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# Endothelial Cell-specific Loss of Breast Cancer Susceptibility Gene 2 Exacerbates Atherosclerosis

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**Abstract**

Despite mounting concern over the increased risk of cardiovascular disease in breast cancer patients, studies evaluating the common genetic/molecular link between these diseases are limited. Mutations in the breast cancer susceptibility genes (BRCA1&2) predispose carriers to breast and ovarian cancers due to compromised DNA damage repair capacity leading to DNA damage accumulation; in cancer cells, and in other cell-types including endothelial cells, causing atherosclerotic hallmarks of endothelial dysfunction/apoptosis. We present a new understanding of BRCA2's protective functionality in the setting of atherosclerosis. Studies have thus far demonstrated that while loss of endothelial BRCA2 in mice does not affect a molecular or functional baseline phenotype, *endothelial cell-specific loss of BRCA2 exacerbates high-fat diet-induced atherosclerosis in ApoE$^{-/-}$ mice*. This study illuminates BRCA2 as a potential therapeutic target in cardiovascular disease and suggests a requirement for studies evaluating the genetic predisposition of BRCA2-mutation carriers for increased risk of atherosclerosis and other cardiovascular diseases.

**Keywords**

Atherosclerosis, BRCA2, DNA damage and repair, BRCA2, Endothelium, Breast cancer

**Summary for Lay Audience**

In Canada, as in most developed countries, the cardiovascular disease atherosclerosis which is the buildup of vessel-occluding plaque, is a leading cause of illness and death. According to the most recent data, one in five deaths in Canada is due to atherosclerosis-associated cardiovascular dysfunction. Despite great medical advances, therapies are urgently needed to improve cardiovascular function in atherosclerosis. Endothelial cells, which line the innermost layer of blood vessels, play important roles in maintaining blood vessel function. Understanding the mechanisms underlying abnormal endothelial function may serve to uncover novel therapeutic approaches to treat atherosclerosis and associated cardiovascular dysfunctions. DNA is the genetic material present in every cell type, and it is prone to damage by various stressors. BRCA2 is a protein molecule, which maintains DNA integrity by repairing the damaged DNA. Loss of BRCA2 function causes breast and ovarian cancer. DNA damage not only causes cancer but also plays important role in the development of cardiovascular diseases. Our aim is to understand if the loss of endothelial BRCA2 results in increased endothelial cell death, endothelial dysfunction, and if it also promotes atherosclerosis-associated cardiovascular diseases. In this thesis, we will use an animal model of atherosclerosis which lacks BRCA2, only in their endothelial cells. We aim to investigate if there will be increased atherosclerosis-associated endothelial dysfunction and endothelial cell death after feeding these animals a high fat diet. Our study will delineate a new role of BRCA2 in atherosclerosis, which may help identify BRCA2 as a new therapeutic target to treat cardiovascular diseases. Our

study may also indicate a cancer-independent increased susceptibility of cardiovascular disease development in carriers of a BRCA2 mutation.

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## Table of Contents

**Abbreviations**

| | |
|---|---|
| **2FLY** | 2-furoyl-LIGRLO-NH2 |
| **ACh** | acetylcholine |
| **A-NHEJ** | alternate non-homologous end joining |
| **ARE** | antioxidant response element |
| **ATR** | ataxia telangiectasia |
| **ATM** | ataxia telangiectasia mutated |
| **BARD1** | BRCA1 associated ring domain 1 |
| **BER** | base excision repair |
| **BRCA** | breast cancer susceptibility gene |
| **BRIP1** | BRCA1 interacting helicase 1 |
| **C-terminal** | carboxyl terminus |
| **cGAS** | cyclic GMP/AMP synthase |
| **CHD4** | chromodomain helicase DNA binding protein 4 |
| **Chk1** | checkpoint kinase 1 |
| **Chk2** | checkpoint kinase 2 |
| **CtIP** | CtBP-interacting protein |
| **CVD** | cardiovascular disease |
| **DBD** | DNA binding domain |
| **DDR** | DNA damage response |
| **DNA** | deoxyribonucleic acid |
| **DSB** | double stranded break |
| **DSS1** | deletion of SUV3 suppressor 1 |
| **EC** | endothelial cell |
| **H2AX** | histone family member X |
| **HIF-1α** | hypoxia-inducible factor 1 alpha |
| **HFD** | high-fat diet |
| **HRR** | homologous recombination repair |
| **$IP_3R$** | inositol 1,4,5-triphosphate receptor |
| **Keap1** | Kelch-like ECH-associated protein 1 |
| **LDL** | low density lipoprotein |
| **LOH** | loss of heterozygosity |
| **MDC1** | mediator of DNA damage checkpoint protein 1 |
| **MMEJ** | microhomology-mediated end joining |
| **MRE11** | meiotic recombination 11 |
| **MRN** | MRE11-Rad50-NBS1 |
| **mtDNA** | mitochondrial DNA |
| **NER** | nucleotide excision repair |
| **NHEJ** | non-homologous end joining |
| **Nrf2** | nuclear factor erythroid 2-related factor 2 |
| **NSB1** | Nijmegen breakages syndrome 1 |
| **oxLDL** | oxidized low density lipoprotein |

| | |
|---|---|
| **$p21^{WAF1/cip1}$** | cyclin dependent kinase inhibitor 1 |
| **PALB2** | partner and localizer of BRCA2 |
| **PAR2** | protease activated receptor 2 |
| **Rad50/51** | Rad50/51 recombinase |
| **RAP80** | receptor-associated protein 80 |
| **ROS** | reactive oxygen species |
| **RNF8** | ring finger protein 8 |
| **SMC** | smooth muscle cells |
| **SNP** | single nucleotide polymorphisms |
| **SNP** | sodium nitroprusside |
| **SSB** | single stranded break |
| **STING** | stimulator of interferon genes |
| **TNFα** | tumor necrosis factor alpha |
| **TP53/p53** | tumor protein 53 |
| **tRNA** | transfer RNA |
| **TUNEL** | terminal deoxynucleotidyl transferase dUTP Nick-End Labeling |
| **UV** | ultraviolet |
| **VGEF** | vascular endothelial growth factor |
| **VSMC** | vascular smooth muscle cell |

## Chapter 1. Introduction

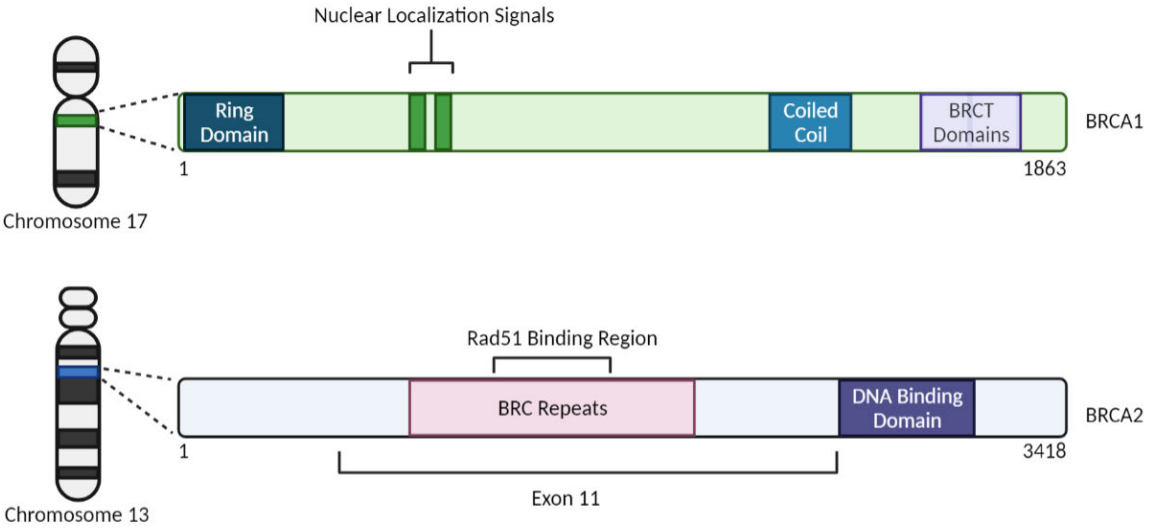
**1.1 Overview:** Cardiovascular disease (**CVD**) is a leading cause of death and morbidity in developed nations, and there are many overlapping characteristics between cancer and CVD. Atherosclerosis is an inflammatory cardiovascular disease of the cells lining the vasculature (endothelial cells), wherein DNA damage and apoptosis occur as disease hallmarks and are overlapping factors with cancer. The breast cancer susceptibility gene 2 (**BRCA2**) repairs DNA damage and protects against apoptosis; this mechanism may play a role in mitigating atherosclerosis – warranting the study of BRCA2 in CVD progression. This research was performed to determine if BRCA2 has anti-atherosclerotic properties and contextualize BRCA2 within the landscape of cardio-oncology. We aim to cultivate progress and a better understanding of the molecular roles and clinical implications of disrupted BRCA2 function. By doing so, we may provide an impetus for expanded BRCA2 screening and the development of novel therapeutics to advance patient care for those carrying a BRCA2 mutation.

**1.2 Thesis Outline:** The work herein seeks to address two main points. 1) We address if mice with endothelial cell-specific Cre-mediated deletion of BRCA2 have a baseline phenotype. Assessing the effect of EC-specific loss of BRCA2 without stress will provide a foundational understanding of this model and will further help delineate the effect of disease-related stress in the same mouse model. 2) We determine if $BRCA2^{endo}$ mice on an $ApoE^{-/-}$ background, fed a high-fat diet, demonstrate a genotype-dependent exacerbation of atherosclerosis compared to the $ApoE^{-/-}$ mice.

## Chapter 2. Literature Review and Background

**2.1 Tumor Suppressor Genes**: Tumor suppressor genes are genes wherein loss of function may promote cancer through unmitigated growth and proliferation. They are subdivided into caretakers, which maintain genomic integrity, and gatekeepers, such as tumor protein 53 (TP53/p53), which control proliferation, differentiation, and apoptosis; thereby regulating tumorigenesis.(Heemst et al., 2007)(Kinzler & Vogelstein, 1997) Inactivation of both types of tumor-suppressor genes synergistically enhances tumorigenic potential. **BR**east **CA**ncer Susceptibility genes **1** and **2** (**BRCA1&2**) are prime examples of caretakers; importantly though, they also provide gatekeeper functionality by virtue of their participation in cell cycle regulation and DNA stabilization, which regulates cellular homeostasis. Thus, BRCA1&2 are key genes of interest in cancer, as well as diseases of disrupted cellular homeostasis.

**2.2 BRCA1&2**: The BRCA1&2 share the tumor suppressor classification primarily as caretakers on the basis of their determined role in a common pathway of homologous recombination repair (**HRR**).(Roy et al., 2012) The BRCA1 gene-product is 1863 amino acids in length; its gene is located on the long arm of chromosome 17 at the position 21.31, encompassing 22 exons coding for a 220kDa protein (**Figure 2-1**). BRCA2 is comprised of 3418 amino acids; its gene is found on the long arm of chromosome 13 at position 13.1 and contains 26 coding exons that produce a 384kDa protein (**Figure 2-1)**. Both BRCA1 and 2 proteins are produced in the cytosol and contain nuclear localization signals for nuclear trans-localization – enabling their function as DNA damage repair proteins.




**2.3 DNA Damage and Repair:** DNA damage represents a constant threat to cellular equilibrium. It arises from a multitude of sources, and, depending on the source and cell type, the number of events range from between less than 1 to over 100,000 times per cell per day.(Ciccia & Elledge, 2010) This ultimately results in an estimated intergenerational mutation rate of $1.1 \times 10^{-8}$ per site.(Roach et al., 2010)

Exogenous DNA damage results from exposures to externally derived environmental factors such as chemicals or ultraviolet (**UV**) and ionizing radiation, causing modifications to the structure of nucleic acids.(Hakem, 2008) Endogenous DNA damage typically occurs as DNA undergoes hydrolysis or engages in chemical reactions with electrophiles or reactive species.(Marnett & Plastaras, 2001) Even the tightly regulated process of DNA replication and repair may generate enzymatically driven polymerization errors.(Sharma

& Chowdhury, 2012) Therefore, cells require different strategies and machinery in the form of the DNA damage response (**DDR**) pathway to recognize and repair the various types of genomic damage and alterations. While there is some redundancy, loss of function of primary repair or sensing protein mechanisms in the DDR pathway results in unchecked damage, mutations, and cellular dysregulation; these events enable the accumulation of DNA damage, which progressively overwhelms cellular repair capacity and results in genomic instability.(Negrini et al., 2010) Repair strategies vary depending on different factors including the type of DNA damage and during which phase of the cell cycle the damage occurs. While some lesions in the DNA may be directly fixed by chemical reaction through a repair strategy known as **direct reversal**,(Ca et al., 2020)(Ragg et al., 2000) extensive genomic insult requires more elaborate repair networks.

Single-stranded break (**SSB**) damage is a discontinuation of one strand of the double helix and commonly results from oxidative stress,(Caldecott, 2008) ionizing radiation, or occurs during the repair of UV radiation-induced damage.(Myllyperkiö et al., 1999) Consequences of SSBs include replication fork collapse and transcriptional blocking.(Caldecott, 2008) SSBs are repaired by excision; specifically, base excision repair (**BER**) or nucleotide excision repair (**NER**) mechanisms, which recognize and remove one to multiple damaged bases, respectively,(Hakem, 2008) before DNA polymerase utilizes the opposite strand as a repair template.

Double-stranded breaks (**DSBs**) are a full cleavage of the double helix structure of DNA. They occur *via* oxidative stress or the introduction of genotoxic agents such as ionizing

radiation and chemotherapeutics. These breaks have the potential to be the most deleterious for genomic integrity, and the outcome of their repair is mechanism and cell cycle dependent.

Non-Homologous End Joining (**NHEJ**) of DSBs takes place without a template, and consequently, the re-attachment of broken DNA occurs through end trimming and resection.(Hakem, 2008) Subsets of this are alternative non-homologous end joining (**A-NHEJ**) and microhomology-mediated end joining (**MMEJ**). In the G1 phase, the absence of a sister chromatid requires that repair of DSBs occurs through NHEJ(Mathiasen & Lisby, 2014)(Zhao et al., 2017) or MMEJ,(Yun & Hiom, 2009) however, DSB repair in the absence of homology is *prone to errors* and deletions and thus, this method will inevitably result in mutation at the site of repair.(Chang et al., 2017)

Homology directed repair, or homologous recombination repair (**HRR**), of DSBs occurs when there is a homologous chromosome or sister chromatid available to be used as the template. HRR is the *error-free* preferred method during the S and G2 phases of the cell cycle.(Zhao et al., 2017)(Mathiasen & Lisby, 2014) Homology directed repair pathway is quintessential for genomic maintenance and is the pathway along which BRCA1&2 operate.

**2.4 Homology Directed Repair Pathway**: Homologous Recombination Repair (**Figure 2-2**) involves a complex network of proteins that sense DNA damage and initiate a signalling cascade that effects the virtually error-free DSB repair mechanism using complementary DNA or a sister chromatid.

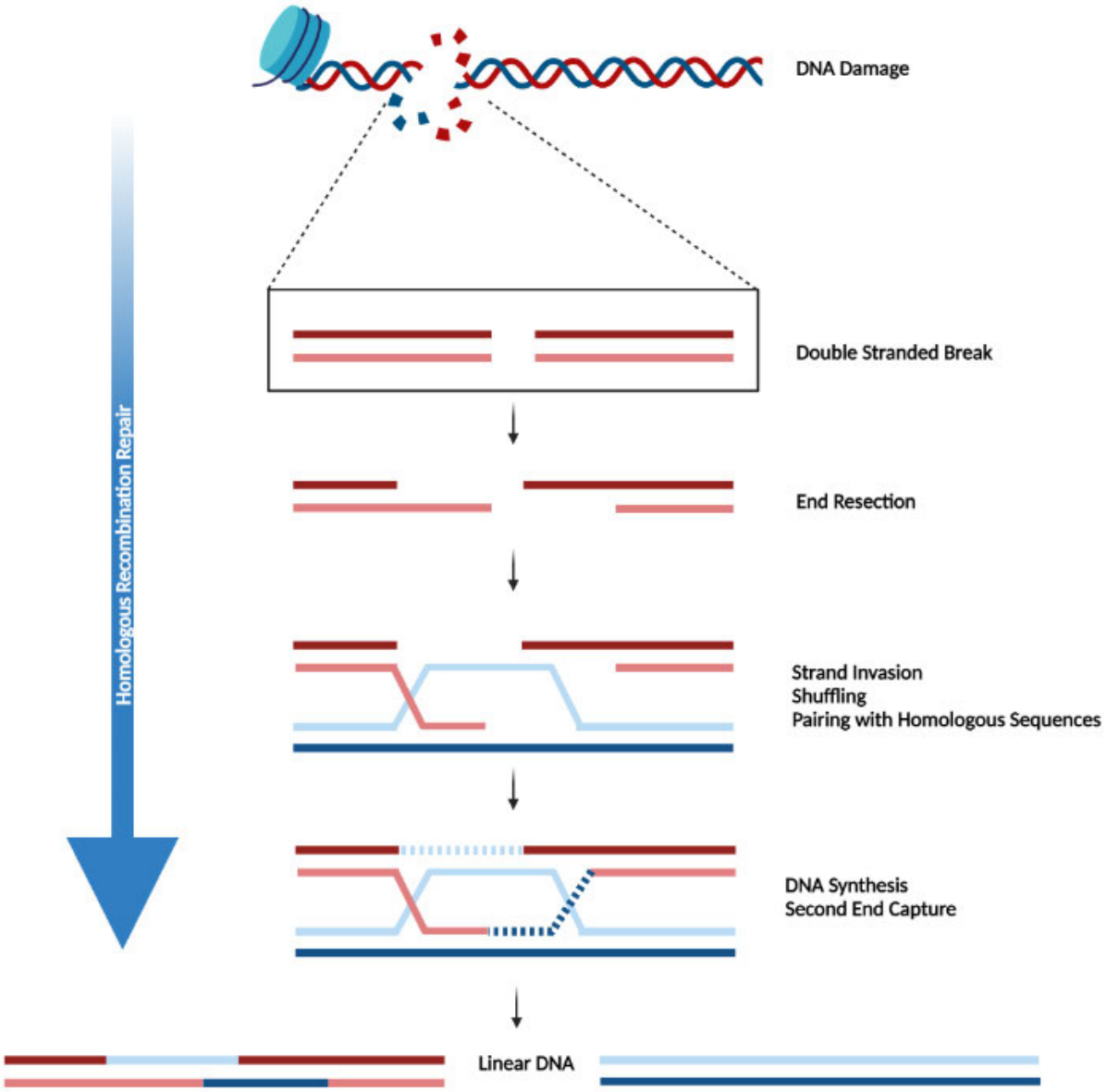



**Figure 2-2. Overview of the Homologous Recombination Repair Pathway.** Upon sensing a double stranded break in the DNA, if a homologous duplex is available as a template, HRR is initiated. 5'-3' end resection creates overhanging ssDNA which invades the template *via* Holiday junctions. The invading strand pairs with homologous sequences in the template DNA; an action facilitated by Rad51. DNA synthesis is followed by second end recapture to anneal the newly synthesized DNA to the original strand resulting in linear DNA (Created with BioRender).

**2.4.1 Pathway Activation (Figure 2-3):** DSBs signal ataxia telangiectasia mutated (**ATM**) dimers to undergo autophosphorylation and disassociation, activating the monomeric ATM kinase.(Kastan & Bakkenist, 2003)(Harper & Elledge, 2007)(Merechal, A., Zou, 2013) ATM activation is further mediated by its interaction with a FXF/Y motif located at the C-terminus of Nijmegen breakages syndrome 1 (NSB1) of the MRE11-RAD50-NSB1 (**MRN**) complex, and upon its recruitment to damaged DNA.(You et al., 2005) Ataxia telangiectasia (**ATR**), another prominent DDR kinase is activated in response to both SSBs and DSBs.(Merechal, A., Zou, 2013)

The MRN complex tethers the damaged DNA, thereby concentrating it, and the ATM kinase phosphorylates a vast number of proteins, including BRCA1, mediator of DNA damage checkpoint 1 (**MDC1**), p53, checkpoint kinase 1 (**Chk1**), checkpoint kinase 2 (**Chk2**), and histone H2AX – a set of reactions that influences repair factors to form at the site of DNA damage and triggers HRR and cell cycle arrest pathways.(Dupré et al., 2006)(Matsuoka et al., 2007)(Podhorecka et al., 2010)(Bonner et al., 2008)(Merechal, A., Zou, 2013)(Harper & Elledge, 2007) MDC1 bridges DNA damage repair and cell cycle checkpoint activation through distinct phospho-dependent interactions; the carboxyl-terminal (**C-terminal**) of BRCA1 C terminus (**BRCT**) region MDC1 associates with yH2AX to aide in DSB repair while MDC1-MRN complex interaction facilitates intra-S-phase checkpoint activation.(Stucki et al., 2005)(Goldberg et al., 2003) The ring finger ubiquitin ligase, ring finger protein 8 (**RNF8**), accumulates alongside NSB1, whereafter they bind to phosphorylated MDC1(Chapman & Jackson, 2008) – RNF8 to its FHA domain – and assemble at the site of DSB DNA damage(Mailand et al., 2007) where, through association

with chromodomain helicase DNA binding protein 4 (**CHD4**), RNF8 mediates chromatin decondesation, providing damage site accessibility by repair factors (Luijsterburg et al., 2012) including BRCA1&2-associated complexes.

**2.4.2 BRCA1-associated Interactions (Figure 2-3)**: The RING domain of BRCA1 ubiquitinates the endonuclease CtBP-interacting protein (**CtIP**) where, under DNA damage conditions, ubiquitinated CtIP is targeted to the chromatin where it aides in end resection and cell cycle checkpoint control of the G2/M phase.(X. Yu et al., 2006) BRCA1-CtIP interaction determines the choice between error-prone MMEJ and the relatively error-free HRR in a cell-cycle phase-dependent manner; CtIP activates MMEJ in the G1 phase while phosphorylation of its serine residue 327 as cells enter S phase allows a switch to HR during S/G2 phase with BRCA1 recruitment.(Yun & Hiom, 2009) The MRN-CtIP-BRCA1 pathway readies DNA for HR-related end resection in S-phase via Topoisomerase-II DNA adduct removal.(Yun & Hiom, 2009)

BRCA1 forms a ubiquitylated histone-associated complex with BRCT domain-interacting abraxas and receptor-associated protein 80 (**RAP80**) that plays a role in G2/M checkpoint mediation, regulates DNA repair of DSBs,(B. Wang et al., 2007) and is a complex that contributes to tumor suppression and genome stability.(Castillo et al., 2014)

Chk2 directly phosphorylates BRCA1 and BRAC2, and directs a preference towards HR over NHEJ(Zannini et al., 2014) and facilitates the effector complex formation that includes BRCA1, partner and localizer of BRCA2 (**PALB2**), and BRCA2.(F. Zhang et al., 2009) The PALB2 protein effectively bridges the gap between BRCA1 and BRCA2 by binding

directly with the N-terminus of BRCA2 and the coiled coil of BRCA1 to facilitate the indirect binding of the two proteins.(F. Zhang et al., 2009)

**2.4.3 BRCA2-associated Interactions (Figure 2-3)**: Deletion of SUV3 suppressor 1 (**DSS1**) binds to BRCA2's DNA binding domain (**DBD**) and enhances molecular stability.(J. Li et al., 2006) PALB2 further stabilizes BRCA2 and promotes nuclear localization(Xia et al., 2006) and is integral for the efficient operation of BRCA2-mediated repair of DSBs by aiding in Rad51 foci localization.(Sy et al., 2009)(Xia et al., 2006)

Following DSBs, BRCA2 directly recruits Rad51 to the site of DNA damage for HRR.(H. Yang et al., 2005)(Jensen et al., 2010)(Michael S.Y. Huen, Shirley M.H. Sy, 2013) Cyclin dependent kinases (**CDKs**) modulate the BRCA2-Rad51 interaction through phosphorylation of serine S3291 on the C terminal domain of BRCA2 which inhibits Rad51 binding, while decreased CDK activity under DNA damage conditions increases BRCA2-Rad51 binding and increases the recombination activity of Rad51.(Esashi et al., 2005) *Rad51 binds to BRC repeats on BRCA2 (encoded by exon 11), an interaction that modulates the choice of localization of Rad51 to either single stranded or double stranded DNA*.(Chatterjee et al., 2016) Differential BRC repeats on BRCA2 alter the binding efficiency of Rad51 and subsequently changes the ability for the complex to form Rad51 nuclear foci.(C. F. Chen et al., 1999) Rad51, a DNA recombinase, forms a nucleoprotein filament on ssDNA known as the presynaptic filament which catalyzes strand invasion and homologous DNA identification, localization, and pairing which is the rate limiting step of HRR and requires BRCA2 mediation as for efficient recombination.(Filippo et al., 2008)(Ml et al., 2018)(Sun et al., 2020) Disruption of Rad51 activity produces a phenotypically

analogous response to a deficiency in BRCA2, indicating that both BRCA2 and Rad51 are critical members that act synergistically late in the HRR pathway.(Schlacher et al., 2011)

After aiding in the formation of the HR replication fork, BRCA2 further acts by stabilizing it against nucleolytic degradation by MRE11.(Schlacher et al., 2011) Through this degradation, MRE11 may be a major cause of instability related to embryonic lethality in BRCA2-deficient development, as blocking MRE11's recruitment rescues this phenotype.(Chaudhuri et al., 2016) Genomic stability is partly maintained through this complex DNA damage response and repair pathway, however, BRCA1 and BRCA2 also engage in complementary participation in cell cycle regulation under both homeostatic and stress conditions.

