast lesions and is associated with resistance to Herceptin and other agents (O'Neill, Shaaban et al. 2004; Kang, Kang et al. 2008). Lastly, the AGR3 gene, also called breast cancer membrane protein 11 (BCMP11), was initially identified in membranes of breast cancer cell lines (Adam, Boyd et al. 2003). AGR3 is implicated in the growth and metastasis of hormone-responsive breast tumors and has been associated with survival and differentiation in ovarian cancer (Fletcher, Patel et al. 2003; King, Tung et al. 2011). Clearly, more functional studies will be necessary in order to make a conclusion about the role of these genes in the response of patients to this treatment.

Finally, the miRNA expression profiling and its integration with gene expression were performed to further complete our understanding of the response of cells to OLP. All three groups of LCLs showed significant OLP-induced changes of miRNome, among which most of the DEm (applying the stringent significance threshold of FDR < 0.05) were common for all LCLs. As the miRNAs are negative regulators of gene expression, we performed an integration study between DEm and DEg and selected those miRNAs and their target genes whose expression negatively correlated. As in the case of gene expression profile, we were initially interested in the common response of WT cells to OLP and then we checked the behaviour of selected targets in mutated cells. What caught our attention was that OLP increased the expression of miR-222-3p which has been previously validated to target FOS gene (Errico, Felicetti et al. 2013), and miR-548ap-5p which is silencing the ERBB2 gene (Chen, Sun et al. 2009). Therefore it seems that, in WT cells, OLP stimulates expression of these two miRNAs which in turns leads to a downregulation of the expression of breast cancer-associated genes ERBB2 and FOS. Surprisingly, we found a completely opposite regulation of miR-222-3p and its target FOS by OLP treatment in cells carrying missense, but not truncating, mutation in BRCA1.
gene. Taking these results into account, we speculate that OLP could induce a favorable effect by FOS downregulation in non-carriers or patients carrying truncating mutation, whereas this effect is not induced in patients with G1706E and A1708E missense mutations in BRCA1.

Overall, our results suggest that carriers of different types of BRCA1 mutations could benefit from the treatment in a distinct way and could show different toxicity to PARP inhibitor OLP. It is also possible that BRCA1 mutation type could be at least partially responsible for the observed huge variability in response to this agent in patients. Our findings are also relevant for the potential use of PARP inhibitors as prophylactic agents in BRCA1 mutation carriers, because such treatment is likely to lead to variable responses, depending on the mutation type of the carrier. In addition, considering our results and that two of the three most recurrent mutations in Spain are missense mutation, further research on differences between BRCA1 mutation types could be of a great importance, particularly in this population.
CONCLUSIONS
1. Carriers of **BRCA1** truncating mutation express less BRCA1 mRNA and protein, most probably due to degradation of the non-stable truncated transcripts by the NMD pathway. On the contrary, cells harboring the missense mutations p.Ala1708Glu and p.Gly1706Glu show similar average mRNA and protein expression levels as controls, which opens up the possibility of a putative dominant-negative effect.

2. At the basal conditions, LCLs established from carriers of heterozygous truncating mutations showed increased level of DNA damage. Both missense and truncating mutants showed impaired DNA repair, indicated by decreased ability to form RAD51 foci. These results suggest haploinsufficiency of the mutated cells in DNA repair and a possible mechanism of increased susceptibility of carriers to cancer.

3. Gene expression profiles vary depending on the **BRCA1** mutation status. Cells carrying missense mutations tend to cluster away from the rest and have the most distinct gene expression profile, showing overrepresentation of downregulated immune response-related genes. These results indicate that different mutations could have different phenotypic effects.

4. Cells derived from carriers of truncating mutations showed similar sensitivity to the PARP inhibitor olaparib as non-mutated controls. However, carrying a missense mutation in the **BRCA1** gene increased the sensitivity of the cells to this agent. Our results strongly indicate that carriers of different types of **BRCA1** mutations could benefit from the treatment in a distinct way and that could show different toxicity to the PARP inhibitor olaparib.

5. Proteomic analysis of BRCA1 BRCT protein interactors revealed downregulation of BRCA1-A complex binding upon treatment with olaparib. This PARP inhibitor also induced phosphorylation of a threonine residue at BRCA1 BRCT domain at position 1834 which has not been previously reported and could therefore be a new marker of response to olaparib.

6. In normal cells, olaparib increased the expression of miR-548ap-5p and miR-222-3p which are targeting **ERBB2** and **FOS** genes, respectively. These genes were significantly downregulated as a response to olaparib treatment. Interestingly, the opposite regulation of miR-222-3p and its target FOS by olaparib treatment in cells carrying missense mutation suggest a possible use of these molecules as markers of sensitivity to the treatment.
CONCLUSIONES
1. Las portadoras de mutaciones de proteína truncada en heterocigosis en el gen BRCA1 expresan niveles más bajos de ARNm y proteína, probablemente debido a la degradación de los transcritos no estables a través del mecanismo NMD. Por el contrario, las células portadoras de mutaciones de tipo cambio de aminoácido (missense) p.Ala1708Glu and p.Gly1706Glu muestran niveles de ARNm y proteína similares a los controles, lo que abre la posibilidad de que ejerzan un posible efecto dominante-negativo.