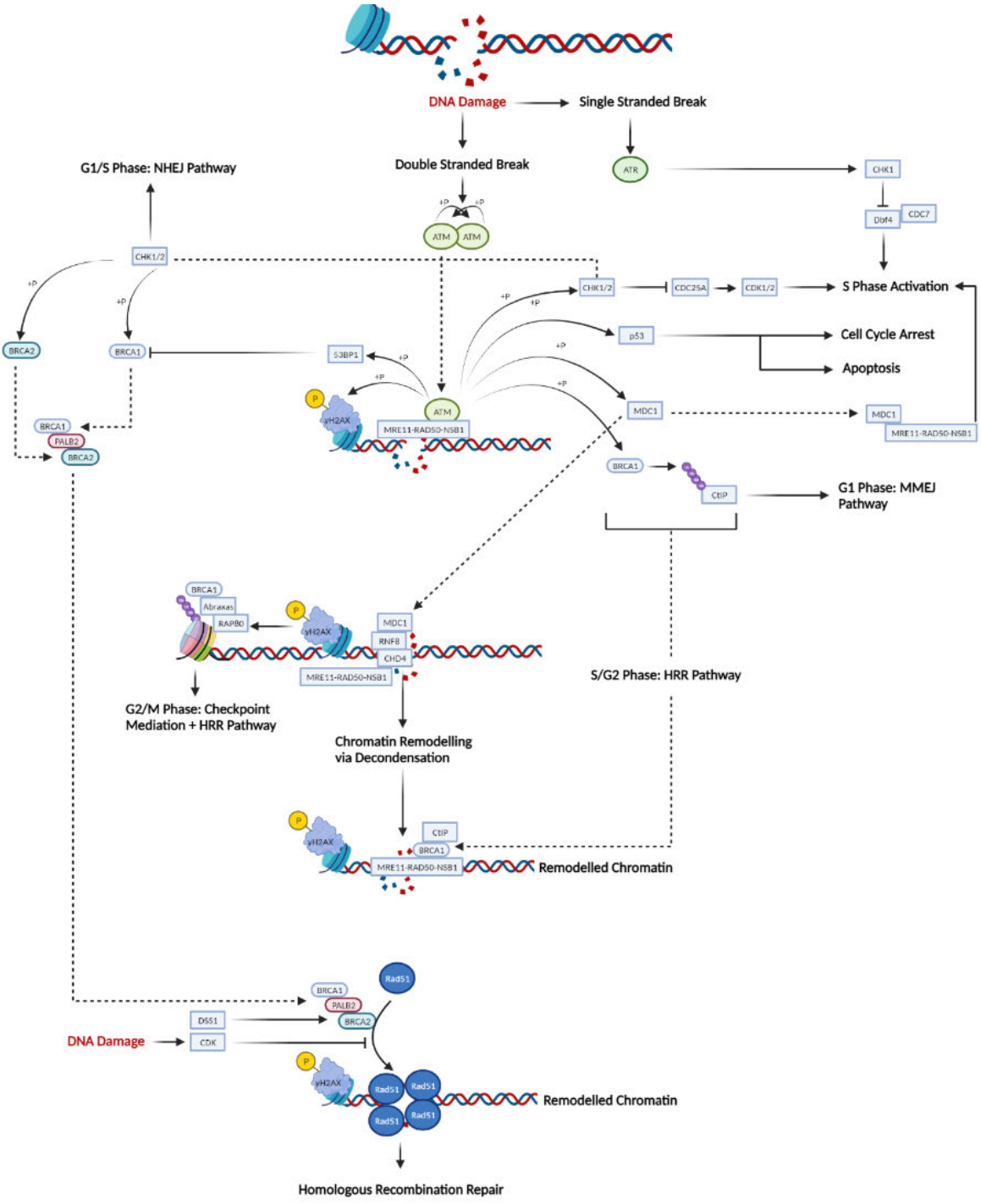



**Figure 2-3. Initiation of HRR.** An overview of the single- and double-stranded break-induced signalling response including BRCA1&2 participation and notable junctures at which the cell cycle is arrested, cell fate is determined, and the choice of repair strategy is made (Created with BioRender).

**2.5 BRCA1&2 Beyond HRR:** The functions of BRCA1 and BRCA2 are distinct from each other and include those that aide in networks conferring stability to cellular processes and integration into the tightly controlled cell cycle.

**2.5.1 Cell Cycle Regulation**: Progression through the cell cycle is highly regulated, and disruptions therein jeopardize cellular homeostasis and bring about aberrant division and cell death, which are increasingly implicated as precipitating factors in diseases of cancer, inflammation, and the cardiovascular system.(Zhivotovsky & Orrenius, 2010) BRCA1&2 mutation cancers are primarily viewed through the lens of impaired DNA damage repair. However, these molecules have other important regulatory functions. Tumors of BRCA1 and BRCA2 mutation are known to occur, in part, due to failure of the cell cycle and chromosomal aberrations which can lead to aneuploidy,(Tirkkonen et al., 1997)(Tomlinson et al., 1998) demonstrating the existence of a regulatory role of these molecules.

**2.5.2 BRCA1 and the Cell Cycle**: BRCA1 aids cell cycle regulation through its HR activity and direct interactions with cell cycle regulatory proteins. BRCA1's deficiency induces genetic instability(Deng & Scott, 2000) *via* pleiotropy, chromosomal defects and aneuploidy,(Paolo et al., 2014) and defects in cell cycle checkpoints.(Deng, 2006) BRCA1's expression and subcellular localization change in response to DNA damage and are dependent on the cell cycle phase and phosphorylation state.(Tibbetts et al., 2000)(Henderson, 2012) BRCA1 functions as part of the G1/S(Vaughn et al., 1996) and intra-S checkpoints(B. Xu et al., 2002) wherein growth arrest is triggered by an abundance of DNA damage which induces increased BRCA1 expression and triggers downstream CDK

inhibition in a cyclin dependent kinase inhibitor 1 (**$p21^{WAF1/cip1}$**)(Somasundaram et al., 1997) or pRb-dependent(Aprelikova et al., 1999) manner.(Mullan et al., 2006) Gadd45 is upregulated with DNA damage and interacts with BRCA1 to support p21/CDK1-mediated cell cycle arrest in the G1 phase(Liebermann & Hoffman, 2018) Additionally, BRCA1 expression acts in modulating G2/M as an upstream stimulator or inhibitor of proteins that mediate the CDC2-CyclinB mitotic kinase to suppress entry into mitosis.(MacLachlan et al., 2000)(Biology, 1999)(Mullan et al., 2006)(Liebermann & Hoffman, 2018) A failure in this pathway leads to an increase in chromosome defects, aneuploidy, and cell cycle breakdown.(Hollander et al., 1999) BRCA1 also performs checkpoint modulation directly by forming a heterodimer with BRCA1 associated ring domain 1 (**BARD1**) via their mutual RING domain that obscures the nuclear export signal – ensuring nuclear retention.(Henderson, 2005) Under stress of DNA damage, this BRCA1-BARD1 ubiquitinates cyclin B and Cdc25C, targeting them for degradation.(Shabbeer et al., 2013) Apoptotic regulation is also performed by BRCA1 binding to ER calcium channel inositol 1,4,5-triphosphate receptor (**$IP_3R$**);(Hedgepeth et al., 2015) additionally establishing the broad governing functions of this molecule.

**2.5.3 BRCA2 and the Cell Cycle**: Beyond its integration in the HR pathway, BRCA2 functions to repair damaged DNA through HR integration but is also a mediator of the cell cycle and its arrest under stress conditions. BRCA2 binds with the centrosome localization signal (**CLS**) cytoplasm dynein 1; thereafter, it localizes to the centrosome where it mediates centrosome pair cohesion, positioning, and duplication and serves as an S phase checkpoint protein.(Malik et al., 2016)(Nakanishi et al., 2007)(Rocca et al., 2015) Without

BRCA2 centrosome localization, there is abnormal centrosome positioning and cell duplication, which results in multinucleate cells, potentially explaining the aneuploidy of many BRCA2-mutation cancers.(Nakanishi et al., 2007) BRCA2 also localizes to telomeres during the S-phase of the cell cycle to enhance telomere replication efficiency(Zimmer et al., 2016) and its absence results in telomere instability, indicating its function in telomere homeostasis.(Min et al., 2012)

Furthermore, BRCA2 appears to play a downstream role to Chk1 in arresting the cell cycle in the S phase upon DNA destabilization.(Rocca et al., 2015) Following DNA damage, BRCA2 and its HR pathway partner PALB2 are also critical G2 checkpoint mediators.(Menzel et al., 2011) BRCA2 also operates during cytokinesis; after phosphorylation by the mitotic polo-like kinase, PLK1, BRCA2 localizes *via* Filamin A to the Flemming body at the centromere midbody, where it stimulates IIC-ring formation.(Takaoka et al., 2014) BRCA2 mutation inhibiting PLK1 binding alters its ability to localize(Takaoka et al., 2014) and causes a deficiency of BRCA2 at the midbody, which provokes a delay of cytokinesis,(Jonsdottir et al., 2009) defects in cell cleavage, and chromosomal instability,(Daniels et al., 2004) indicating the need for functional BRCA2 to maintain this stage of the cell cycle. Insufficiency or altered localization of BRCA2 to the midbody is another possible explanation for the chromosomal aneuploidy in many BRCA2-related tumors.(Dupré et al., 2006) If properly localized, BRCA2 facilitates abscission through the mediation of pro-abscission protein complexes, and if these interactions are disrupted by a BRCA2 mutation, cancer due to cytokinetic dysregulation may result independent of BRCA2's ability to perform DNA damage repair.(Mondal et al.,

2012) Altered BRCA2 expression dysregulation at the midbody due to RAS oncogene mutation may also induce genetic instability regardless of BRCA2 functionality.(G. Yang et al., 2013)

**2.5.4 BRCA1&2, Oxidative Stress, Hypoxia, and Inflammation:** Oxidizing molecules are injurious at high cellular concentrations and can be produced from a multitude of sources, including NADPH oxidase, mitochondrial or other cellular metabolic processes, and by external origins; the deleterious effects of which encompass lipid, protein, and DNA oxidation.(Weng et al., 2018) Complications of oxidative injuries are inflammation and tissue injuries which, if chronic, are strong contributing factors in many diseases,(Mittal et al., 2014) including atherosclerosis and cancer.(Hasselbalch, 2012)(Mittal et al., 2014) In addition, these complications are themselves sources of reactive oxygen species (**ROS**) generation.(Ohnishi et al., 2013)

BRCA1 expression is positively correlated with the antioxidant response and cellular protection, while its deficiency results in hypersensitivity to oxidizing agents.(Bae et al., 2004) BRCA1 stabilizes and activates nuclear factor erythroid 2-related factor 2 (**Nrf2**) to reinforce its antioxidant signalling,(Gorrini et al., 2013)(Bae et al., 2004) and direct interaction with p21 further stabilizes Nrf2 by diminishing the ubiquitination by the Kelch-like ECH-associated protein 1 (**Keap1**) dimer.(W. Chen et al., 2009) Nrf2 and BRCA1 are complementary effectors against xenobiotic stress(Kang et al., 2012) and work in tandem with p21 and p53 in the antioxidant response. Moreover, Nrf2 binding to an antioxidant response element (**ARE**) directly regulates hypoxia-inducible factor 1 alpha (**HIF-1a**) by

transcriptional activation.(Lacher et al., 2018) HIF-1a is crucial for regulating the hypoxic response and BRCA1 may interact directly with HIF-1a to stabilize it in hypoxic conditions.(Hyo et al., 2006) *Most importantly, we recently demonstrated that loss of BRCA2 enhances ROS production*.(S. Singh et al., 2020)

Cellular hypoxia promotes an increase in ROS, leading to oxidative stress and exacerbating local inflammation.(McGarry et al., 2018) Hypoxia, alongside oxidative stress and inflammation, is a hallmark of many diseases, including cancer(Van Der Groep et al., 2008) and atherosclerosis.(Marsch et al., 2013) Specific BRCA1 mutation may positively influence the inflammatory cytokine response.(Woolery et al., 2015) Our group has previously shown that loss and gain of BRCA1 exacerbates and promotes against cytokine insults, respectively;(K. K. Singh et al., 2009) and also that BRCA1 overexpression improves survival by reducing organ failure and inflammation in murine sepsis.(Teoh et al., 2013)

Loss of both BRCA1 and BRCA2 increases cellular sensitivity to tumor necrosis factor-alpha (**TNF$\alpha$**), with BRCA2 inactivation resulting in micronuclei production and a pro-inflammatory cytokine response, including the production of TNF$\alpha$ in a cyclic GMP/AMP synthase/stimulator of interferon genes (**cGAS/STING**)-mediated fashion.(Heijink et al., 10 C.E.) The myriad ways in which BRCA1&2 control cellular process and respond to numerous cellular stresses independently and through synergistic activity indicate that these molecules are critical for cellular homeostasis. Without their proper function, the resulting dysregulation of cellular processes, increased inflammation and oxidative stress, and reduced genomic stability and DNA repair capacity are disruptions that confer a propensity towards diseases.

**2.6 BRCA1&2 in Cancer**: A person's cancer risk is contingent on their susceptibility factors, including those that lie within their genetic makeup.(Cox, 2014) A primary example of this is how germline mutations in the HRR pathway diminish DNA damage repair functionality, thereby promoting carcinogenesis, as evidenced by HR-associated gene mutations occurring in a high percentage of malignancies.(Riaz et al., 2017)

Within the scope of the HRR pathway, two of the cornerstone genes are BRCA1 and BRCA2. Normally women face an approximately 12% risk of breast cancer development. However, mutations in BRCA1 increase a woman's lifetime risk of ovarian cancer to 44% and breast cancer to 72%, while BRCA2 mutations carry a 17% and 69% lifetime risk of ovarian and breast cancer, respectively.(Kuchenbaecker et al., 2017) Albeit, BRCA1&2 mutation is relatively rare at about 1 in 400 in North America, in certain populations such as the Ashkenazi Jewish, germline mutation may occur in approximately 1 in 40 women.(Tennen et al., 2020) Males, however, have a lifetime risk of breast cancer of 0.1%, which increases to 1% or 7-8% with BRCA1 or BRCA2 mutation, respectively.(Ibrahim et al., 2018)

Approximately 5-10% percent of all breast and 10-15% of all ovarian cancers are due to BRCA1&2 mutation and at an earlier onset than sporadic cancers.(Mehrgou & Akouchekian, 2016) Both breast and ovarian cancer dispositions are dependent on where within the sequences of each genes the mutation emerges; specific site mutations in either BRCA1 or BRCA2 have been identified to predispose significantly towards either breast or ovarian cancer.(Rebbeck et al., 2015)(Thompson & Easton, 2002)

Due to the many possible mutations that may differentially affect the function of BRCA1&2, breast and other cancer penetrance vary with population and other statuses, including any prophylactic measures as well as age, child-bearing history, the parental origin of germline mutation, and comorbidities – particularly those that exacerbate DNA damage.(Kramer et al., 2005)(Bernholtz et al., 2011)(Friebel et al., 2014) Analysis of penetrance demonstrated that among families with a strong history of breast cancer, 52% were BRCA1 positive and 32% BRCA2 positive and while breast-ovarian cancer is highly predictive of a BRCA1 mutation, families with a male and female history of breast cancer exhibit a high propensity towards BRCA2 mutation.(Ford et al., 1998)

Beyond breast and ovarian cancers, BRCA1/2 mutation predisposes carriers to cancer of the digestive system,(Cavanagh & Rogers, 2015) prostate,(Cavanagh & Rogers, 2015) fallopian tube, peritoneum, and possibly melanomas(Ginsburg et al., 2010)(Gumaste et al., 2015) and other cancers.(Mersch et al., 2015)(B., 2005) However, BRCA1/2 mutations are benign in many cancers, and the full mechanism for their specificity is still being elucidated.(Cullinane et al., 2020)(Jonsson et al., 2019)

Cancer-type rates, while unilaterally heightened for men and women, exhibit sex and tissue specificity due to hormone-responsive gene expression-related stress(X. Zhang et al., 2017) and modulation of BRCA1&2 leading to differential cellular development and proliferation.(Van Asperen et al., 2005)(Rajan et al., 1997) Proliferative regulation by BRCA2 of cancer cells may further exacerbate tumorigenesis in the case of BRCA2 mutation.(S. C. Wang et al., 2002) Moreover, low overall BRCA2 expression level is independently associated with cancer severity in sporadic breast cancers(Sarkar, 2018), and higher cytoplasmic

expression of BRCA1&2 is indicative of better outcomes in cancers of the digestive system, whereas BRCA1 nuclear expression is correlated with poor survival rates.(G. H. Wang et al., 2018)

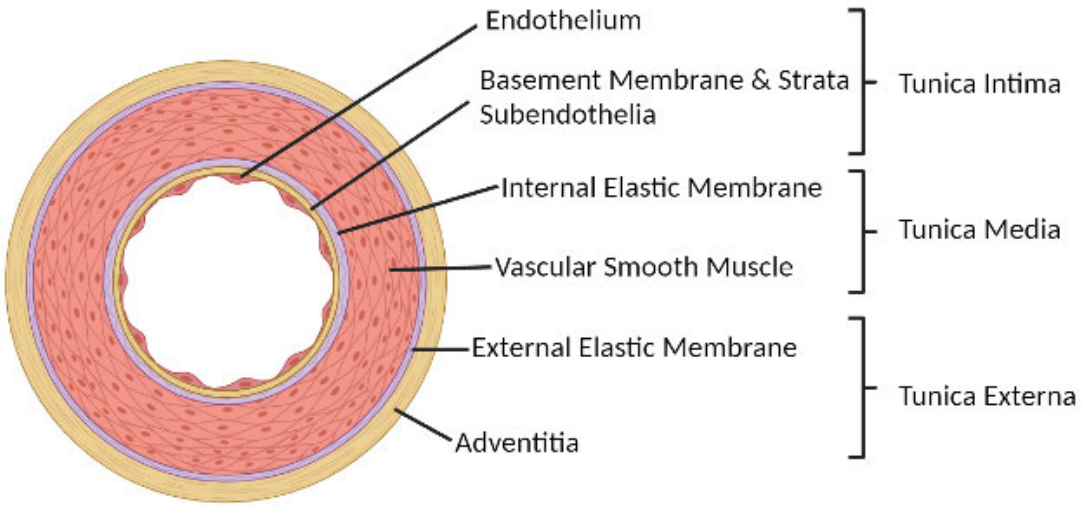
BRCA1&2 do not act only within the scope of DNA damage repair, but instead function along a complex pathway of homologous recombination, so unsurprisingly, loss-of-function mutations in molecules along this common pathway may also result in cancer predisposition by failure to mitigate DNA damage leading to accumulation and mutation.

**2.7 Homologous Recombination Repair Pathway and Cancer**: Increased cancer risks have been observed with HR molecule mutations such as in BARD1, BRCA1's N terminal interactor, which is associated with breast cancer;(De Brakeleer et al., 2010)(Ratajska et al., 2012) abraxas which has known tumor suppressor functions;(Castillo et al., 2014) DSS1 that acts with BRCA2 to confer stability to the protein such that mutations in it cause hypersensitivity to DNA damage and could be implicated in breast and ovarian cancer;(J. Li et al., 2006) and PALB2 mutations which impair its operation with BRCA2 leading to a failure to properly recruit Rad51 to the site of DNA damage.(Sy et al., 2009)(Xia et al., 2006) While this is not an exhaustive list, other genes that have been implicated in carcinogenesis range from DNA damage sensors, mediators, and effectors and include ATM,(Choi et al., 2016) BRCA1 interacting helicase 1 (**BRIP1**),(Weber-Lassalle et al., 2018) NSB1,(Lamarche et al., 2010) and H2AX.(Celeste et al., 2003)

If proteins critical for the efficient function of the HR repair pathway have their function hindered or lost, a common effect is the promotion of carcinogenesis. However, the

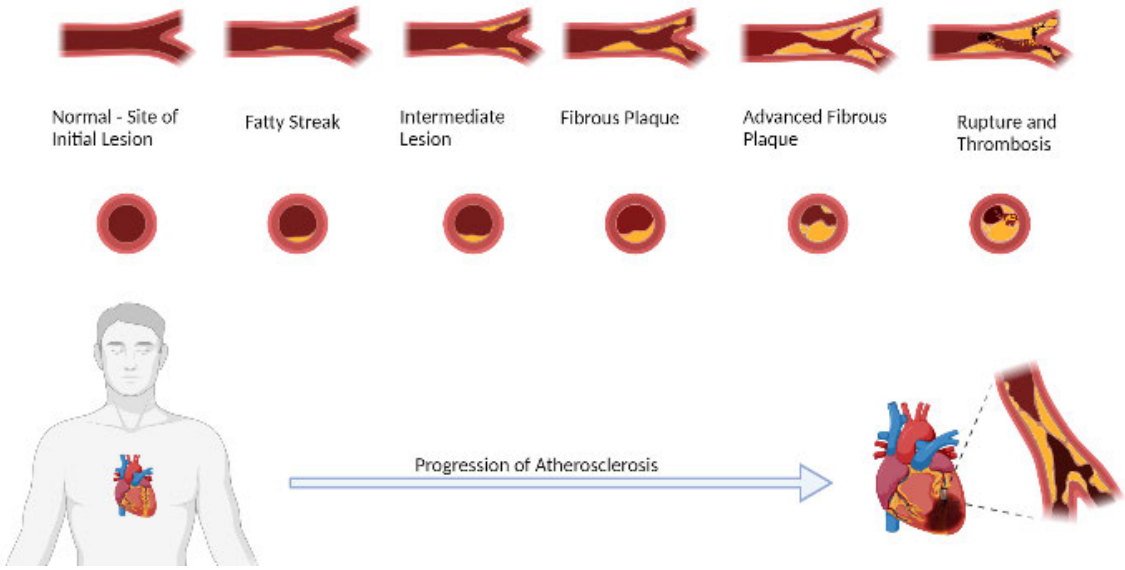
development and proliferation of cancerous cells often require loss of the synergistic tumor suppression capacity that is conferred by molecules like p53.(Jonkers et al., 2001) p53 is a tumor suppressor gene that responds to DNA damage, signals cell cycle arrest, and can also initiate apoptosis in a context-dependent manner.(Efeyan & Serrano, 2007)(Perri et al., 2016)(Agrawal et al., 2018)

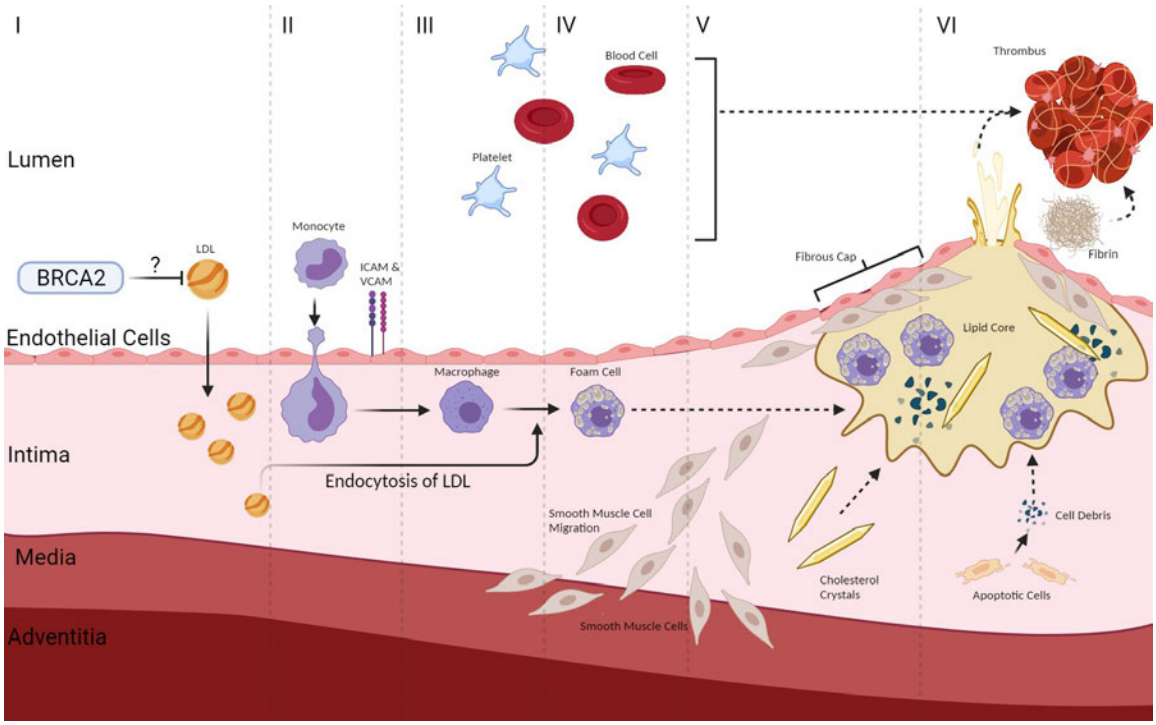
**2.8 Atherosclerosis and Endothelial Dysfunction:** The endothelium (**Figure 2-4**) is comprised of a single layer of cells lining blood vessels that exhibit tissue- and organ-type heterogeneity(Marcu et al., n.d.)(Rafii, 2018) with greatly differing tissue-dependent turnover rates(Hobson & Denekamp, 1984)(Heart et al., 2015) and composition.(Gehr, 1982)(Bowden, 1981) Endothelial cells are the direct interface between the blood and other vascular cells and play a direct role in maintaining vascular smooth muscle cell (**VSMC**) homeostasis in healthy vascular tissue.(Microrna- et al., 2013)



Viscous blood flowing through the varied and branching geometry of the vasculature subjects the endothelium to differential hemodynamic forces, including wall shear stress and hydrostatic pressure.(Davies, 2009) Branch points and curved regions are areas of turbulence and reduced laminar flow; these areas are prone to endothelial injury, cholesterol deposition, and are vulnerable to developing atherosclerotic lesions(Michael A. Gimbrone Jr., 2013) due to reduced shear stress.(Foteinos et al., 2008)(Tricot et al., 2000)

In atherosclerosis (**Figure 2-5**), endothelial cells undergo apoptosis, senescence, and release stimulus-dependent, pro-inflammatory cytokines.(Suzuki et al., 2013) Cytokines recruit leukocytes to the endothelium, where they become plaque constituents and participate in endothelial stress. As atherosclerosis progresses, arterial narrowing results in even further shear stress reduction in downstream plaque regions, leading to high rates of endothelial apoptosis and may spur plaque erosion and thrombus.(Tricot et al., 2000)(Kockx, 1998)


Normal - Site of Initial Lesion
Fatty Streak
Intermediate Lesion
Fibrous Plaque
Advanced Fibrous Plaque
Rupture and Thrombosis
Progression of Atherosclerosis


I
II
III
IV
V
VI
Lumen
Endothelial Cells
Intima
Media
Adventitia
BRCA2
?
LDL
Monocyte
ICAM & VCAM
Platelet
Blood Cell
Thrombus
Fibrin
Fibrous Cap
Lipid Core
Macrophage
Foam Cell
Endocytosis of LDL
Smooth Muscle Cell Migration
Smooth Muscle Cells
Cholesterol Crystals
Cell Debris
Apoptotic Cells

(**V, VI**) As atherosclerosis progresses, the intimal lipid core is formed by constituents including cholesterol crystals, cellular debris, lipid-laden macrophages, SMCs, and connective tissue. (**VI**) Intimal and lipid core expansion exacerbated by increasing shear stress as the lumen narrows may cause fibrous cap rupture and the release of clot-forming plaque. Clinical outcomes of such a thrombus include peripheral artery disease, myocardial infarction, and stroke. (Created in BioRender)

**2.9 DNA Damage and Repair in Cardiovascular System**: A persistent source of DNA damage is the generation of reactive oxygen species (**ROS**), including hydroxyl radicals, hydrogen peroxide, singlet oxygen, and peroxynitrite, which are generated throughout the course of cell metabolism, or by exogenous sources such as drugs, sunlight, and ionizing radiation.(Hakem, 2008) ROS interact with DNA, causing nucleotide alterations, and importantly, they have the capacity to break the DNA chain resulting in single- and double-stranded breaks.(MacLachlan et al., 2000)(Altieri et al., 2008) Cells under oxidative stress and undergoing accelerated DNA damage experience dysregulation of critical cellular processes, arrest of the cell cycle, cell death, and mutations – leading to phenotypic abnormalities such as accelerated aging, cancer development, cardiovascular diseases, and pathologies stemming from genomic instability.(Errico et al., 2021)(Deng & Scott, 2000)

**2.9.1 DNA damage and Atherosclerosis:** DNA damage levels are positively correlated with the extent of atherosclerosis and are an integral component of coronary artery disease.(Botto et al., 2001) VSMCs constitute a significant portion of atherosclerotic regions and may undergo phenotype switching via trans-differentiation into macrophage-like cells, foam cells, and cells that produce the extracellular matrix of the fibrous cap.(Feil

et al., 2014)(Bennett et al., 2016) Given their ubiquity in atherosclerotic plaque, VSMC stability is integral to stable lesions, while high rates of apoptosis induced by DNA damage destabilizes plaque, leading to rupture and the consequential atherosclerotic cascade culminating in vessel occlusion and ischemia.(Clarke & Bennett, 2007)(Bennett et al., 1995)(Gray et al., 2015) Moreover, the increased oxidative stress in atherosclerotic VSMCs compromises these cells' DNA damage repair pathways, (Shah et al., 2018) and causes RAS protein-induced VSMC senescence and inflammation; all of which accelerate atherosclerosis.(Minamino et al., 2003) Endothelial cells, which line the inner-most layer of every blood vessel, play an important role in the pathogenesis of atherosclerosis.(M. Li et al., 2018)

We have previously demonstrated that accumulation of DNA damage in endothelial cells exacerbates endothelial dysfunction and apoptosis.(S. Singh et al., 2020) DNA damage is found ubiquitously in atherosclerotic patients, including in circulating cells, which exhibit a high micronuclei index correlating with CAD severity,(Botto et al., 2001) as well as in the mitochondrial DNA (mtDNA) within atherosclerotic regions – indicating an extensive link between DNA damage and atherogenesis.

**2.9.2 mtDNA Damage and Atherosclerosis:** Mitochondria are responsible for oxidative phosphorylation, a by-product of which is the generation of ROS. Mitochondrial DNA (**mtDNA**) exists as a single circular chromosome, and DNA damage to it is found extensively throughout the vessel walls and circulating cells of atherosclerotic mice. mtDNA damage is associated with greater CVD risk.(E. Yu et al., 2013) Evidence supports

the link between mtDNA damage and atherogenesis and has provided elucidation of its role in inflammation, apoptosis, and oxidative stress.(E. P. K. Yu & Bennett, 2014) mtDNA damage results in mtDNA mutations,(Mohamed et al., 2004) which cause mitochondrial metabolic dysfunction in VSMCs and, to an even greater extent, the endothelial cells of atherosclerotic patients(Ballinger et al., 2000) due to impaired function of genes that support the electron transport chain and code for ribosomes and transfer RNA (**tRNA**).(Volobueva et al., 2019) ROS-induced mtDNA damage to vascular tissues may thus be an early initiating factor in atherogenesis that compromises the metabolic process and perturbs mitochondrial lipids, proteins, and DNA, all of which culminate in vascular dysfunction and disease. mtDNA-related disruptions have been linked to defects and lesions of the vasculature, and are congruent with the atherosclerotic hallmarks of endothelial dysfunction and inflammation.(Ballinger et al., 2002)

**2.10 DNA Modification and Atherosclerosis**: DNA and mtDNA damage are readily apparent among the atherosclerotic *milieus*. However, CVD's progression and severity may also be influenced by genetic and epigenetic modifications to DNA, including single nucleotide polymorphisms (**SNPs**), methylation states, and dysregulated telomere length.

**2.10.1 Genetic Associations and Epigenetic Regulation in Atherosclerosis:** Genome-wide association analysis has revealed a connection between SNPs and subclinical CVD, including increased coronary artery calcium and greater carotid intimal media thickness.(Vargas et al., 2016) SNP functional variants associated with atherosclerosis risk have been found in Caucasian, Hispanic, and Chinese people.(Howard et al., 2013)(Chu et al., 2017)(Liu et al., 2013) Furthermore, quantitative microarray profiling of

atherosclerotic patients has detected differential methylation of CpG-sites in both the aortic and carotid arteries in humans, as well as in animal models.(Nazarenko et al., 2011)(Aavik et al., 2019)(Wierda et al., 2015)(Papers et al., 2004) Interestingly, the BRCA2 locus has been verified to contain alterations associated with increased coronary artery disease and stroke.(Miao et al., 2017)

**2.10.2 Telomere Shortening and Atherosclerosis**: Differential genomic modifications in atherosclerosis have been observed in telomeric shortening rates. Telomeres naturally exhibit progressive attrition upon each round of cellular division; however, premature shortening exists as one facet of many pathologies, including metabolic syndrome and those involving CVD.(Obana et al., 2003)(Salpea & Humphries, 2010) Telomere dysfunction occurs alongside activation of the DNA damage response pathway,(Takai et al., 2003) which exhibits continuous activation at telomere damage sites that resist repair, thus initiating the cell into senescence irrespective of otherwise sufficient telomere length.(Fumagalli et al., 2012) Telomeric shortening is associated with increased oxidative stress, cellular senescence, and DNA damage in VSMCs.(Sampson et al., 2001)(Matthews et al., 2006) VSMC senescence, activated along DDR, p21 and p16 pathways, is a known exacerbator of CVD.(Andreassi, 2008) VSMC senescence may be mitigated through statin treatment that induces an increase in DNA damage repair and NSB1 stabilization, thereby preventing the attrition of telomeres.(Mahmoudi et al., 2008) Exacerbators of CVD associated with shorter telomere length include age, smoking, and high levels of low-density lipoprotein (**LDL**) cholesterol(Koriath et al., 2019) – co-risk factors with cancer (**Figure 2-6**).

**2.11 BRCA1&2 in Cardiovascular Diseases:** Cancer risks for BRCA1&2 mutation carriers are well defined; however, significantly higher non-neoplastic morbidities have been reported in BRCA1&2 mutation-positive cancer patients.(Mai et al., 2009) Moreover, negative effects on the cardiovascular system may occur independently of cancer; for those with cancer, complications be compounded due to a potentially increased BRCA mutation-related sensitivity to CVD.

### 2.11.1 Intersections Between Cardiovascular Disease and Cancer

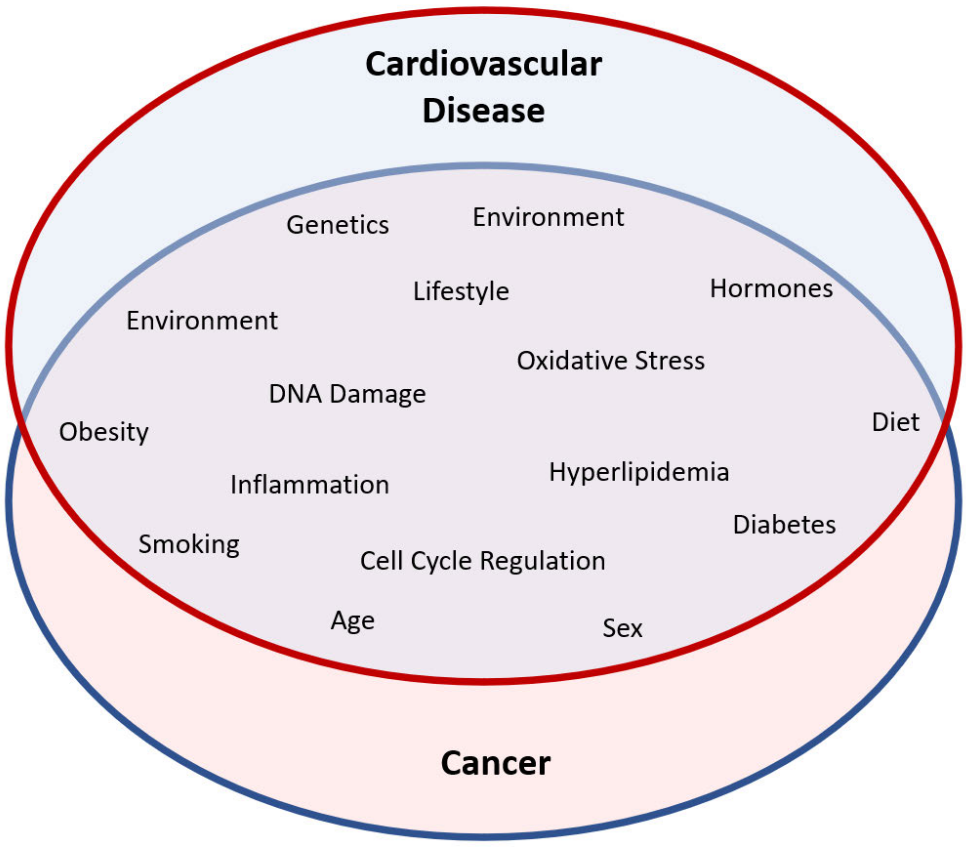



**Figure 2-6. Cardio-oncological Overlaps**: common risk factors and links between cardiovascular disease and cancer.

**2.11.2 Risk Reducing Salpingo-oophorectomy and Mastectomy and CVD**: BRCA1/2 mutation carriers may opt to protect themselves against the high likelihood of cancer by undergoing the prophylactic surgeries of risk-reducing salpingo-oophorectomy (**RRSO**) or mastectomy (**RRM**). While these surgeries confer a high degree of protection against cancer development,(Kramer et al., 2005) they are associated with increased non-cancer mortality in BRCA-positive women.(Anna Öfverholm, Zakaria Einbeigi, Antonia Wigermo, 2019) This phenomenon may be the result of cardiovascular complications due to early menopause. The hormone and lipid changes due to natural menopause are associated with a higher CVD predisposition,(Kat et al., 2017)(Fairweather, 2014) an effect exaggerated by more acute changes, and a high incidence of metabolic syndrome, particularly in those who have undergone RRSO, further exacerbating the rate of type II diabetes, heart disease, and stroke,(Dørum et al., 2008) due to arterial stiffening,(Abbas et al., 2018) hyperlipidemia, and hypertension(Cohen et al., 2012) in these patients. If below the age of 50, RRSO recipients also exhibit a higher risk of osteoporosis,(Cohen et al., 2012) a syndrome that overlaps with CVD in risk factors and may be implicated in CVD development.(Cauley & Cauley, 2008)

**2.11.3 Anthracyclines and Cardiotoxicity:** After cancer diagnosis, treatment options are often centered around surgery and chemotherapeutics, such as a class of drugs known as anthracyclines.(Tan et al., 2017) Anthracyclines are genotoxic as they intercalate into the DNA minor grooves and also pose a high risk to cardiovascular health.(Schmidt et al., 2016)(Aleman et al., 2007)(Belt-dusebout et al., 2021) Anthracyclines are a known factor in the development of congestive heart failure(Lefrak, A., Pit'ha, J., Rosenheim, S., 1973)

and cardiomyopathy.(Blanco et al., 2021) Children, in particular, are subjected to a high risk of these deleterious effects, (Mulrooney & Yeazel, 2009) which may be intensified by the use of radiation therapy(Mueller et al., 2013); these complications may not be apparent until many years after treatment completion.(Skitch et al., 2017)

An analysis of BRCA mutation carriers supports the idea that these individuals are indeed at an increased risk of cardiotoxicity and non-neoplastic mortality due to anthracycline treatment,(Sajjad et al., 2017)but this connection is not always observed.(Pearson et al., 2017) The risk could be partly explained by the marked increase in cardiomyocyte apoptosis under anthracycline stress and BRCA2-deficient conditions.(K. K. Singh et al., 2012) Moreover, hypertension is a common comorbidity in BRCA1&2-positive patients with breast cancer, leaving these patients particularly vulnerable to cardiotoxicity.(Antone et al., 2017)

**2.11.4 Cardiovascular Disease Intrinsic Risk with BRCA1&2 Mutation**: The ubiquitous nature of DNA damage occurring alongside cardiovascular diseases, such as atherosclerosis, has spurred interest in the study of an intrinsic risk of CVD for BRCA1&2 mutation carriers. Hallmarks of atherosclerosis include oxidative stress, cellular dysregulation, and DNA damage, all of which are known to be intensified under a BRCA deficit. As previously discussed, differential methylation is a hallmark of atherosclerotic regions, and BRCA1 is known to be affected alongside CVD with methylation changes that occur with subclinical atherosclerosis.(Geoffrey et al., 2017) Further, the study of SNPs as potential biomarkers for disease revealed specific BRCA2 variants to be associated with CVD incidence,(Zbuk et al., 2012)(Miao et al., 2017) and a large-scale meta-analysis

identified an association between BRCA2 SNP variants and increased LDL cholesterol.(Asselbergs et al., 2012)

Lipid dysregulation is a hallmark of cardiovascular disease and cancer, and the proper modulation of lipogenesis is important for disease protection. BRCA1 affects this by acetyl coenzyme A carboxylase binding(Shen & Tong, 2008) – stabilizing its phosphorylation site – thereby inhibiting increased lipogenesis(Moreau et al., 2006) and insulin signalling.(Jackson et al., 2014) Further evidence exists for lipid(Genetic et al., 2015)(Oliverio et al., 2020) and metabolic(Oliverio et al., 2020) dysregulation in both BRCA1&2 mutation carriers, suggesting the existence of a metabolically active role carried out by these genes and their potentially protective role against a proatherogenic lipid profile.

**2.11.4.1 BRCA1&2 in the Heart**: BRCA1 is shown to provide cardio-protective qualities, as its loss in cardiomyocytes exacerbates myocardial infarction-induced heart failure;(Shukla et al., 2011) cardiomyocyte-specific loss of BRCA1 is also associated with glucose and fatty-acid metabolic changes, resulting in an energy-starved heart.(K. K. Singh et al., 2013) This loss further leads to DSB accumulation; apoptosis due to activation of the pro-apoptotic p53; and eventual cardiac failure as a result of impaired cardiac remodelling.(Shukla et al., 2011) Inefficient or reduced expression of BRCA1 may therefore present as cardiomyocyte stress and lead to a higher incidence of cardiac failure under ischaemic stress in BRCA1 mutation carriers. Association between heart failure and BRCA1 is also demonstrated by a significantly increased expression of BRCA1 observed in

failing human heart/cardiomyocytes, arguably to promote DNA damage repair and survival in the stressed heart.(Shukla et al., 2011) Interestingly, cardiomyocyte-specific loss of BRCA2 also promotes heart failure following genotoxic stress.(Cite J Biol Chem, 2012) Taken together, it appears that BRCA1, BRCA2, and DNA damage repair mechanisms are cardioprotective in nature and loss of any of these leads to adverse cardiac remodelling under stress.

**2.11.4.2 BRCA1&2 in Thrombosis**: The most critical clinical outcome of atherosclerosis is thrombogenic plaque rupture and release, which quickly progresses to clot formation and ischemia. BRCA1 mutation carriers exhibit altered expression of proteins known to participate in thrombogenesis, including elevated levels of fibrinogen gamma chain isotypes 2 and 3,(Custodio et al., 2012) which, interestingly, are known to be associated with poor outcomes in some cancers.(J. M. Jones et al., 2006)(Qingqu Guo et al., 2009) Similarly, BRCA2 mutation results in expression alterations to thrombo-coagulating related proteins, not dependent on breast cancer development.(Perez-segura et al., 2016) Additionally, independent of carcinogenesis, those with a BRCA gene mutation are at increased risk of pulmonary embolism after abdominal flab breast construction surgery(Timman & Ph, 2013). This shift to a potentially pro-thrombotic plasma environment may leave BRCA1&2 mutation carriers particularly vulnerable to plaque rupture, thrombosis, thromboembolism, and vessel occlusion, especially concerning for mutation carriers who develop atherosclerosis, even if cancer-free.

**2.11.4.3 BRCA1&2 in the Vasculature**: Cellular regulation is essential for the impedance of atherogenesis at the level of inflammatory cells and, in particular, the cells of the

vasculature, which include vascular smooth muscle cells and endothelial cells.(Foteinos et al., 2008; Michael A. Gimbrone Jr., 2013; Microrna- et al., 2013)(M. Li et al., 2018) BRCA1&2 act independently to mediate genomic- and cell-stability,(Roy et al., 2012) thus may be integral in hindering atherosclerotic progression via these mechanisms.

**2.11.4.4 BRCA1&2 in the Vascular Smooth Muscle Cells**: Vascular smooth muscle cells are structurally significant and are integral to vascular constriction and relaxation. They are also responsible for proliferative and vascular remodelling under cyclic and hypotensive conditions. BRCA1 has been shown to have an effect on protecting VSMCs by mitigating oxidative stress through NADPH Nox1-dependent ROS production inhibition.(Lovren et al., 2014) Moreover, hypertensive rats with induced BRCA1 expression via adenovirus exhibit vascular protection due to decreased blood pressure and ROS production.(Chessex et al., 2013)

**2.11.4.5 BRCA in the Endothelium, Endothelial Dysfunction, and Atherosclerosis**: A cohort of male BRCA1&2 mutation carriers was observed with significantly higher levels of endothelial progenitor cells when measured as a fraction of CD34+/VEGF or CD133+/VEGF, suggesting a state of accumulated endothelial damage and indicating increased cardiovascular risk in BRCA mutation carriers in addition to their established cancer susceptibility.(Witberg et al., 2019) Our group previously demonstrated that protection against atherosclerosis was provided by BRCA1 through upregulation of endothelial nitric oxide synthase and attenuated ROS production, inflammation, and apoptosis. In addition, limited atherogenesis, and improved capillary density was observed after ischemic injury.(K. K. Singh et al., 2009) Similarly, BRCA2 protected

endothelial cells under oxidized LDL (**oxLDL**)-induced oxidative stress; BRCA2 loss was associated with a reduction in DNA damage repair capacity and an increase in apoptosis *in vitro*.(S. Singh et al., 2020) These findings have demonstrated an important role for BRCA1 and BRCA2 in endothelial regulation and suggest a protective function against atherosclerosis.

In atherosclerosis, the oxidative environment causes DNA damage and alteration to the vasculature. To blunt these effects, endothelial cells produce proteins to repair DNA damage and maintain stability. As previously discussed, the breast cancer susceptibility genes 1 and 2 represent a major component of error-free HR and confer stability to many cell types. The BRCA1/2-integrated HR pathway broadly mitigates the deleterious effects of endothelial dysfunction, including altered DNA damage, apoptosis, inflammation, and proliferation. However, only recently has there been an appreciation for BRCA2-induced cardioprotective and anti-atherogenic roles.

**2.12 Thesis Motivation**: There is now extensive evidence for DNA damage and alterations occurring within the atherosclerotic *milieu*. As the evidence mounts, a closer look at how DNA damage relates to the cellular disturbances that beget atherosclerosis may open new therapeutic avenues. Moreover, DNA damage and markers of functional or dysfunctional repair may be used as subclinical diagnostic biomarkers, allowing for enhanced screening in vulnerable populations.

However, DNA damage repair proteins are not autonomous actors apart from other cellular processes. They often interact with many partners, forming complexes that have

the regulatory capacity to maintain cellular stability on multiple levels. Therefore, the study of these proteins and their interconnected pathways is likely to bring about a richer understanding of their role in DNA damage repair, aging, and diseases, including those of the cardiovascular system.

Pioneering work on assessing the role of the HR proteins BRCA1 and BRCA2 has already provided evidence for their likely protective quality of maintaining vascular and cardiac health. Yet, further *in vivo* studies and translational research should be carried out to confirm the magnitude of their involvement in CVD. Carriers of mutations in these genes may very well be more susceptible to or have a higher, yet unrecognized, independent risk of developing cardiovascular disease. Beyond these efforts, an expansive look into the functional HR pathway proteins may provide insight into other culprits of cellular dysregulation and CVD initiation.