2. En condiciones basales, las LCLs establecidas a partir de portadoras de mutaciones de proteína truncada mostraron los mayores niveles de daño en el ADN. Tanto las LCLs portadoras de mutaciones de proteína truncada, como las portadoras de mutaciones missense mostraron defectos en la reparación del ADN, indicada por una reducción en la formación de focos de la proteína RAD51. Estos resultados sugieren que las células portadoras de mutaciones en heterocigosis en BRCA1 son haploinsuficientes para la reparación del daño en el ADN.

3. Los perfiles de expresión génica de las LCLs portadoras de mutaciones en heterocigosis en el gen BRCA1 varían dependiendo del tipo de mutación. Las portadoras de mutaciones missense mostraron el perfil de expresión más diferente, observándose una sobrepresentación de genes implicados en la respuesta inmune con niveles de expresión reducidos. Estos resultados sugieren que las diferentes mutaciones pueden tener distintos efectos fenotípicos.

4. Las células derivadas de portadoras de mutaciones de proteína truncada en heterocigosis mostraron la misma sensibilidad que los controles al inhibidor de PARP olaparib. Sin embargo las células portadoras de mutaciones missense mostraron una sensibilidad incrementada al fármaco. Nuestros resultados sugieren que portadores de diferentes tipos de mutaciones en BRCA1 podrían beneficiarse de forma distinta del tratamiento con olaparib y presentar distinta toxicidad al mismo.

5. El análisis de las proteínas que interaccionan con el dominio BRCT de BRCA1 reveló una regulación negativa del complejo BRCA1-A tras el tratamiento con olaparib. El tratamiento también indujo la fosforilación de un residuo treonina en la posición 1834 de BRCA1 que no ha sido previamente descrito. Estas modificaciones podrían ser importantes de cara a entender el mecanismo de sensibilidad de las células a olaparib.

6. En las células normales, el tratamiento con olaparib aumenta la expresión de miR-548ap-5p y miR-222-3p que actúan sobre los genes ERBB2 y FOS respectivamente. La expresión de estos genes disminuyó de una forma estadísticamente significativa tras el tratamiento con olaparib. Encontramos un efecto contrario en la regulación de estos genes en las células con mutaciones missense, las más sensibles al tratamiento, sugiriendo que estos miRNAs y genes podrían ser marcadores de sensibilidad al mismo.
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APPENDIX
Supplementary figures
Supplementary Figure S1. Effect of the BRCA1 germline mutation on the level of DNA damage and repair. (A) High-throughput microscopy quantification of gamma-H2AX signal intensity in nuclei of WT LCLs or cells harboring monoallelic missense or truncating mutation in BRCA1. The data represent a signal intensity detected in 240 individual nuclei of each LCL in the group and the grey line indicates mean intensity of the gamma-H2AX signal. The differences between groups were evaluated using Mann-Whitney U test (*** represents P<0.001) (B) Average number of RAD51 foci per nucleus in control cells or cells with heterozygous missense or truncating mutation in BRCA1. The data represent a mean from 240 analyzed nuclei of each LCL in the particular group +/- SEM. The P-value was calculated using Mann-Whitney U test (** represents P<0.01).
Supplementary Figure S2. Involvement of the differentially expressed genes in cells with missense mutation in cell death and inflammatory response pathways. The network shows interaction of 27 (cell death pathway) and 16 (inflammatory response pathway) genes. Underlined genes belong to our set of differentially expressed genes between WTs and cells with missense mutation in BRCA1. The legend specifies the molecule type and type of interaction between molecules.
Supplementary Figure S3. Differentially expressed miRNAs in groups of LCLs carrying WT or mutated \textit{BRCA1}. Venn diagram and a table illustrating the number and names of miRNAs being differentially expressed (unadjusted P-value ≤ 0.05) between LCLs harboring WT, missense (MIS) or truncated (TRUN) \textit{BRCA1}.
Supplementary Table S1. List of human GIPZ lentiviral shRNAmir set against BRCA1

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Supplementary Table S2. Molecular and cellular functions related to genes differentially expressed in WTs and cells with missense mutation in BRCA1.

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Supplementary Table S3. Molecular and cellular functions related to genes differentially expressed in WTs and cells with truncating mutation in BRCA1.

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Supplementary Table S4. BRCA1 BRCT protein interactors identified by mass spectrometry in DMSO- or OLP-treated HEK293FT cells.

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First author publication summarizing the “Results – Part I”, submitted to Genetics in Medicine in December 2014.

TITLE: Impaired DNA repair capacity and gene expression indicate haploinsufficiency in healthy heterozygous BRCA1 mutation carriers

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