Mutation in BRCA1 or BRCA2 increases the risk of breast cancer and both BRCA1&2 play an integral but non-redundant role in HRR pathway. However, it is important to note that BRCA1 and BRCA2 are structurally distinct proteins; they interact with different protein partners, play a non-redundant role in different stages of genome protection, have different cancer predisposition rates, lifetime risk, estimated frequency, and types and characteristics of cancers. (Roy et al., 2012) In contrast to BRCA1, BRCA2 is also associated with lipid regulation and inflammation. (Genetic et al., 2015)(Oliverio et al., 2020)(Woolery et al., 2015) Moreover, BRCA2 gene resides on the human chromosome 13q12.3, a region linked to CVDs in humans. (Miao et al., 2017)(Jie et al., 2013) It is also

important to note that BRCA2, but not BRCA1 expression, is significantly affected by oxLDL treatment in endothelial cells.(S. Singh et al., 2020) Therefore, identifying mechanisms linked to BRCA2 would provide new and distinct information related to BRCA2's role in the endothelium, and how BRCA2 may pertain to cardiovascular health and disease. In summary, the BRCA2 protein responds to DNA damage by participating in complex cellular pathways of DNA damage repair. (Roy et al., 2012) BRCA2 provides genome stability and limits apoptosis, whereas mutations in BRCA2 cause breast and ovarian cancer.(Roy et al., 2012) In humans, potential higher incidence of non-neoplastic deaths have been observed in BRCA2-mutants,(Mai et al., 2009) and BRCA2 SNPs are correlated with plasma-lipid levels,(Asselbergs et al., 2012) and deregulation of both lipid and metabolites in BRCA2-mutants.(Genetic et al., 2015) Loss of endothelial BRCA2 exacerbates oxLDL-induced endothelial dysfunction.(S. Singh et al., 2020) Abnormal lipid metabolism and endothelial dysfunction are the central pathways leading to atherosclerosis;(M. Li et al., 2018) however, BRCA2's role within the scope of endothelial dysfunction and atherosclerosis remains unknown.

Accordingly, the goal of this thesis is to evaluate the pathophysiological role of endothelial BRCA2 using a clinically relevant *in vivo* model of atherosclerosis. Specifically, *we hypothesize that endothelial cell-specific loss of BRCA2 augments DNA damage, apoptosis, and endothelial dysfunction, and thereby exacerbates atherosclerosis.*

My thesis will delineate a critical and novel role of BRCA2 limiting endothelial cell apoptosis and dysfunction. In addition to identifying a potentially new therapeutic target, our data may point towards a heightened susceptibility of BRCA2 mutation carriers

toward atherosclerosis and other CVDs, thereby providing an evidence-based framework for future studies to support translational research applications.

**2.13 Thesis Objectives:** This thesis is conducted to determine the pathophysiological role of BRCA2 using loss-of-function approach with the following objectives.

(i) Generate and characterize an endothelial cell-specific BRCA2 knockout mouse utilizing Cre-LoxP technology.

(ii) Generate and characterize endothelial cell-specific BRCA2 knockout mouse on $ApoE^{-/-}$ background and evaluate high-fat diet-induced atherosclerosis.

## Chapter 3. Methods

**3.1 Animal Studies**: Studies herein were approved by the Western University Animal Care Committee and all associated animal procedures adhered to the guidelines set out by the Canadian Council on Animal Care. Animal endpoints were determined pursuant to approved experimental parameters and early endpoints by veterinarian recommendation. Experiments were performed on mice aged 8 weeks and older which were housed in a sterile, environment-controlled facility (40-60% humidity, 22-24°C temperature, cage ventilation with HEPA filtration) on a 12hr light/dark cycle. Each cage housed up to four mice and were supplied with reverse osmosis water chlorinated to 2-3ppm ad libitum, and the mice were maintained on an excess of standard laboratory chow-type diet (NIH-31, Cat# 7013, Open Formula Mouse/Rat diet, irradiated – Envigo) or experimental, high-fat diet ("Western" purified atherogenic diet TD.88137, irradiated – Envigo).

**3.2 Generation of Single Knockout Mice**: Endothelial cell-specific BRCA2 knockout was achieved by virtue of the Cre-LoxP system of tissue-specific gene excision (**Figure 3-1, B**). Mice homozygous for the exon 11 floxed BRCA2 allele with a BALB/cJ background (NCI; Strain #: 01XB9; Common name: Brca2 floxed; Strain Nomenclature: STOCK *Brca2*$^{tm1Brn}$/Nci) were crossed with mice hemizygous for Cre-recombinase expression under control of the vascular endothelial (VE) Cadherin 5 promoter and on a C57BL/6J background (The Jackson Laboratory; Stock #: 006137; Common Name: VE-Cadherin-Cre (VE-CRE; Strain Nomenclature: B6.FVB-Tg(Cdh5-cre)7Mlia/J). Breeding was performed in accordance with the schematic in **Figure 3-1, A**. Heirs from the crossing of the two described strains were bred to generate single knockout mice that are homozygous

BRCA2 floxed and hemizygous for VE-Cre: BRCA2$^{fl/fl}$;VE-Cre$^{tg/-}$ (BRCA2$^{endo}$). Heterozygote knockout BRCA2$^{fl/wt}$;VE-Cre$^{tg/-}$ (BRCA2$^{het}$) were used for to gauge the dose dependency of BRCA2-loss in endothelial cells. Wildtype littermates – BRCA2$^{fl/fl}$;VE-Cre$^{-/-}$ and BRCA2$^{fl/wt}$;VE-Cre$^{-/-}$ and BRCA2$^{wt/wt}$;VE-Cre$^{tg/-}$ (BRCA2$^{wt}$) – were used as controls. Wherever possible, the latter Cre+ wildtype was analyzed independently, then if no differences were observed, all wildtype mice were pooled together. Functional and molecular assays were utilized to elucidate the baseline effect of the loss of function of endothelial cell specific BRCA2 in young (8–16-week-old) mice to ascertain the emergence of early developmental effects. These mice are inbred for multiple generations and the strain is currently being maintained by breeders.

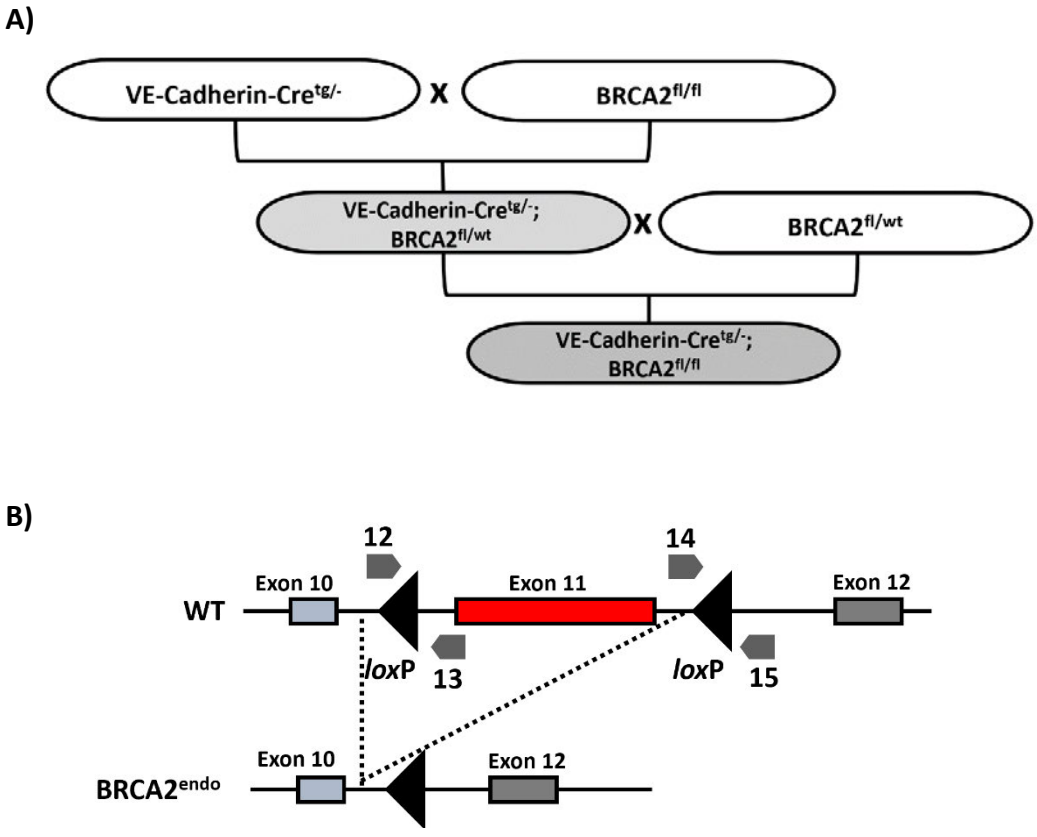


**Figure 3-1. Breeding Schematic and the Cre-LoxP System. (A)** Cre-LoxP method-based breeding strategy for generating endothelial cell specific BRCA2 knockout mice ($BRCA2^{endo}$). **(B)** Cre-LoxP system schematic representation at the genomic level illustrating Cre-mediated, BRCA2 exon 11 deletion.

**3.3 Generation of Double Knockout Mice:** Generation of the *in vivo* model of atherosclerosis was achieved by crossing the existing $BRCA2^{endo}$ strain with mice homozygous for an ApoE loss of function mutation on a C57BL/6J background (The Jackson Laboratory; Stock #: 002052; Common Name: ApoE KO; Strain Nomenclature: B6.129P2-*Apoe*$^{tm1Unc}$/J). ApoE is a requisite molecule for hepatic LDL receptor-mediated lipoprotein clearance, and its deficiency results in high levels of circulating cholesterol.(Curtiss, 2000) These mice are well described for their propensity towards plaque development and vascular lesions under high fat diet and are a standard model for atherosclerotic studies.(Curtiss, 2000)(Greenow et al., 2005) The progenies of this crossing ($BRCA2^{fl/+}$;$ApoE^{-/+}$;VE-$Cre^{tg/-}$ X $BRCA2^{fl/+}$;$ApoE^{-/+}$;VE-$Cre^{-/-}$) were interbred, resulting in the generation of the double knockout experimental target genotype ($BRCA2^{fl/fl}$;$ApoE^{-/-}$;VE-$Cre^{tg/-}$) (Figure 3-2). Both this double knockout and their wildtype control littermates were fed a high-fat diet starting at 10 weeks of age. Monitoring of these mice was done weekly for gross phenotype, and they were weighed at intervals of 2 weeks. After 8, 12, and 16 weeks of high fat diet, tissues from these mice were collected for RNA, protein, and histological analysis and the aortas and aortic roots of these mice were assessed for atherosclerotic magnitude. This strain is currently being maintained by breeders.

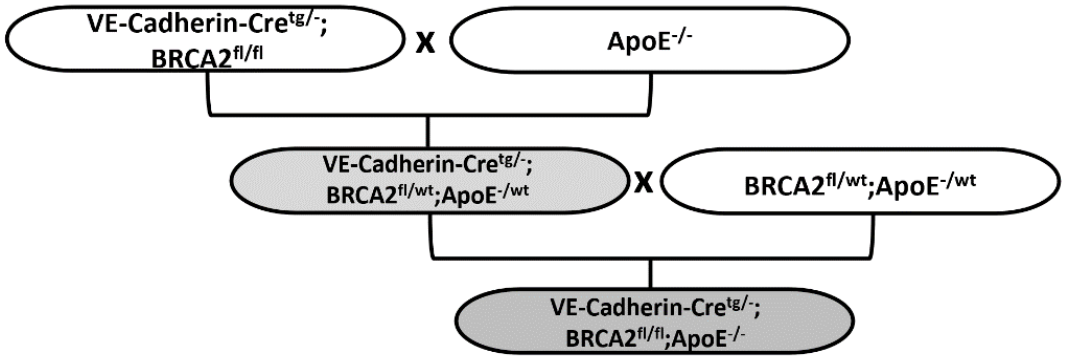



**Figure 3-2. Breeding Schematic for Double Knockout Mouse.** Previously generated BRCA2$^{endo}$ single knockout mice are bred in conjunction with ApoE$^{-/-}$ mice to generate ApoE$^{-/-}$;BRCA2$^{endo}$ double knockout mice.

**3.4 Genotyping**: The WISNET ADVANCED$^{TM}$ DNA fast extract kit was used to extract genomic DNA from ear clippings pursuant to the manufacturers recommended guidelines. The supernatant of this extract was used in conjunction with primers for BRCA2, VE-Cre, or the ApoE mutant gene where appropriate. Amplification of these genes was performed via polymerase chain reaction using 2x HS-Red Taq mix, and the product was run on 2% agarose gel formed with 1x Tris-Borate-EDTA (TBE) or Tris-acetate-EDTA (TAE) buffer containing Red Safe (FroggaBio, Cat#21141) for visualization with UV transillumination with the LICOR Odyssey FC imaging system.

List of Mouse Specific Genotyping Primers

| Gene | Primer Name | Primer Sequence | Primer Type | Product Sizes |
|---|---|---|---|---|
| | **Genotyping PCR** | | | |
| **BRCA2** | B012 | 5' -GGC TGT CTT AGA ACT TAG GCT G- 3' | Forward | 376 bp (5' Lox) |
| | B013 | 5' -CTC CAC ACA TAC ATC ATG TGT C- 3' | Reverse | 298 bp (Wildtype) |
| | B014 | 5' -CTC ATC ATT TGT TGC CTC ACT TC- 3' | Forward | 529 bp (3' Lox) |
| | B015 | 5' -TGT TGG ATA CAA GGC ATG TAC AC- 3' | Reverse | 450 bp, (Wildtype) |
| **VE-Cre** | oIMR1084 | 5' -GCG GTC TGG CAG TAA AAA CTA TC- 3' | Transgene Forward | 100 bp (Transgene) |
| | oIMR1085 | 5' -GTG AAA CAG CAT TGC TGT CAC TT- 3' | Transgene Reverse | |
| | oIMR7338 | 5' -CTA GGC CAC AGA ATT GAA AGA TCT- 3' | Internal Control Forward | 324 bp (Internal Positive Control) |
| | oIMR7339 | 5' -GTA GGT GGA AAT TCT AGC ATC ATC C- 3' | Internal Control Reverse | |
| **ApoE** | oIMR0180 | 5' -GCC TAG CCG AGG GAG AGC CG- 3' | Common Forward | 245 bp (Mutant) |
| | oIMR0181 | 5' -TGT GAC TTG GGA GCT CTG CAG C- 3' | Wildtype Reverse | 155 bp and 245 bp (Heterozygote) |
| | oIMR0182 | 5' -GCC GCC CCG ACT GCA TCT- 3' | Mutant Reverse | 155 bp (Wildtype) |

**3.5 Tissue Collection**: Mice were placed under deep anesthesia using bell jar isoflurane method. Mice were anchored at their extremities in a supine position under maintenance anesthesia. Fur and skin were removed, and incisions were made into the parietal peritoneum for abdominal access and further lateral cuts were made into the peritoneum and bilateral ascending cuts were made along the ribcage. Tissue forceps were used to secure the xyphoid process and peel back the sternum and ribcage to access the thoracic cavity. Lateral or descending cuts along the parietal peritoneum were performed to further open the abdominal cavity as necessary for organ and abdominal aorta collection. Exsanguination was performed *via* right ventricle cardiac puncture using a 27-gauge needle. Perfused and non-perfused tissue collection was performed based on the needs of individual experiments as further noted. The head was separated and placed on ice while organs were collected including the heart, aorta, lung, liver, kidney, and spleen, followed by the brain. Tissues were washed in 1x cold PBS and immediately flash frozen

in liquid nitrogen then stored in -80°C for RNA and protein extraction or placed in 4% PFA or 10% formalin for immunohistochemical work.

**3.6 RNA Extraction**: Whole tissues were snap frozen in liquid nitrogen and triturated using a mortar and pestle. Tissues were homogenized in 1mL Trizol (ambion, Cat# 15596018, Lot# 318303) and allowed to incubate at room temperature for 5-10 minutes then on ice for another 15 minutes. Phase separation was performed by adding 200μL of chloroform and vigorously shaken by hand for 15 seconds followed by 3-minute incubation at RT before centrifuging at 12,000 RCF and 4°C for 15 minutes. The clear, upper RNA phase was transferred to a new RNase free tube. RNA precipitation was performed by adding 500μL of isopropyl alcohol and inverted 5 times before incubating for 10 minutes at RT and centrifuging at 12,000 RCF at 4°C for 10 minutes to pellet the RNA. The supernatant was poured off and the pellet was left to partially air dry before adding 1mL of 75% ethanol then lightly vortexed to wash the pellet prior to centrifuging at 7,500 RCF at 4°C for 5 minutes; this step is repeated once if the pellet is visible. The ethanol is poured off and the pellet is allowed to partially air dry and the RNA is subsequently dissolved in 25-40μL of DEPC water and reconstituted with a pipette then vortexed and 65°C incubation for 3 minutes, followed by 2-3 minutes on ice, high speed vortex for 5 seconds, centrifuge, and storage in -80°C. This method was used for RNA isolation unless otherwise indicated.

**3.7 cDNA Synthesis**: cDNA synthesis was performed using QIAGEN's QuantiTect reverse transcription kit (Cat# 205311) and pursuant to the recommended guidelines using 1μg RNA/sample and Eppendorf PCR Mastercycler. Samples were stored in -20°C.

**3.7.1 PCR on Lung cDNA and Agarose Gel Electrophoresis**: Whole lung RNA was extracted followed by cDNA synthesis as described. PCR amplification was carried out using 12.5µL 2x HS-Red Taq 2-6µL cDNA extracted from BRCA2$^{endo}$, BRCA2$^{het}$, and BRCA2$^{WT}$ samples; with primers for BRCA2 exon 10F/14R, BRCA2 exon 11F/11R, and GAPDH F/R at a final concentration of 0.4µM per primer; and balanced to 25µL with DEPC H2O on Eppendorf PCR Mastercycler. 10µL were loaded into wells of 2% agarose (VWR Life Science Agarose I, Cat# 0710-500g, Lot# 18K2756730) gel and run at 60V for 1-2hrs until desired resolution was reached.

**3.7.2 Real-time PCR or Quantitative (q)PCR:** Whole lung tissue RNA was extracted followed by cDNA synthesis as described. qPCR was effectuated with SYBR Green master mix on the Quantstudio 3$^{TM}$ with standard protocol using primers indicated in **Table 3-2**.

**Table 3-2**

**Primers for Mouse Specific mRNA Amplification**

| Gene | Lab Nomenclature | Sequence |
|---|---|---|
| **BRCA2-Ex10 F** | Mm-BRCA2-Ex10-F1 | 5'-TTCAGTGAGGAGACTTGTGGTAGA-3' |
| **BRCA2-Ex11 F** | Mm-BRCA2-Ex11F | 5'-CCCATCCTTCACGCACTTAT-3' |
| **BRCA2-Ex11 R** | Mm-BRCA2-Ex11R | 5'-TGTGACCAGTTTTCCACCTG-3' |
| **BRCA2-Ex14 R** | Mm-BRCA2-Ex14-R2 | GGACGAAGACTTTGGTGGATT |
| **GAPDH-R** | mGAPDHAS | 5'-TGCACCACCAACTGCTTAGCC-3' |
| **GAPDH-F** | mGAPDHS | 5'-TGGATGCAGGGATGATGTTCT-3' |
| **p21-F** | Mm-Cdkn1a-F1 | 5'-CGGTGTCAGAGTCTAGGGGA-3' |
| **p21-R** | Mm-Cdkn1a-R1 | 5'-ATCACCAGGATTGGACATGG-3' |
| **p53-F** | Mm-Tp53-F1 | 5'-CTAGCATTCAGGCCCTCATC-3' |
| **p53-R** | Mm-Tp53-R1 | 5'-TCCGACTGTGACTCCTCCAT-3' |

PCR reactions consisted of 5µL SYBR, 0.5µM final concentration of forward and reverse primers, 2-3µL cDNA (synthesized from 1µg RNA and diluted 1:5) and balanced to 10µL

with DEPC H2O. Each sample and primer combination were performed in triplicates and fold-change expression was calculated on triplicate means by the delta-delta CT method. Statistical analysis was carried out in Graphpad Prism using ordinary one-way ANOVA with Tukey's post-hoc.

**3.8 Immunoblotting**: Tissues or isolated cells were extracted as previously described. Tissues were mechanically broken down either using a mortar and pestle and liquid nitrogen or a homogenizer. Homogenized tissues or cells were suspended in ice-cold RIPA (Fisher Scientific, Cat# J62524, Lot# U14F514) buffer with protease inhibitor (PI) cocktail and incubated on ice for 30 minutes . Samples were centrifuged at 15,000 RCF and 4°C for 15 minutes and the supernatant was extracted for further processing. Total protein was quantified in triplicates by standard Bradford assay using Coomassie Plus (ThermoScientific, Cat# 1856210, Lot# UC277715) reagent and absorbance at 595nm was measured by microplate reader (Bio-Rad iMark Microplate Reader).

Protein samples were prepared and normalized to 5-50μg. Loading sample preparation consisted of normalized protein supernatant, ¼ total volume loading dye made by diluting 1:9 2-Mercaptoethanol (Cat#1610710) and 4x Laemmli Sample Buffer (Bio Rad, Cat# 161047) loading dye and RIPA+PI to balance volume. Samples were resolved via SDS-polyacrylamide gel electrophoresis. Gel percentage and running voltage varied based on the intended targets. Gels were transferred to methanol-activated 0.22μm or 0.45μm pore size polyvinylidene difluoride (PVDF) membrane overnight (16-20hrs) at 25V. The membrane was blocked for 1hr in 5% skim-milk (Criterion, Cat# C6961, Lot# 471903) or 2-5% BSA (Multicell, Cat# 800-095-EG, Lot# 800095043) diluted in 1x TBS to limit non-

specific binding then probed with primary antibodies for BRCA2 (Proteintech, Cat# 19791-1-AP, 1:500), yH2AX (Cell Signalling, Cat# 9718S, 1:1000), p53 (Cell Signalling, Cat# 2524S), GAPDH (Cell Signalling, Cat# D16H11, 1:1000), or β-Tubulin (Proteintech, Cat# 66240-1-Ig, 1:5000) in accordance with the manufacturer's recommendations. Horseradish peroxidase secondary antibody incubation was performed with anti-rabbit (Cell Signalling, Cat# 7074, 1:3000 or Enzo, Cat# BML-SA204-0100, 1:5000) and anti-mouse (Enzo, Cat# BML-SA204-0100) specificity.

Membranes were washed in 1xTBST (0.5% Tween20) and signal was generated by the enhanced chemiluminescent (Bio-Rad, Clarity Western ECL) detection system. Images were procured using the LICOR Odyssey FC imaging system and protein quantified by densitometry using the associated analysis software (Image Studio v.5.2.5). Relative abundance of target proteins was determined as the mean of target/loading control.

**3.9 Endothelial Cell Isolation**: Aortic segments were harvested and cleaned of periadventitial adipose tissue *via* microdissection under stereoscopic microscope using Vannas scissors and Dumont forceps. These segments were cut longitudinally and further divided into 1-3mm x 1-3mm square pieces before they were immersed in 4°C endothelial cell isolation buffer (ECIB [pH 7.4, 50mM NaCl, 80mM Sodium Glutamate, 6mM KCl, 2mM $MgCl_2$, 1mM $CaCl_2$, 10mM Hemisodium HEPES, $H_2O$, 0.2μm filtered and stored at 4°C, 10mM Glucose added on day of use]).

400μL of 4°C vascular smooth muscle cell isolation buffer (VSMCIB [pH 7.4, 55mM NaCl, 80mM Sodium Glutamate, 5mM KCl, 2mM $MgCl_2$, 10mM Hemisodium HEPES, 0.1mM

EGTA, H2O, 0.2µm filtered and stored at 4°C, 10mM Glucose added on day of use]) was added to 1.5mL Eppendorf tubes. Tissues were added to the tubes and gently rocked to resuspend. 50µL of Papain (Cedarlane-LS003119, 10µg/µL) and 50µL of DTT (Sigma-D9779, 10µg/µL) were added to each tube, flicked to resuspend tissues, and incubated at 37°C for 30min, flicking every 10min.

290µL VSMCIB, 10ul CaCl2 (20mM), 100µL Collagenase IV (Cedarlane-LS004188, 10µg/µL), and 100µL neutral protease (Cedarlane-LS02100, 10µg/µL) was added to each tube, resuspended, and incubated at 37°C for 25min, flicking every 10min. Contents were mixed by flicking and rocking for 1min and centrifuged at 120 RCF for 5min. 800µL supernatant was removed and replaced by fresh ECIB and the pellet triturated with a 45° cut 1mL pipette tip. Suspended endothelial cells were confirmed for viability under 10x microscopy. Endothelial cells were pelleted and washed in 1x cold PBS prior to being lysed in RIPA or Trizol for protein and RNA extraction respectively.

**3.10 Immunohistochemistry**: Mice tissues were fixed *in situ* – they were perfused with 10-20mL cold PBS followed with 4% paraformaldehyde (PFA) where applicable. Whole tissues of the heart, aorta, lung, brain, kidney, liver, and spleen were stored in 4% PFA or 10% formalin for >48hrs. Prior to histological processing, these tissues were washed and stored in 1x PBS. Tissues were embedded in paraffin wax and sectioned on a microtome at 5um.

Gross histological examinations were performed on the heart, lung, liver, and aorta stained with haematoxylin and eosin (H&E). Target proteins were revealed via standard

immunohistochemical methods for horseradish peroxidase (HRP)-catalyzed 3,3'-Diaminobenzidine (DAB) staining in conjunction with antibodies directed against BRCA2 (Proteintech, Cat# 19791-1-AP), yH2AX (Cell Signalling, Cat# 9718S), and Rad51 (Cell Signalling, Cat# 8875S) in accordance with manufacturers guidelines for further tissue section analysis.

TUNEL staining was performed on deparaffinized tissues sectioned at 5 microns and counterstained with DAPI for nuclear identification. These images were captured at 60x magnification and merged to illuminate endothelial apoptosis of the aorta and lung, while the liver was used for positive control of apoptosis.

**3.11 Myography**: Functional myographic assays were performed on isolated aortic arterial segments of $BRCA2^{endo}$ and $BRCA2^{WT}$ control male mice. Aortas were extracted from mice euthanized by cervical dislocation and maintained in ice-cold Krebs buffer. Aortas were cleaned of adipose and surrounding tissues aortic rings were mounted under isometric tension on pins in myograph chambers (DMT620M; Danish Myograph Technologies, Aarhus, DK). Vessel segments were equilibrated in Krebs buffer (pH 7.4, 37°C, continuously bubbled at 95 $O_2$/5% $CO_2$) with constitutes of 114 mM NaCl, 4.7 mM KCl, 0.8 mM, $KH2PO_4$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 2.5 mM $CaCl_2$, 11mM D-glucose, and 20 mM $NaHCO_3$, 25mM HEPES.

Baseline contractile dose response to KCl (30, 60, 90 mM) was used for normalization calculations and to determine tissue viability. Contractile response curve generation was performed using phenylephrine ($1^{-9}$-$3^{-6}$ M) (PE). PE was used as a contractile agent prior

to the generation vasorelaxation response curves induced by acetylcholine (Ach) ($1^{-9}$-$3^{-6}$ M), sodium nitroprusside (SNP) ($1^{-9}$-$3^{-6}$ M), and 2-furoyl-LIGRLO-NH2 (2FlI) ($1^{-9}$-$3^{-6}$ M), each separated by a 20–40-minute washout period. Data were analyzed by testing for linear correlation and statistical significance was determined by two-way ANOVA with Bonferroni post-hoc test.

**3.12 Echocardiography**: Two-dimensional echocardiography (Visualsonics Vevo 2100 imaging system) was performed by a blinded investigator on sedated mice (4% induction, 1.5-2% maintenance isoflurane; body temperature maintained at 37-38°C). Their chests were shaved, and residual fur was removed with Nair hair removal cream. B-Mode was used to identify the area of interest and acquire 2-dimensional images of the heart while short-axis M-Mode was utilized for a mono-dimensional, high temporal resolution images of the mid-papillary muscle. Subsequent measurement of muscle relaxation and actuation was done at three separate intervals each and averaged. Fractional shortening was calculated as (LVEDD-LVESD)/LVEDD, and LV ejection fraction was determined by ((LVEDD^3-LVESD^3)/LVEDD^3) x 100.

**3.13 Metabolic Caging**: Metabolic measurements were performed *via* the Comprehensive Lab Animal Monitoring System.(Guzman et al., 2013)(Janickova et al., 2017) Metabolic chambers were maintained at room temperature (24 ± 1°C) wherein $BRCA2^{WT}$ and $BRCA^{endo}$ mice were kept individually for 48 hours and provided water and food (standard chow - powdered) *ad libitum*. Mice were allowed 24 hours of acclimation, after which measurements for respiration (O2 intake and CO2 production), energy output

at rest, food and water intake, and activity were measured at 10-minute intervals (Opto-M3 Activity Monitor, Columbus Instruments) over a 24-hour period (12/12hr; light/dark).

**3.14 Oil Red-O Staining of Aorta**: Aorta Collection: Exsanguination was performed *via* right ventricle cardiac puncture using a 27-gauge needle. 2-3 incisions were made in the liver for drainage and perfusion was performed by left ventricle cardiac puncture using a 23-gauge syringe with 10mL of 1x cold PBS.

Tissues were collected as previously described with the exception of the heart, aortic arch, thoracic aorta, and abdominal aorta which were extracted in contiguous segments and cleaned of periadventitial adipose tissue *via* microdissection under stereoscopic microscope using Vannas scissors and Dumont forceps. The aorta was transversely cut in the atrial plane near the heart to separate the aorta including the aortic arch from the heart containing the aortic root which was placed in 10% formalin for fixation and histological analysis.

Plaque Staining: The aorta segment was placed in 10% formalin overnight up to 24hrs to facilitate the removal of the remaining adventitia. These segments were washed 3 times for 5min in 1mL of 78% methanol before staining for 50-60min in Oil Red-O solution (10mL methanol, 4mL 1M NaOH, 0.028g Oil Red-O [Cat# O0625-25G, Lot# SHBM5455) powder. Stained aortas were washed 2x in 1mL 78% methanol and placed in 1mL 4°C 1x PBS. The following procedures were performed under cold PBS submersion aortas while avoiding excessive manipulation to minimize plaque dislodgement and loss. Segments were further cleaned of residual, stained adipose tissue. Aortic arch branches were trimmed,

and the aorta was cut in the coronal plane along the outer curvature of the ascending arch, hemisecting the remaining brachiocephalic, left common carotid, and left subclavian arterial branches, then following along the descending thoracic aorta, cutting the full length of the aorta. The aorta was pinned down on a black background using 10mm x 0.10mm minutien pins and exposed intimal surface was photographed at magnification for *en face* plaque visualization.

The magnitude of plaque burden was calculated for the aortic arch using ImageJ (Fiji) *via* manual segmentation (**Figure 3-3**) as percent plaque area (yellow dotted line) as a percent of total vessel area (white dashed line) in a blinded fashion. Branches and plaque within are not included in total vessel area or plaque calculations.


**Figure 3-3. Aortic Arch Plaque Staining and Quantification.** Representative *en face* image of high-fat diet-fed mouse aorta exhibiting moderate to high plaque burden as stained by Oil Red-O (magenta; encompassed by yellow dotted line) manually segmented and calculated as a percent of total vessel area (white dashed line).

# Chapter 4. Results Part I

## *Baseline characterization of BRCA2$^{endo}$ mice*

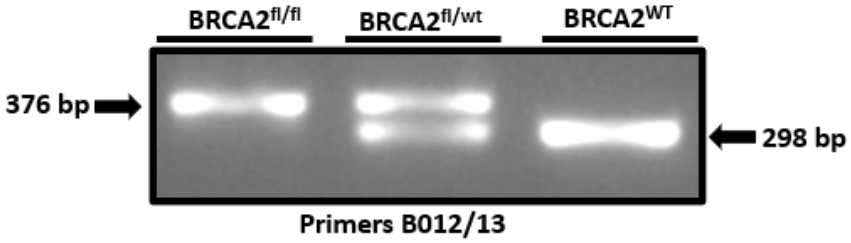
### 4.1 Generation of Endothelial Cell-specific BRCA2 knockout (BRCA2$^{endo}$) Mice

To circumvent the embryonic lethality associated with systemic BRCA2 knockout mice, the *Cre-Lox P* method was used to generate BRCA2$^{endo}$ mice as described in the methods section (**Figure 3-1, A**). Initial crossing of stock mice was performed between homozygous BRCA2 floxed and homozygous VE-Cre mice. Their progeny was heterozygous BRCA2 floxed and hemizygous for VE-Cre expression. These mice were back-crossed with homozygous BRCA2 floxed mice to generate BRCA2$^{fl/wt}$ and BRCA2$^{fl/wt}$;VE-Cre$^{Tg/-}$ mice. To minimize the possibility of a Cre phenotype including Cre toxicity,(Silver & Livingston, 2001) and unintended DNA alterations/damage,(Loonstra et al., 2001)(Huh et al., 2010) only mice hemizygous for Cre were used for experiments. Genotyping does not produce results allowing for the resolution of one or two copies of VE-Cre, higher Cre recombinase activity is toxic,(Company of Biologists, 2013) thus breeding of experimental mice was performed between the crossing of BRCA2$^{fl/wt}$ and BRCA2$^{fl/wt}$;VE-Cre$^{Tg/-}$ mice. Experimental mice generated were BRCA2$^{WT}$ (BRCA2$^{fl/fl}$;VE-Cre$^{-/-}$, BRCA2$^{fl/wt}$;VE-Cre$^{-/-}$, BRCA2$^{wt/wt}$;VE-Cre$^{-/-}$), BRCA2$^{WT;Cre+}$ (BRCA2$^{fl/fl}$;VE-Cre$^{Tg/-}$), BRCA2$^{het}$ (BRCA2$^{fl/wt}$;VE-Cre$^{Tg/-}$) and BRCA2$^{endo}$ (BRCA2$^{fl/fl}$;VE-Cre$^{Tg/-}$) mice. Where possible, BRCA2$^{WT;Cre+}$ mice were evaluated separately to ascertain the presence of a Cre phenotype. If not significantly different than Cre- BRCA2$^{WT}$ mice, results were grouped.

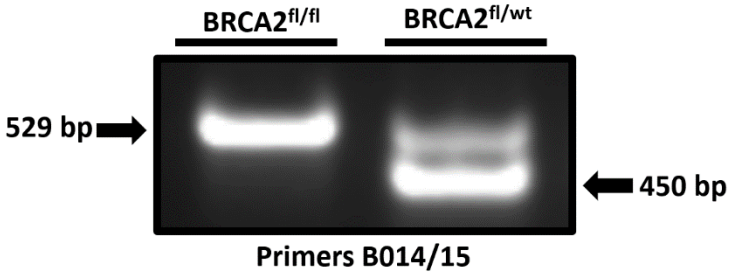
### 4.1.1 Genotyping

The genotype of each animal was determined *via* DNA extract and amplification for the genes of interest. The results of which allowed for directed breeding pursuant to the schematics in **figure 3-1, A,** and **figure 3-2** and the selection of experimental and control animals. The genotype of animals used for data generation was validated by tail DNA extraction. BRCA2 *flox* status was determined primarily by testing for the presence of the upstream loxP site (**Figure 3-1, B**) as resolved by primers B012/13 (**Table 3-1**), while primers B014/15 (**Table 3-1**) were used to test for the presence of the downstream *lox* site (**Figure 3-1, B**) as a redundant confirmation in a smaller subset of these animals.

**A)**




**B)**



**(C)**

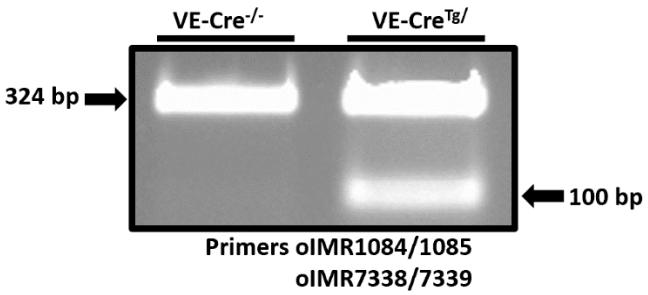
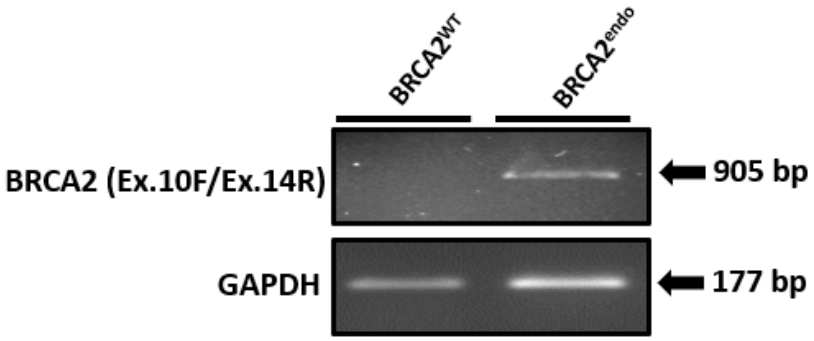



**Figure 4-1. BRCA2 and VE-Cre Genotyping.** Representative agarose gel images of genotype variants as amplified by PCR for the **(A)** BRCA2 upstream lox site, **(B)** BRCA2 downstream lox site and **(C)** Vascular Endothelial Cre (VE-Cre).

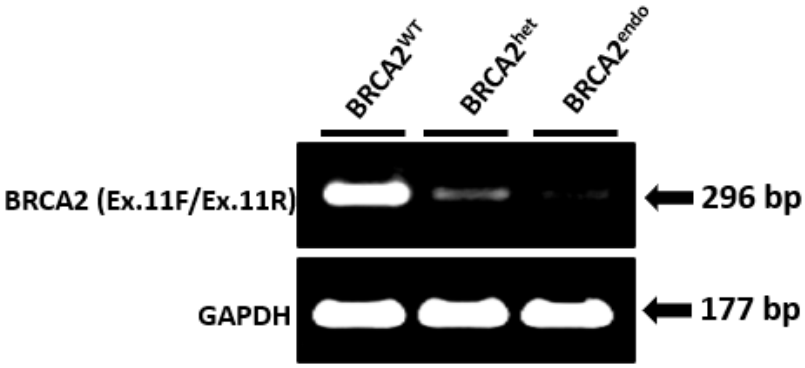
### 4.1.2 Successful Deletion of BRCA2 at Transcript Level in the Endothelium of Endothelial Cell-specific BRCA2 Knockout Mice

Isolated aortic endothelial cell or whole lung RNA was extracted, and cDNA was synthesized. cDNA was used to ascertain BRCA2 deletion and the loss of BRCA2 transcript using exon 11-specific primers. Results were visualized by PCR product run on agarose gel electrophoresis. First, in isolated aortic endothelial cells, **figure 4-2, A** demonstrates PCR product for BRCA2 after exon 11 deletion in $BRCA2^{endo}$ mice which is not visible in the $BRCA2^{WT}$ mice as full length BRCA2 transcript is far longer and not able to be amplified under these limited PCR conditions. **Figure 4-2, B** shows the overall transcript level in whole lung tissue of BRCA2 exon 11 diminished in a genotype dependent manner and **figure 4-2, C** demonstrates the same via quantitative polymerase chain reaction analysis of BRCA2 exon 11.

**A)**


BRCA2WT
BRCA2endo
BRCA2 (Ex.10F/Ex.14R)
905 bp
GAPDH
177 bp

**B)**


BRCA2WT
BRCA2het
BRCA2endo
BRCA2 (Ex.11F/Ex.11R)
296 bp
GAPDH
177 bp

**C)**

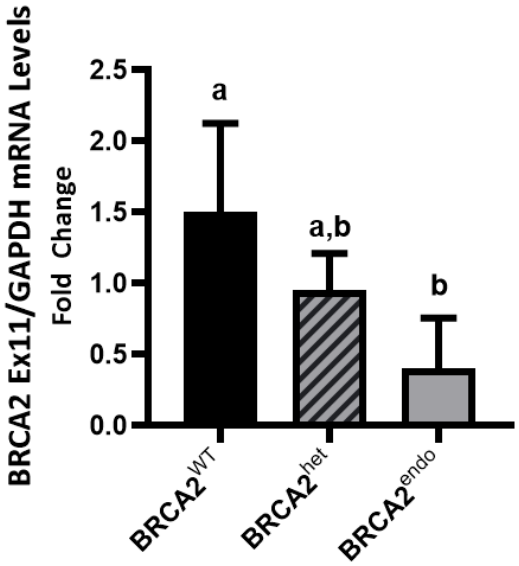
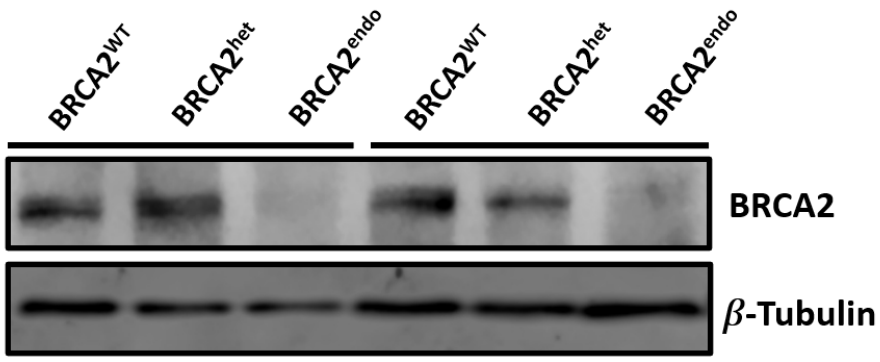

BRCA2 Ex11/GAPDH mRNA Levels
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
a
a,b
b
BRCA2WT
BRCA2het
BRCA2endo

**Figure 4-2. Confirmation of endothelial cell specific BRCA2 Deletion. (A)** Agarose gel image exhibiting the truncated BRCA2 (905 bp) PCR product produced by BRCA2 Exon 11 deletion only present in $BRCA2^{endo}$ mice. **(B)** PCR amplification of BRCA2 Exon 11 in whole-lung RNA extract demonstrating the genotype-dependent reduction of exon 11 in $BRCA2^{endo}$ and $BRCA2^{het}$ mice relative to their $BRCA2^{WT}$ counterparts. **(C)** qPCR results from the amplification of BRCA2 Exon 11 and GAPDH in whole-lung RNA extract revealed a significant difference between $BRCA2^{WT}$ (fold change = 1.5 ± 0.62, n=5), and $BRCA2^{endo}$ (fold change = 0.4 ± 0.36, n=5) (p=0.0035), while $BRCA2^{het}$ (fold change = 1.0 ± 0.26, n=6) was not significantly different from either. Data are presented as mean ± SD.

### 4.1.3 Loss of BRCA2 Protein in the Endothelium of Endothelial Cell-specific BRCA2 Knockout Mice

Immunoblotting of whole lung protein extract demonstrated a trend of decreased BRCA2 expression in $BRCA2^{endo}$ mice (**Figure 4-3, A**). Immunohistochemistry for BRCA2 presence confirmed loss of BRCA2 protein in the endothelium of the lung and aorta of $BRCA2^{endo}$ mice (**Figure 4-3, B**).

**A)**



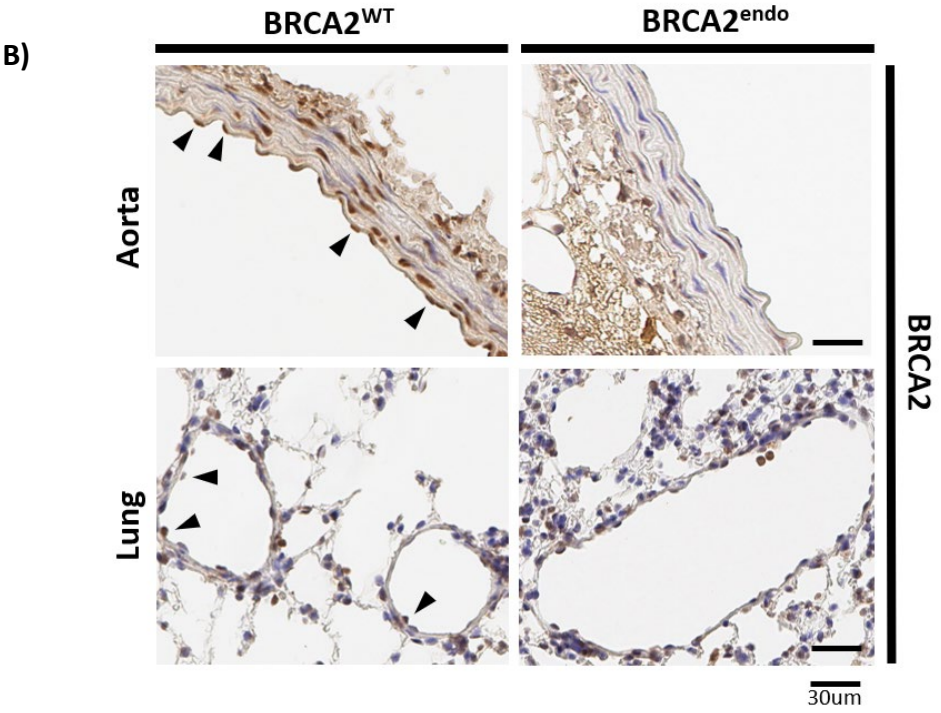



**Figure 4-3. (A)** Immunoblot of protein extracted from whole-lung tissue lysate for BRCA2 with Beta-Tubulin as a loading control (n=2). **(B)** Histological images of BRCA2 antibody-incubated DAB-stained aorta and lung of $BRCA2^{WT}$ and $BRCA2^{endo}$ mice (5um, n=2).

### 4.2 Characterization of Endothelial Cell-specific BRCA2 Knockout Mice

Endothelial cell specific BRCA2 knockout mice were characterized at baseline (8-20 weeks) for mendelian ratio, weight, DNA damage, cardiac and vascular function, and for metabolism.

#### 4.2.1 Endothelial Cell-specific BRCA2 Knockout Mice are Born in Expected Mendelian Ratio

Evaluating the Mendelian ratio of progeny born in transgenic crossing showed no deviation from the expected ratio in males (**Table 4-1**), females (**Table 4-2**), or in total progeny (**Table 4-3**).

### Table 4-1. Mendelian Ratio of Male Progeny

Genotype ratio of male progeny from $BRCA2^{fl/wt};VE\text{-}Cre^{Tg/}$ X $BRCA2^{fl/wt}$

| Genotype: | $BRCA2^{fl/fl};VE\text{-}Cre^{Tg/}$ | $BRCA2^{fl/fl};VE\text{-}Cre^{-/-}$ | $BRCA2^{fl/wt};VE\text{-}Cre^{Tg/}$ | $BRCA2^{fl/wt};VE\text{-}Cre^{-/-}$ | $BRCA2^{wt/wt};VE\text{-}Cre^{Tg/}$ | $BRCA2^{wt/wt};VE\text{-}Cre^{-/-}$ |
|---|---|---|---|---|---|---|
| expected no (%) | 5.875 (12.5%) | 5.875 (12.5%) | 11.75 (25%) | 11.75 (25%) | 5.875 (12.5%) | 5.875 (12.5%) |
| observed no (%) | 8 (17.0%) | 3 (6.4%) | 10 (21.3%) | 15 (31.9%) | 7 (14.9%) | 4 (8.5%) |

### Table 4-2. Mendelian Ratio of Female Progeny

Genotype ratio of female progeny from $BRCA2^{fl/wt};VE\text{-}Cre^{Tg/}$ X $BRCA2^{fl/wt}$

| Genotype: | $BRCA2^{fl/fl};VE\text{-}Cre^{Tg/}$ | $BRCA2^{fl/fl};VE\text{-}Cre^{-/-}$ | $BRCA2^{fl/wt};VE\text{-}Cre^{Tg/}$ | $BRCA2^{fl/wt};VE\text{-}Cre^{-/-}$ | $BRCA2^{wt/wt};VE\text{-}Cre^{Tg/}$ | $BRCA2^{wt/wt};VE\text{-}Cre^{-/-}$ |
|---|---|---|---|---|---|---|
| expected no (%) | 2.875 (12.5%) | 2.875 (12.5%) | 5.75 (25%) | 5.75 (25%) | 2.875 (12.5%) | 2.875 (12.5%) |
| observed no (%) | 0 (0.0%) | 2 (8.7%) | 10 (43.5%) | 6 (26.1%) | 4 (17.4%) | 1 (4.3%) |

### Table 4-3. Mendelian Ratio of Total Progeny

Genotype ratio of total progeny from $BRCA2^{fl/wt};VE\text{-}Cre^{Tg/}$ X $BRCA2^{fl/wt}$

| Genotype: | $BRCA2^{fl/fl};VE\text{-}Cre^{Tg/}$ | $BRCA2^{fl/fl};VE\text{-}Cre^{-/-}$ | $BRCA2^{fl/wt};VE\text{-}Cre^{Tg/}$ | $BRCA2^{fl/wt};VE\text{-}Cre^{-/-}$ | $BRCA2^{wt/wt};VE\text{-}Cre^{Tg/}$ | $BRCA2^{wt/wt};VE\text{-}Cre^{-/-}$ |
|---|---|---|---|---|---|---|
| expected no (%) | 5.875 (12.5%) | 5.875 (12.5%) | 11.75 (25%) | 11.75 (25%) | 5.875 (12.5%) | 5.875 (12.5%) |
| observed no (%) | 8 (17.0%) | 3 (6.4%) | 10 (21.3%) | 15 (31.9%) | 7 (14.9%) | 4 (8.5%) |

**Mendelian Ratio:** Endothelial cell specific BRCA2 heterozygous ($BRCA2^{het}$) and homozygous knockout male (**Table 4-1**, n=47), female (**Table 4-2**, n=23), and total (**Table 4-3**, n=70) mice are born in expected Mendelian Ratio. Chi-Squared analysis showed no significant difference from the expected ratio.

### 4.2.2 Endothelial Cell-specific BRCA2 Knockout Mice Display Similar Weight at 8 Weeks of Age

Weight was not found to be genotype-dependent at 8 weeks of age in males (**Figure 4-4, A**) or females (**Figure 4-4, B**).

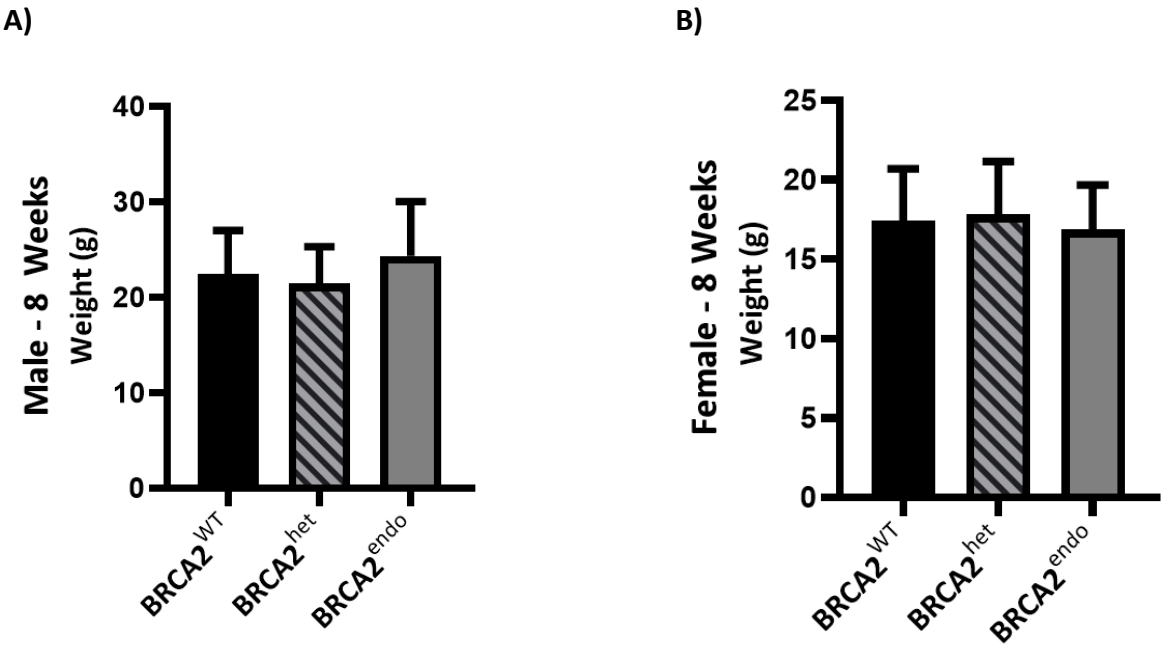
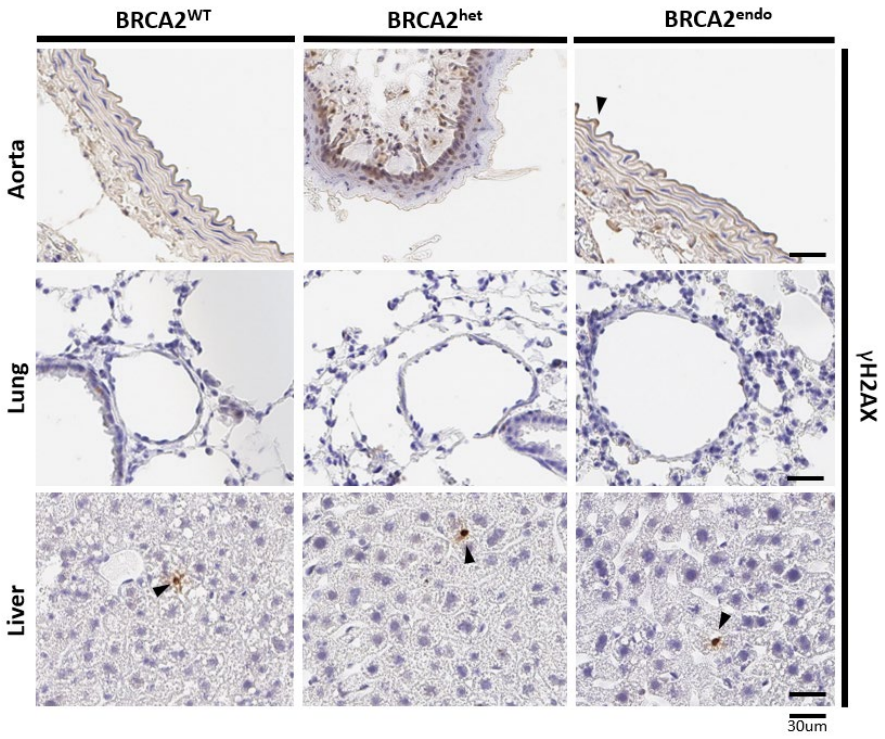



**Figure 4-4. Weight at 8 Weeks of Age**. Data are presented as means ± SD. Weight at 8 weeks was not significantly different between **(A)** male $BRCA2^{WT}$ (n= 34, 22.4g ± 4.59), $BRCA2^{het}$ (n= 20, 22.4g ± 3.87), and $BRCA2^{endo}$ (n=18, 24.3g ± 5.71) mice; or **(B)** female $BRCA2^{WT}$ (n= 27, 17.4g ± 3.26), $BRCA2^{het}$ (n= 19, 17.8g ± 3.32), and $BRCA2^{endo}$ (n=14, 16.9g ± 2.82) mice.

### 4.2.3 Endothelial Cell-specific BRCA2 Knockout Mice Demonstrate No Signs of DNA Damage and DNA Damage Repair in Endothelial Cells at Baseline

Aortas, lungs, and livers were collected from perfused mice. Tissues were fixed, cut, and stained for γH2AX (**Figure 4-5, A**) and Rad51 (**Figure 4-5, B**). H2AX is phosphorylated to γH2AX after the occurrence of DNA damage, while Rad51 is recruited by BRCA2 to the site of DNA damage to initiate DNA damage repair. No difference was observed between genotypes for γH2AX or Rad51 expression, indicating a similar extent of DNA damage and repair between the groups.

**A)**


BRCA2WT
BRCA2het
BRCA2endo
Aorta
Lung
Liver
γH2AX
30um

**B)**

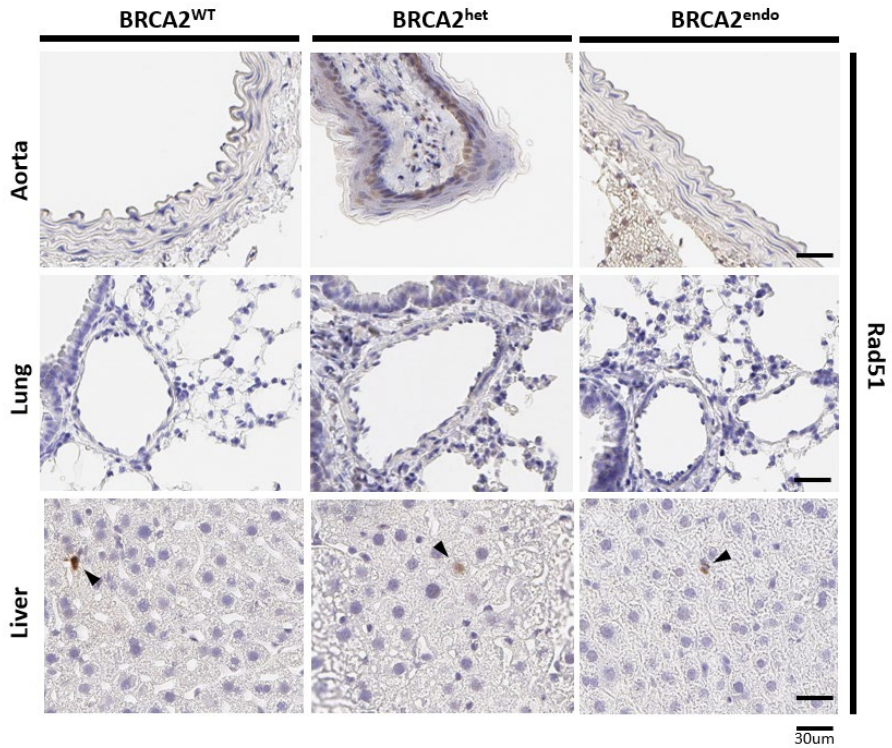
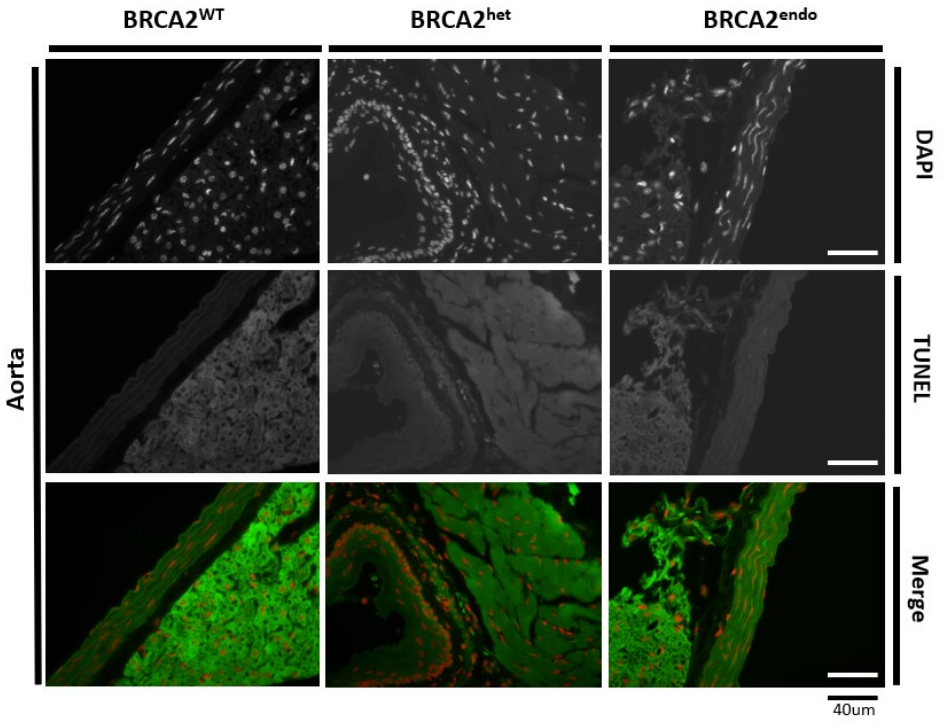

BRCA2WT
BRCA2het
BRCA2endo
Aorta
Lung
Liver
Rad51
30um

**Figure 4-5. Representative Histological Images.** Aorta, lung, and liver were dissected from perfused BRCA2$^{endo}$, BRCA2$^{het}$, and BRCA2$^{WT}$ littermates. Tissues were cut at 5 microns and sections were stained with antibodies for **(A)** yH2AX and **(B)** Rad51. Nuclei are stained blue by hematoxylin and antibody signal is visible as brown precipitate formed by horseradish peroxidase (HRP)-catalyzed Diaminobenzidine (DAB). Scale bar is 30um (n=2/group).

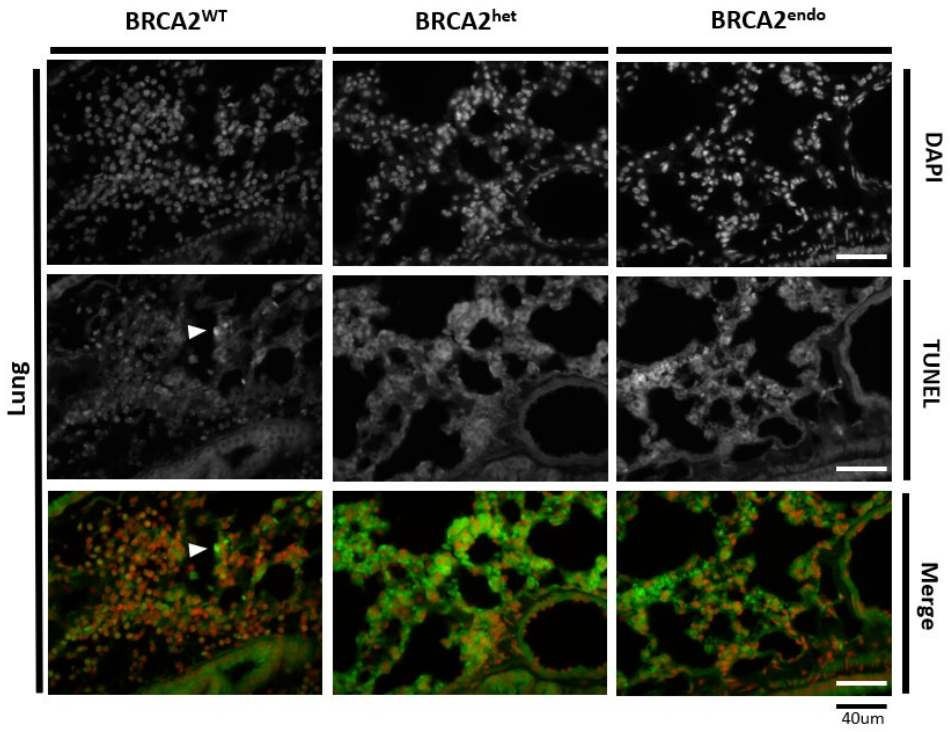
### 4.2.4 Endothelial Cell-specific BRCA2 Knockout Mice Demonstrate No Signs of Apoptosis in Endothelial Cells at Baseline

Sections of the aorta, lung, and liver were stained by terminal deoxynucleotidyl transferase dUTP nick end labeling (**TUNEL**), which detects DNA fragmentation generated during apoptosis. These nuclei of the same tissue segments were counterstained by DAPI. No TUNEL-positive cells were detected across genotypes in the aorta (**Figure 4-6, A**), and virtually no TUNEL-positive cells were detected across genotypes in the lung (**Figure 4-6, B**) or liver (**Figure 4-6, C**), with the only isolated incidence of TUNEL positivity in BRCA2$^{WT}$ lung and BRCA2$^{het}$ liver shown as experimental validation.

**A)**


BRCA2WT
BRCA2het
BRCA2endo
Aorta
DAPI
TUNEL
Merge
40um

**B)**


BRCA2WT
BRCA2het
BRCA2endo
Lung
DAPI
TUNEL
Merge
40um

**C)**

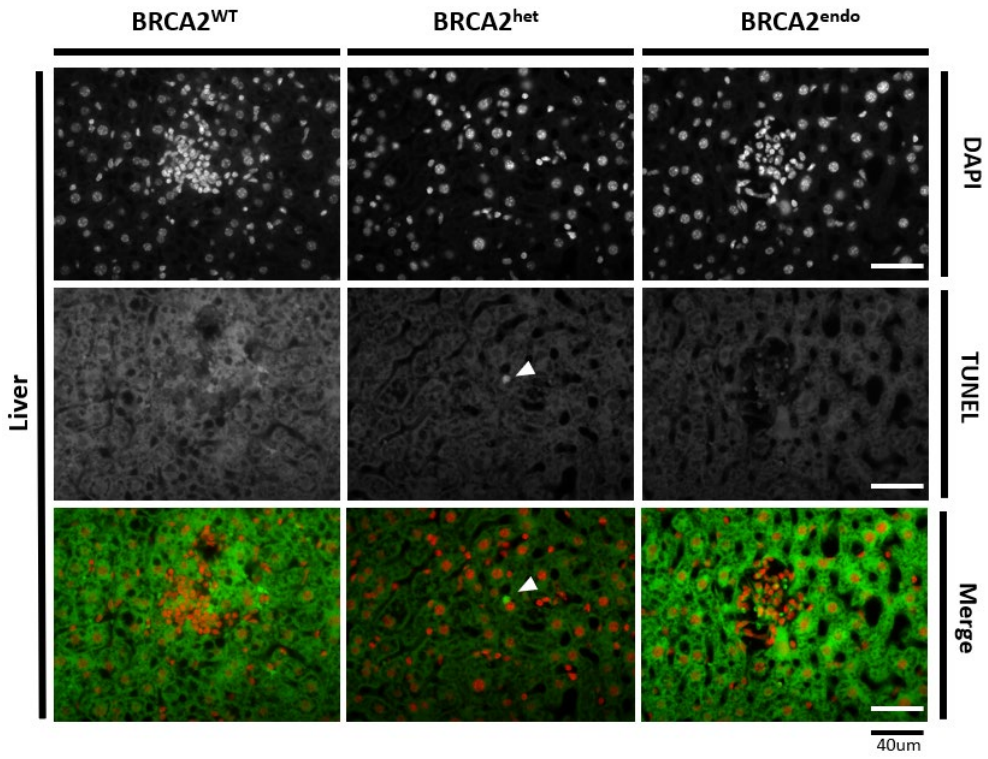
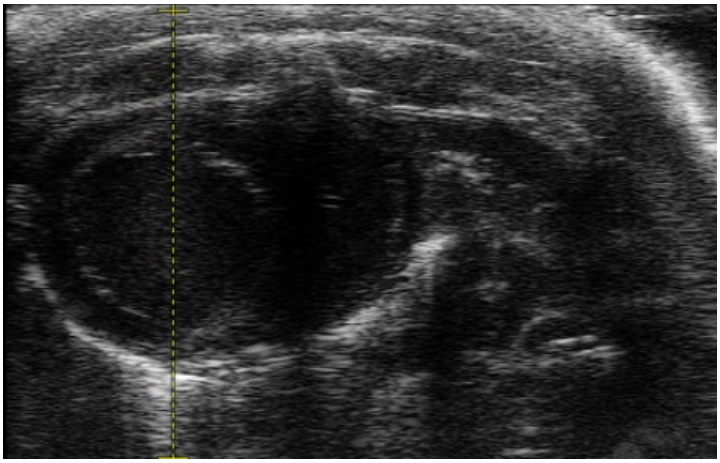



**Figure 4-6. No Apoptosis in Endothelial Cells of BRCA2$^{WT}$ and BRCA2$^{endo}$ Mice.** Representative DAPI, terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL), and colourized merged images of the **(A)** aorta and **(B)** lung show no apoptosis in the endothelium of BRCA2$^{WT}$, BRCA2$^{het}$, or BRCA2$^{endo}$ mice. **(C)** Liver sections stained by TUNEL were used as a positive control. n=2.

### 4.2.5 Endothelial Cell-specific BRCA2 Knockout Mice Display Similar Cardiac Function as WT Mice at Baseline

Ultrasound was performed on male (**Figure 4-8, A-B**) and female (**Figure 4-8, C-D**) mice and no significant genotype-dependent differences were found in left ventricle ejection fraction or fractional shortening indicating similar cardiac function at baseline. Echocardiographic images of B-mode showing the mouse chest cavity and heart in transverse (**Figure 4-7, A**) and M-mode, which visualizes the diameter during oscillations of the left ventricle used to determine LVEDD and LVESD (**Figure 4-7, B**).

**A)**



**B)**

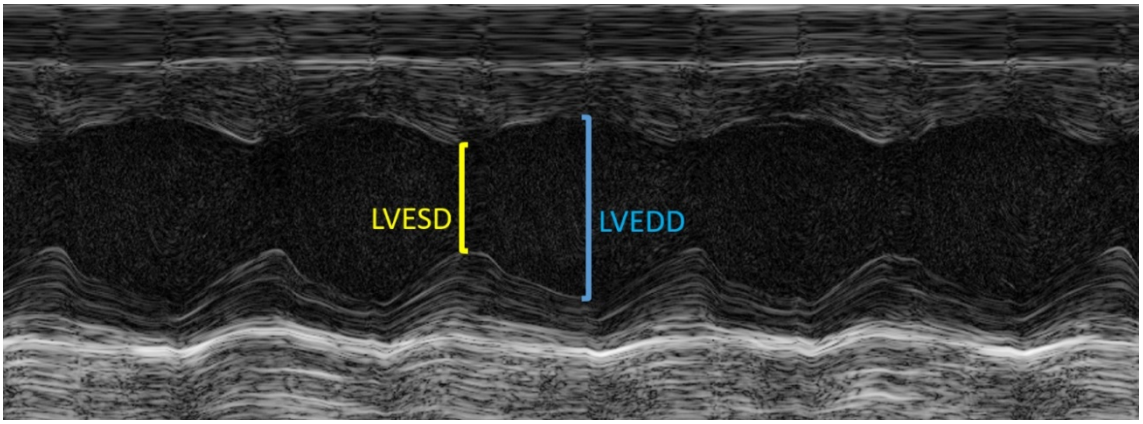



**Figure 4-7. Echocardiographic Images and Measurements.** Sample image of echocardiograph in **(A)** B-mode delineating the cardiac region being probed by **(B)** M-mode displaying left ventricle end diastolic diameter (LVEDD) and left ventricle end systolic diameter (LVESD).

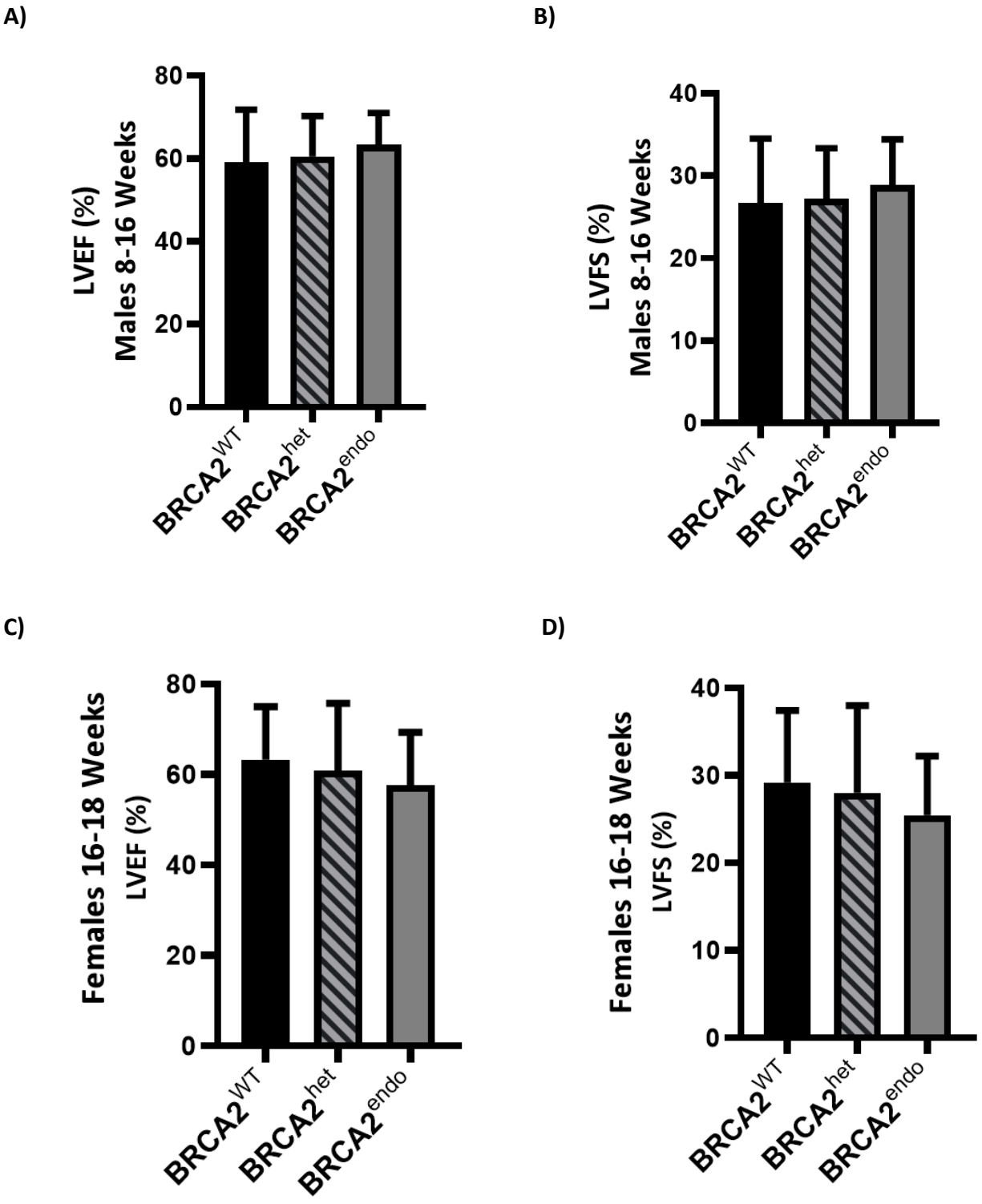
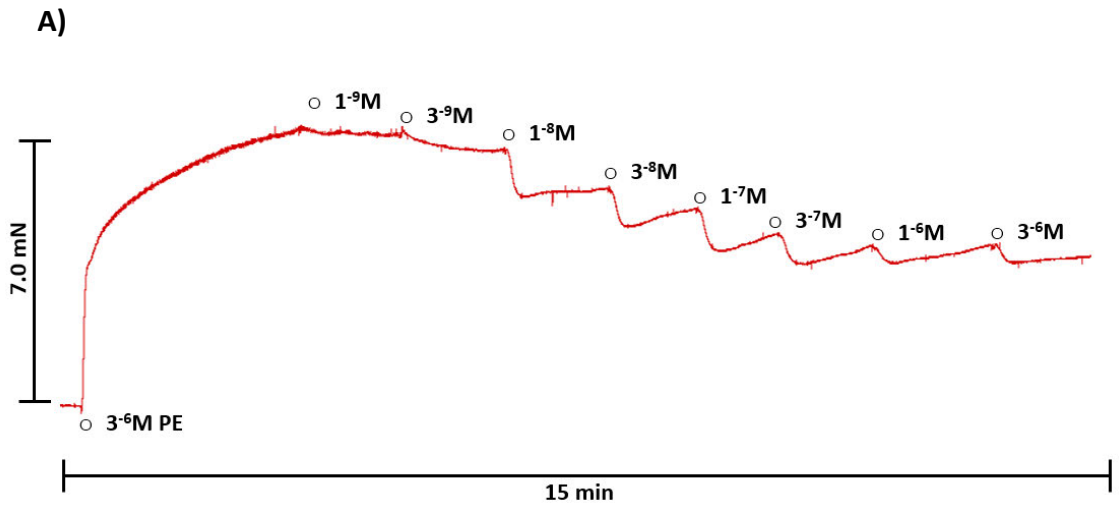


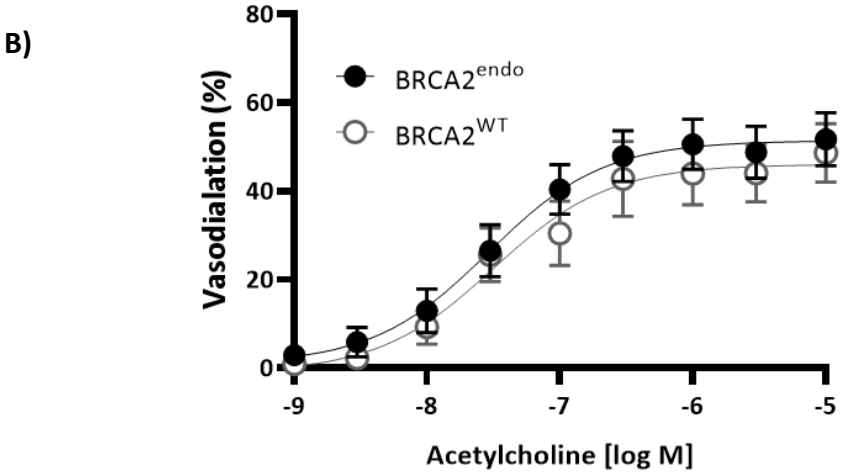

**Figure 4-8. Similar Baseline Cardiac Function in Male and Female Mice at 8-16 Weeks of Age.** Echocardiography was performed on male and female mice. Data for male mice at 8-10 weeks and 14-16 weeks were not significantly different and these data sets are combined to 8-16 weeks. No significant difference between genotypes was observed for

**(A)** male left ventricle ejection fraction (LVEF) in $BRCA2^{WT}$ (n=11, 59.2% ± 12.64), $BRCA2^{het}$ (n= 9, 60.5% ± 9.81), and $BRCA2^{endo}$ (n=9, 63.3% ± 7.67) or **(B)** left ventricle fractional shortening (LVFS) in $BRCA2^{WT}$ (n=11, 26.2% ± 7.78), $BRCA2^{het}$ (n=9, 27.2% ± 6.17), and $BRCA2^{endo}$ (n=9, 28.9% ± 5.51); or in in females for **(C)** LVEF in $BRCA2^{WT}$ (n=6, 63.3% ± 11.78), $BRCA2^{het}$ (n=5, 60.9% ± 14.83), and $BRCA2^{endo}$ (n=5, 57.7% ± 11.70) or **(D)** LVFS in in $BRCA2^{WT}$ (n=6, 29.2% ± 8.22), $BRCA2^{het}$ (n=5, 28.0% ± 9.97), and $BRCA2^{endo}$ (n=5, 25.4% ± 6.78). Statistical analysis was performed by one-way ANOVA with Tukey's post hoc. Data are presented as mean ± SD.

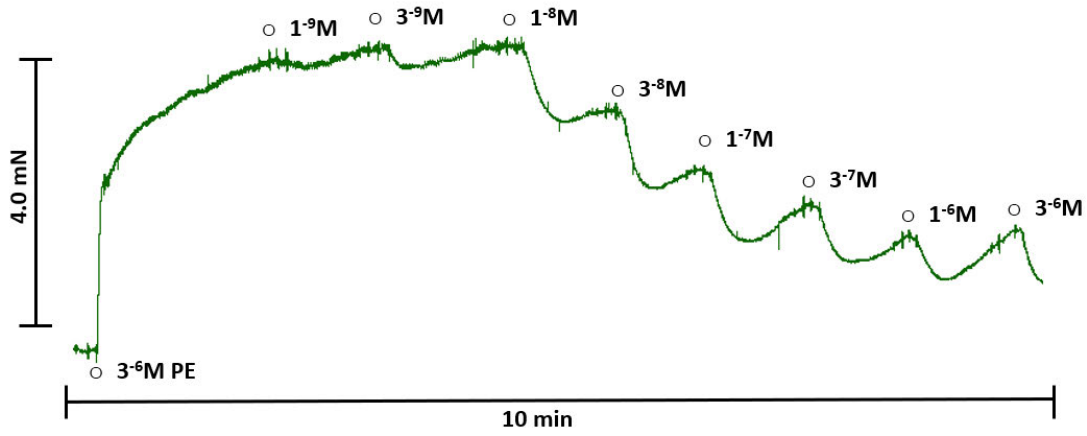
### 4.2.6 Endothelial Cell-specific BRCA2 Knockout Mice Display Similar Vascular Function as WT Mice at Baseline

Aortic segments were extracted, mounted on pins in Krebs buffer, and challenged with increasing doses of the endothelial dependent vasodilators acetylcholine (**Figure 4-9, A-B**) and 22-furoyl-LIGRLO-NH2 (**Figure 4-9, C-D**), and the endothelium independent vasodilator sodium nitroprusside (**Figure 4-9, E-F**). Similar vasodilatory response to each drug was observed across genotypes, indicating a lack of endothelial dysfunction in the BRCA2$^{endo}$ mice.

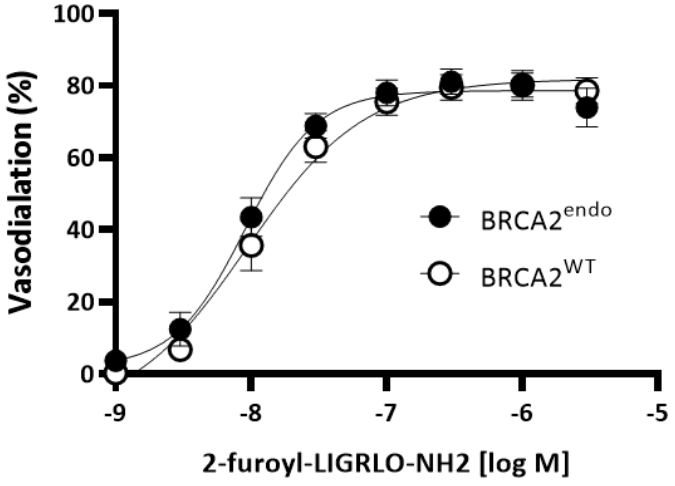


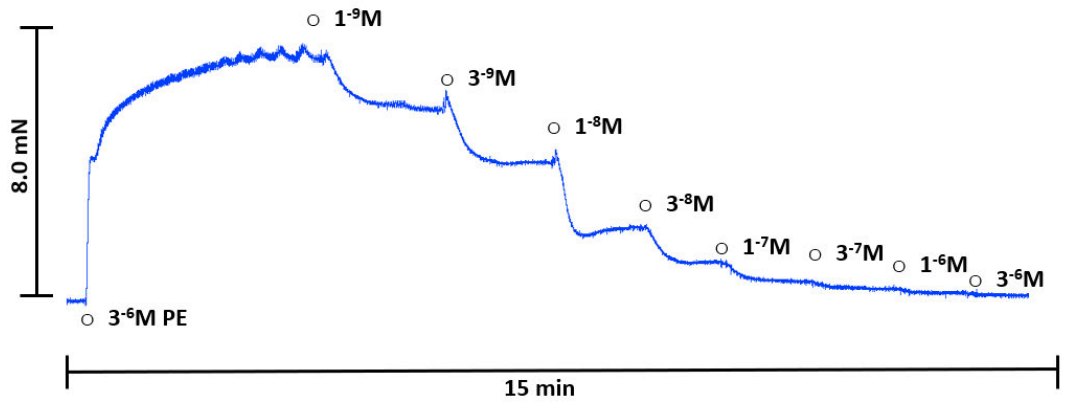
**C)**


1-9M
3-9M
1-8M
3-8M
1-7M
3-7M
1-6M
3-6M
4.0 mN
3-6M PE
10 min

**D)**


Vasodialation (%)
100
80
60
40
20
0
-9
-8
-7
-6
-5
BRCA2endo
BRCA2WT
2-furoyl-LIGRLO-NH2 [log M]

E)




F)

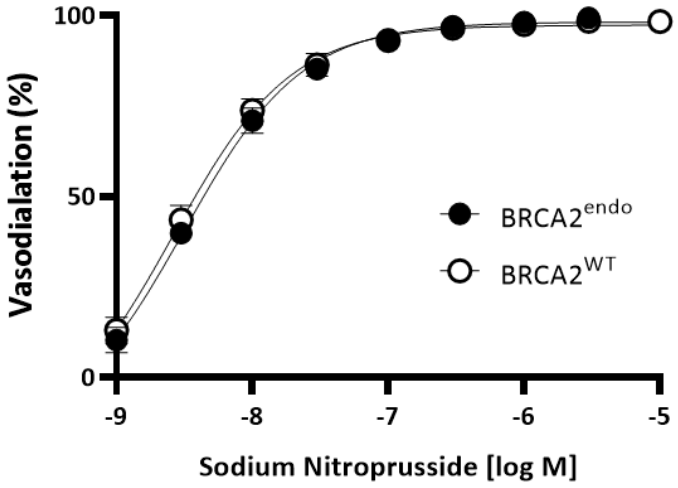



**Figure 4-9. Similar Vasoreactivity at Baseline in WT and EC-specific BRCA2 Knockout Mice**. Representative myographic tracings are shown for each vasodilator. Four parameter sigmoidal dose response curve demonstrates similar dose-dependent vasorelaxation response when vessels were subjected to **(A-B)** acetylcholine (ACh) (n=5, p>0.05), **(C-D)** 2-furoyl-LIGRLO-NH2 (2FLY), or **(E-F)** sodium nitroprusside (SNP) $BRCA2^{endo}$. n=5, data are presented as mean ± SEM.

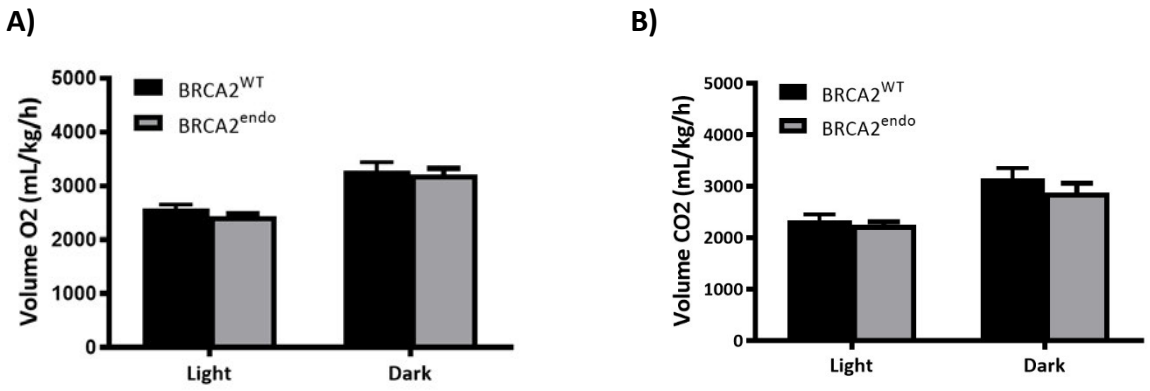
**Table 4-4**

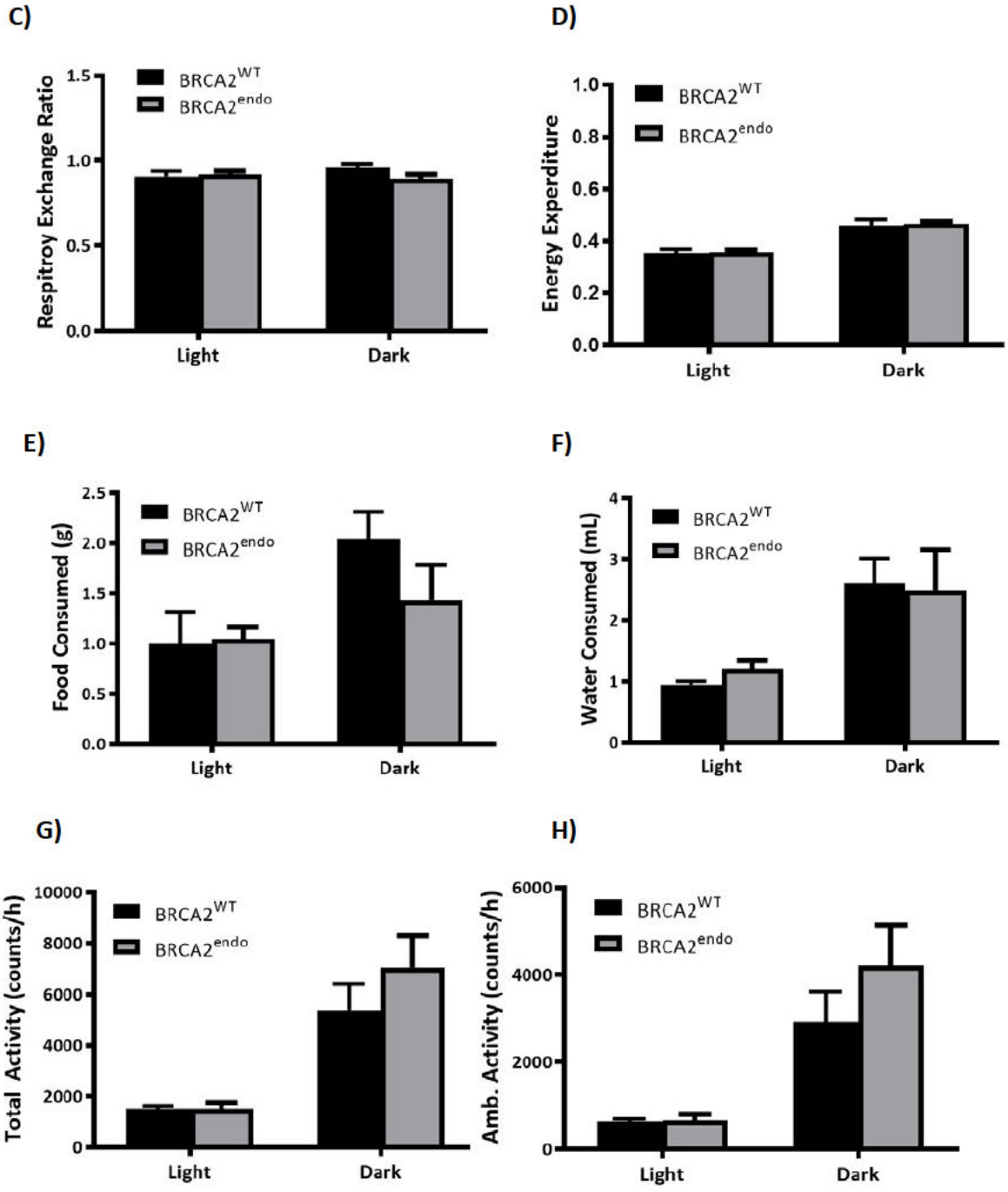
| Vasodilator | Genotype | -logEC$_{50}$ | E$_{max}$ | Hill Slope |
|---|---|---|---|---|
| **ACh** | BRCA2$^{endo}$ | 7.52 | 50.85 | 1.12 |
| | BRCA2$^{WT}$ | 7.52 | 47.00 | 0.85 |
| **2-fly** | BRCA2$^{endo}$ | 8.04 | 78.64 | 1.66 |
| | BRCA2$^{WT}$ | 7.95 | 79.52 | 1.45 |
| **SNP** | BRCA2$^{endo}$ | 8.68 | 99.32 | 0.83 |
| | BRCA2$^{WT}$ | 8.72 | 98.11 | 0.90 |

**Table 4-4. Similar Vasoreactivity at Baseline in WT and EC specific BRCA2 Knockout Mice**. Vasoreactivity to acetylcholine (ACh), 2-furoyl-LIGRLO-NH2 (2FLY), and sodium nitroprusside was not significantly different between BRCA2$^{endo}$ and BRCA2$^{WT}$ mice as indicated by $E_{max}$ and shared hill slope and -logEC$_{50}$ best-fit values as calculated by the generated four parameter sigmoidal dose response curve. n=5, data are analyzed by two-way ANOVA with Bonferroni's post-hoc.

### 4.2.7 Endothelial Cell-specific BRCA2 knockout mice Display Similar Metabolic Parameters as WT Mice at Baseline

Mice were placed in metabolic caging and monitored for respiration, activity, and food and water intake. Similar metabolic function was observed between genotypes across all metabolic parameters.




C)
Respitroy Exchange Ratio
1.5
1.0
0.5
0.0
BRCA2WT
BRCA2endo
Light
Dark
D)
Energy Experditure
1.0
0.8
0.6
0.4
0.2
0.0
BRCA2WT
BRCA2endo
Light
Dark
E)
Food Consumed (g)
2.5
2.0
1.5
1.0
0.5
0.0
BRCA2WT
BRCA2endo
Light
Dark
F)
Water Consumed (mL)
4
3
2
1
0
BRCA2WT
BRCA2endo
Light
Dark
G)
Total Activity (counts/h)
10000
8000
6000
4000
2000
0
BRCA2WT
BRCA2endo
Light
Dark
H)
Amb. Activity (counts/h)
6000
4000
2000
0
BRCA2WT
BRCA2endo
Light
Dark

**I)**

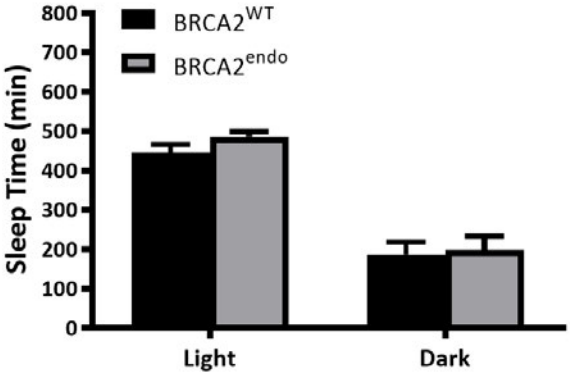



**Figure 4-10. Similar Metabolic Function at Baseline in BRCA2$^{WT}$ and BRCA2$^{endo}$ mice**. No significant difference was observed during a 50/50 light/dark cycle over 24 hours in **(A)** O2 consumption between BRCA2$^{WT}$ (light=2567mL/kg/h ± 80; dark=3279 mL/kg/h ± 154) and BRCA2$^{endo}$ (light=2435mL/kg/h ± 49; dark=3205mL/kg/h ± 118) mice; or **(B)** CO2 emission between BRCA2$^{WT}$ (light=2332mL/kg/h ± 116; dark=3144 mL/kg/h ± 203) and BRCA2$^{endo}$ (light=2250mL/kg/h ± 57; dark=2867mL/kg/h ± 187); or **(C)** in respiratory exchange ratio between BRCA2$^{WT}$ (light=0.91 ± 0.031; dark=0.96 ± 0.020) and BRCA2$^{endo}$ (light=0.92 ± 0.017; dark=0.89 ± 0.027); or **(D)** energy expenditure between BRCA2$^{WT}$ (light=0.35 ± 0.015; dark=0.46 ± 0.024) and BRCA2$^{endo}$ (light=0.36 ± 0.011; dark=0.47 ± 0.007); or **(E)** food consumption between BRCA2$^{WT}$ (light=1.0g ± 0.31; dark=2.0g ± 0.28) and BRCA2$^{endo}$ (light=1.0g ± 0.12; dark=1.4 ± 0.35); or **(F)** in water consumption between BRCA2$^{WT}$ (light=0.9mL ± 0.06; dark=2.6mL ± 0.40) and BRCA2$^{endo}$ (light=1.2mL ± 0.13; dark=2.5 ± 0.67); or **(G)** total activity between BRCA2$^{WT}$ (light=1819counts/h ± 99; dark=5357counts/h ± 1044) and BRCA2$^{endo}$ (light=1524counts/h ± 239; dark=7051 ± 1243); or **(H)** ambulatory activity between BRCA2$^{WT}$ (light=624counts/h ± 62; dark=2916counts/h ± 700) and BRCA2$^{endo}$ (light=656counts/h ± 145; dark=4211counts/h ± 914); or **(I)** time asleep between BRCA2$^{WT}$ (light=446min ± 21; dark=186min ± 31) and BRCA2$^{endo}$ (light=486min ± 13; dark=198min ± 36) mice. Data are presented as mean ± SEM, n=5.

## Chapter 4. Results Part II

***HFD-exacerbated atherosclerosis in endothelial cell-specific BRCA2 deficient atherosclerotic mouse (ApoE-/-) model***

### 4.3 Generation and Characterization of ApoE$^{-/-}$;BRCA2$^{endo}$ – Atherosclerotic Mouse Model Characterization

Breeding was executed pursuant to the schematic in **Figure 3-2** to produce a cohort of mice all lacking a functional ApoE protein (ApoE$^{-/-}$) and of the same experimental BRCA2;VE-Cre genotypes as described in **section 4.1** (BRCA2$^{wt/wt}$;VE-Cre$^{tg/-}$, BRCA2$^{fl/fl}$;VE-Cre$^{-/-}$, BRCA2$^{fl/wt}$;VE-Cre$^{-/-}$, BRCA2$^{wt/wt}$;VE-Cre$^{-/-}$, BRCA2$^{fl/wt}$;VE-Cre$^{tg/-}$, and BRCA2$^{fl/fl}$;VE-Cre$^{tg/-}$,)**.** Again, where possible, BRCA2$^{WT;Cre+}$ mice were evaluated separately and grouped if results did not significantly differ from Cre- BRCA2$^{WT}$ mice.

#### 4.3.1 Genotype Identification of ApoE Mutant Allele

Pro-atherosclerotic mice were generated by the addition of another transgene; an ApoE mutation causing lipid dysregulation. The desired double mutation genotype was identified to inform breeding. The possible permutations of the ApoE mutation are shown below in **Figure 4-11**. The PCR product of 155bp represent WT and 245bp represents the mutant ApoE allele.

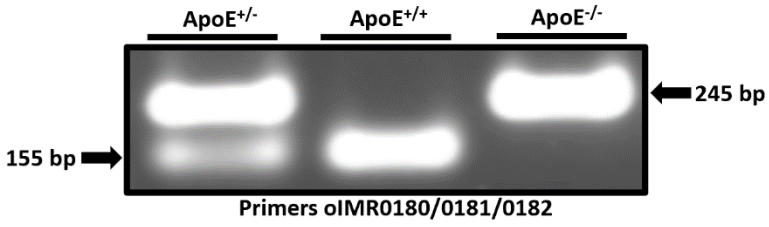



**Figure 4-11. ApoE Mutation Genotype.** Representative agarose gel image of genotype variants of ApoE mutation. Identification performed by ear tissue digest and amplified by PCR using primers for the ApoE mutant allele (**Table 3-1**).

### 4.3.2 ApoE$^{-/-}$;BRCA2$^{endo}$ Mice are Born in Expected Mendelian Ratio

Evaluating the Mendelian ratio of progeny born in transgenic crossing aof ApoE$^{-/-}$ mice showed no deviation from the expected ratio in males (**Table 4-5**), females (**Table 4-6**), or in total progeny (**Table 4-7**).

**Table 4-5**

Genotype ratio of male progeny from Apoe$^{null}$;BRCA2$^{fl/wt}$;VE-Cre$^{Tg/}$ X Apoe$^{null}$;BRCA2$^{fl/wt}$

| Genotype: | Apoe$^{null}$;BRCA2$^{fl/fl}$;VE-Cre$^{Tg/}$ | Apoe$^{null}$;BRCA2$^{fl/fl}$;VE-Cre$^{-/-}$ | Apoe$^{null}$;BRCA2$^{fl/wt}$;VE-Cre$^{Tg/}$ | Apoe$^{null}$;BRCA2$^{fl/wt}$;VE-Cre$^{-/-}$ |
|---|---|---|---|---|
| expected no (%) | 12.5 (25%) | 12.5 (25%) | 12.5 (25%) | 12.5 (25%) |
| observed no (%) | 9 (18%) | 13 (26%) | 14 (28%) | 14 (28%) |

**Table 4-6**

Genotype ratio of female progeny from Apoe$^{null}$;BRCA2$^{fl/wt}$;VE-Cre$^{Tg/}$ X Apoe$^{null}$;BRCA2$^{fl/wt}$

| Genotype: | Apoe$^{null}$;BRCA2$^{fl/fl}$;VE-Cre$^{Tg/}$ | Apoe$^{null}$;BRCA2$^{fl/fl}$;VE-Cre$^{-/-}$ | Apoe$^{null}$;BRCA2$^{fl/wt}$;VE-Cre$^{Tg/}$ | Apoe$^{null}$;BRCA2$^{fl/wt}$;VE-Cre$^{-/-}$ |
|---|---|---|---|---|
| expected no (%) | 5.25 (25%) | 5.25 (25%) | 5.25 (25%) | 5.25 (25%) |
| observed no (%) | 3 (14.3%) | 8 (38.1%) | 5 (23.8%) | 5 (23.8%) |

**Table 4-7**

Genotype ratio of progeny from Apoe$^{null}$;BRCA2$^{fl/wt}$;VE-Cre$^{Tg/}$ X Apoe$^{null}$;BRCA2$^{fl/wt}$

| Genotype: | Apoe$^{null}$;BRCA2$^{fl/fl}$;VE-Cre$^{Tg/}$ | Apoe$^{null}$;BRCA2$^{fl/fl}$;VE-Cre$^{-/-}$ | Apoe$^{null}$;BRCA2$^{fl/wt}$;VE-Cre$^{Tg/}$ | Apoe$^{null}$;BRCA2$^{fl/wt}$;VE-Cre$^{-/-}$ |
|---|---|---|---|---|
| expected no (%) | 17.75 (25%) | 17.75 (25%) | 17.75 (25%) | 17.75 (25%) |
| observed no (%) | 12 (16.9%) | 21 (29.6%) | 19 (26.8%) | 19 (26.8%) |

**Tables 4-5 – 4.7. Mendelian Ratio for 2 ApoE$^{-/-}$;BRCA2$^{endo}$ Mice:** Male (**Table 4-5**, n=50), female (**Table 4-6**, n=21), and total (**Table 4-7**, n=71) mice are born in expected Mendelian Ratio. Chi-Squared analysis demonstrated no statistical deviation from expected ratio.

### 4.3.3 ApoE$^{-/-}$;BRCA2$^{endo}$ and Endothelial Cell-specific BRCA2 Knockout Mice Show Similar Weight at 10 Weeks of Age

The weight of ApoE$^{-/-}$ mice at 10 weeks of age was not found to significantly differ based on genotype in males (**Figure 4-12, A**) or females (**Figure 4-12, B**).

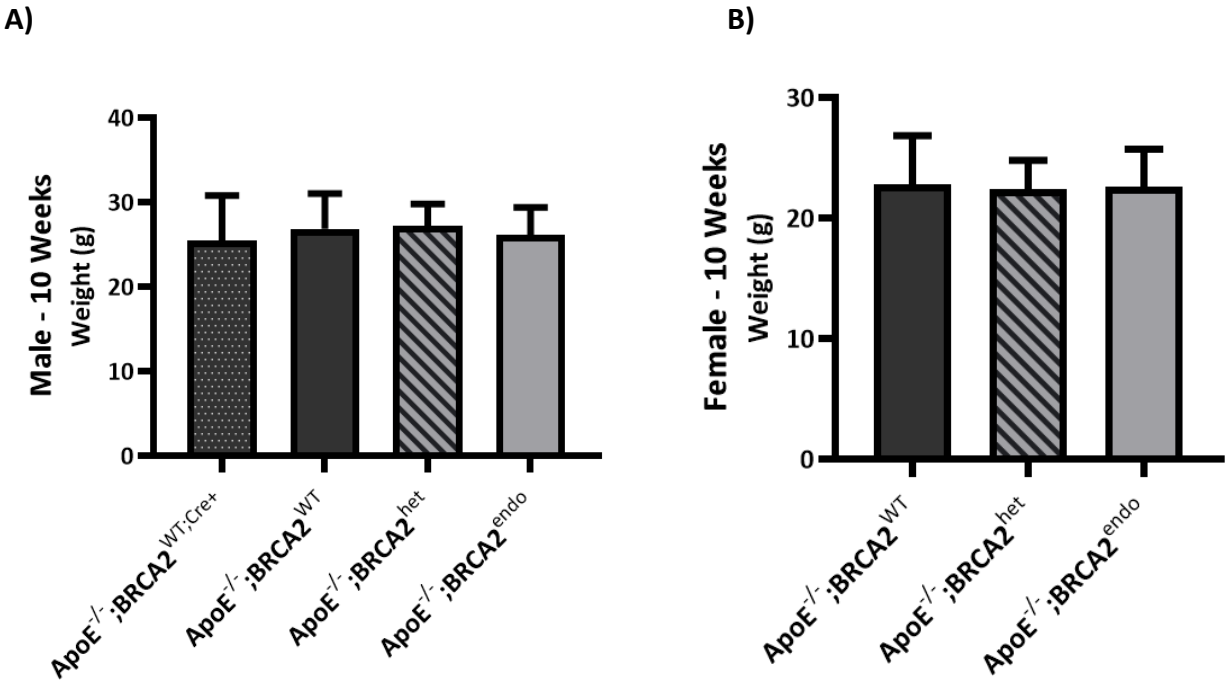



**Figure 4-12 Weight at 10 Weeks of Age.** Weight at 10 weeks was not significantly different between **(A)** male ApoE$^{-/-}$; BRCA2$^{WT;Cre+}$ (n=4, 25.6g ± 5.30), ApoE$^{-/-}$;BRCA2$^{WT}$ (n= 40, 26.9g ± 4.15), ApoE$^{-/-}$;BRCA2$^{het}$ (n= 19, 27.2g ± 2.61), and ApoE$^{-/-}$;BRCA2$^{endo}$ mice (n=29, 26.2g ± 3.27); or **(B)** female ApoE$^{-/-}$;BRCA2$^{WT}$ (n= 30, 22.9g ± 4.04), ApoE$^{-/-}$;BRCA2$^{het}$ (n= 13, 22.5g ± 2.39), and ApoE$^{-/-}$;BRCA2$^{endo}$ (n=13, 22.7g ± 3.10) mice. Data are presented as mean ± SD.

### 4.3.4 High-fat Diet Induced Weight-gain in ApoE$^{-/-}$;BRCA2$^{endo}$ Mice

Weight at 10 weeks of age was similar between all tested genotypes. However, upon the administration of high-fat diet, male and female ApoE$^{-/-}$;BRCA2$^{endo}$ mice appear to be undergoing accelerated net-weight gain up to 8 and 10 weeks HFD respectively, with male

mice of this genotype losing weight after 8 weeks HFD. Moreover, when weight gain was interpreted as a cumulative percent of body weight, this weight loss seems more drastic and begins to emerge as early as 6 weeks in male ApoE$^{-/-}$;BRCA2$^{endo}$ mice.

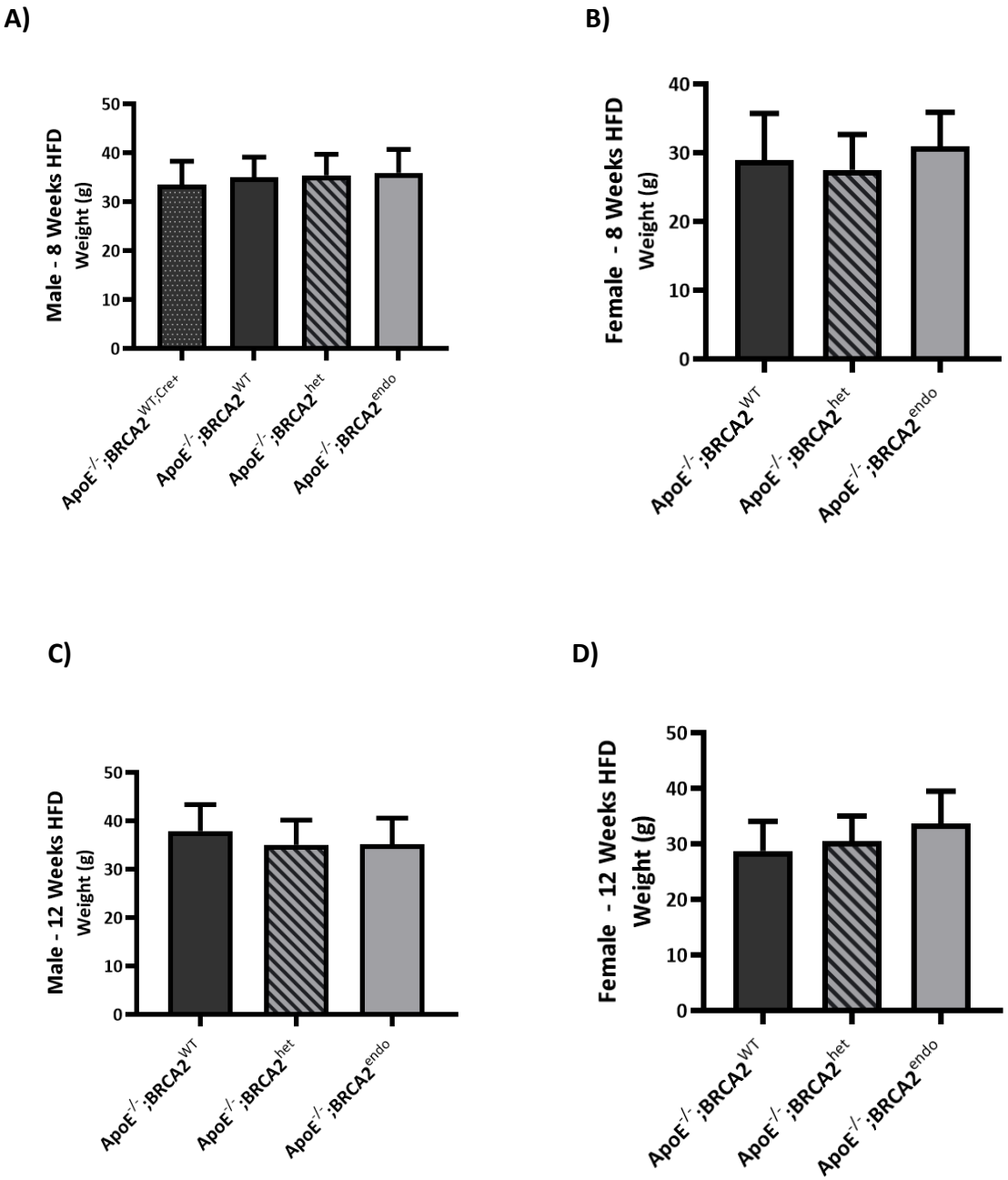


**Figure 4-13. Weight after 8 Weeks of High-fat Diet**. Weight at 10 weeks was not significantly different between **(A)** male ApoE$^{-/-}$;BRCA2$^{WT;Cre+}$ (n=4, 33.6g ± 4.75), ApoE$^{-/-}$;BRCA2$^{WT}$ (n=22, 35.9g ± 4.35), ApoE$^{-/-}$;BRCA2$^{het}$ (n=15, 35.5g ± 4.52), and ApoE$^{-/-}$;BRCA2$^{endo}$ (n=9, 37.1g ± 5.45); or **(B)** female ApoE$^{-/-}$;BRCA2$^{WT}$ (n=21, 31.9g ± 7.20), ApoE$^{-/-}$;BRCA2$^{het}$ (n=17, 29.3g ± 5.07), and ApoE$^{-/-}$;BRCA2$^{endo}$ (n=7, 32.4g ± 5.27) mice. Mouse weight after 12 weeks HFD was similar in **(C)** male ApoE$^{-/-}$;BRCA2$^{WT}$ (n=12, 38.0g ± 5.44), ApoE$^{-/-}$;BRCA2$^{het}$ (n=7, 35.2g ± 5.07), and ApoE$^{-/-}$;BRCA2$^{endo}$ (n=4, 35.3g ± 5.36); or **(D)** female ApoE$^{-/-}$;BRCA2$^{WT}$ (n=13, 28.8g ± 5.35), ApoE$^{-/-}$;BRCA2$^{het}$ (n=9, 30.6g ± 4.52), and ApoE$^{-/-}$;BRCA2$^{endo}$ (n=4, 33.7g ± 5.88) mice. Data are presented as mean ± SD and analyzed via one way ANOVA with Tukey's post-hoc.

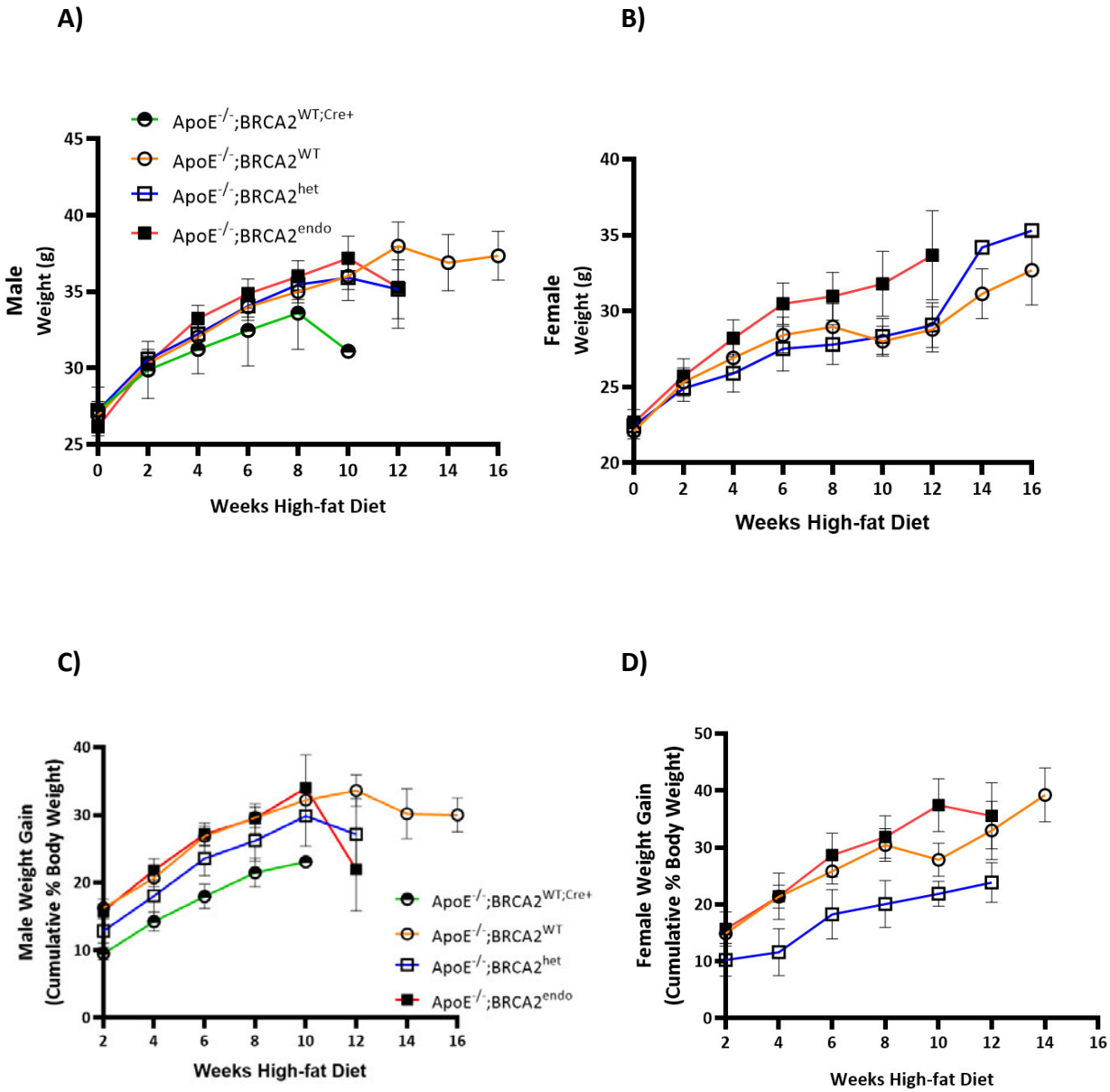
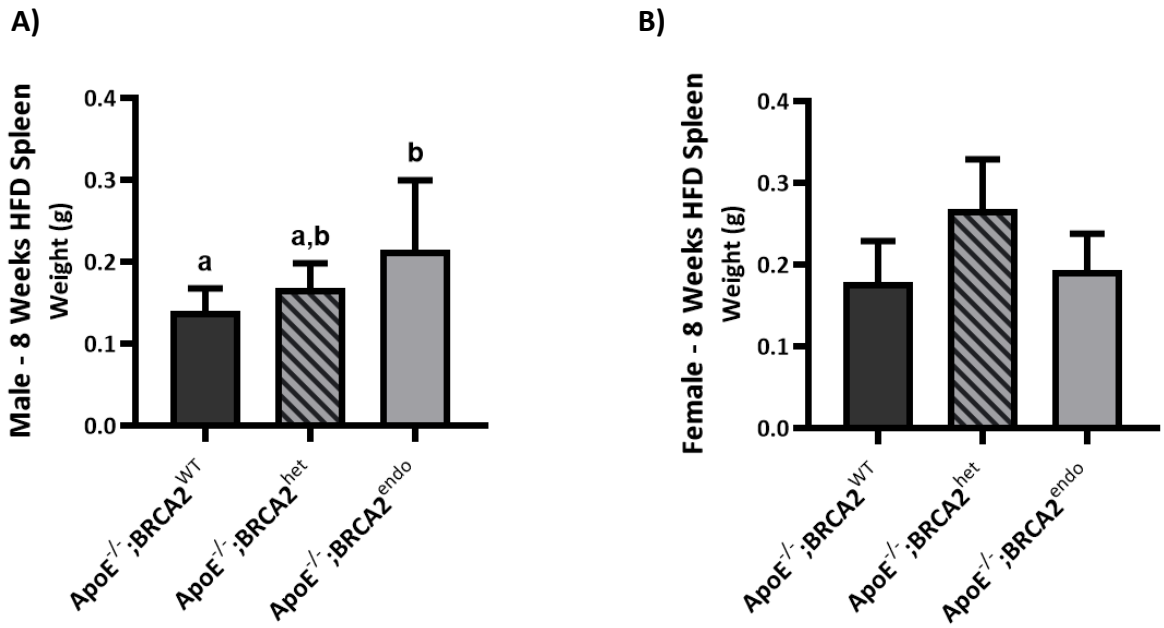


**Figure 4-14. Weight Gain on High-fat Diet.** Graphs illustrating net weight from the initiation of HFD and every two weeks in **(A)** males and **(B)** females as well as graphs showing cumulative weight gain as a percentage of body weight from the initiation of HFD and at every two-week interval in **(C)** males and **(D)** females. Data are presented as mean ± SEM.

### 4.3.5 High-fat Diet Induced Spleen Enlargement in ApoE$^{-/-}$;BRCA2$^{endo}$ Mice

Mice were observed to have a high incidence of splenomegaly, with an overarching trend towards increased spleen weights in the heterozygous and homozygous knockouts vs wildtype control mouse spleen weight (**Figure 4-15**). Spleen-weight was significantly higher in male 8-week-old homozygous (ApoE$^{-/-}$;BRCA2$^{endo}$) male spleens (**Figure 4-15, A**).



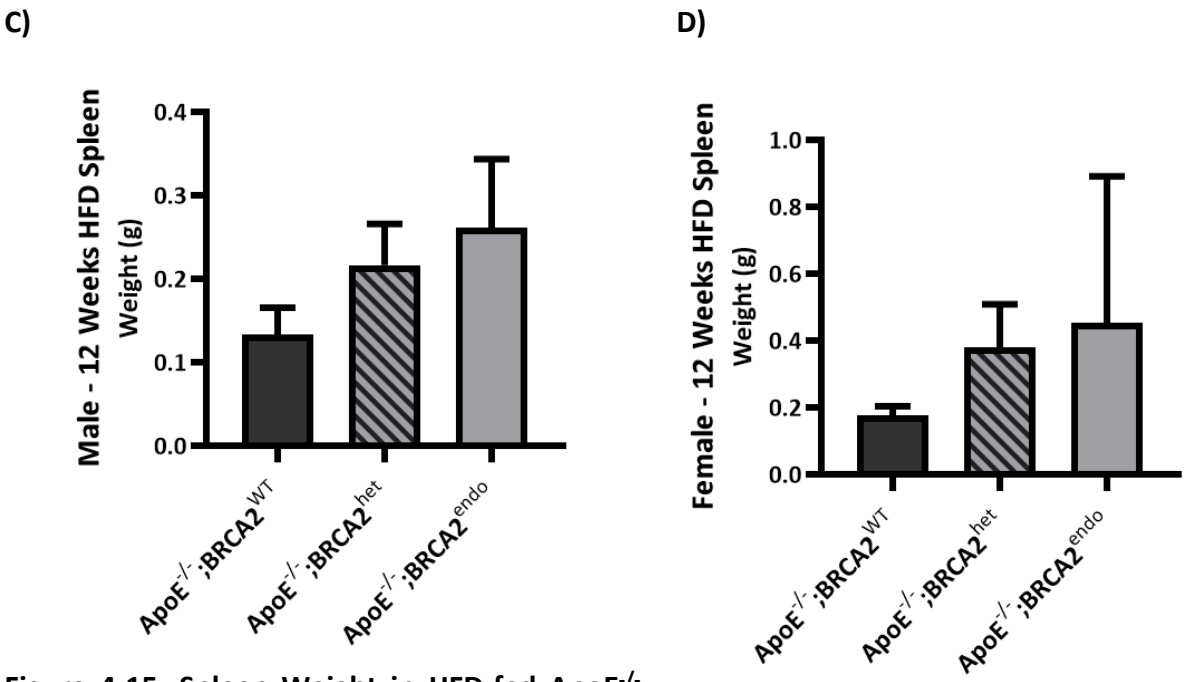



**Figure 4-15. Spleen Weight in HFD-fed ApoE$^{-/-}$ mice.** Significant difference was found in the spleen weight of **(A)** males fed HFD for 8 weeks between ApoE$^{-/-}$;BRCA2$^{WT}$ (n=10, 0.141g ± 0.0271) and ApoE$^{-/-}$;BRCA2$^{endo}$ (n=7, 0.216g ± 0.0848) (p=0.0227). While in this group, ApoE$^{-/-}$;BRCA2$^{het}$ (n=6, 0.169g ± 0.0303) was not significantly different than either. No significant difference was found in (**B)** females fed HFD for 8 weeks between ApoE$^{-/-}$;BRCA2$^{WT}$ (n=4, 0.179g ± 0.0506), ApoE$^{-/-}$;BRCA2$^{het}$ (n=6, 0.268g ± 0.0612), or ApoE$^{-/-}$;BRCA2$^{endo}$ (n=3, 0.194g ± 0.0441) mice; or **(C)** males fed HFD for 12 weeks between ApoE$^{-/-}$;BRCA2$^{WT}$ (n=3, 0.0134g ± 0.0321), ApoE$^{-/-}$;BRCA2$^{het}$ (n=4, 0.217g ± 0.0494), or ApoE$^{-/-}$;BRCA2$^{endo}$ (n=3, 0.262g ± 0.0823); or **(D)** females fed HFD for 12 weeks between ApoE$^{-/-}$;BRCA2$^{WT}$ (n=6, 0.179g ± 0.0260), ApoE$^{-/-}$;BRCA2$^{het}$ (n=3, 0.381g ± 0.1292), or ApoE$^{-/-}$;BRCA2$^{endo}$ (n=4, 0.454g ± 0.4394). Data are analyzed by one-way ANOVA with Tukey's post hoc and are presented as mean ± SD.

### 4.3.6 High-fat Diet Induced Significantly Higher Plaque Burden in the Aortic Arch of ApoE$^{-/-}$;BRCA2$^{endo}$ Mice

After feeding ApoE$^{-/-}$ mice a Western-type HFD for 8 and 12 weeks, a trend towards increased atherosclerotic burden in the ApoE$^{-/-}$;BRCA2$^{het}$ and ApoE$^{-/-}$;BRCA2$^{endo}$ mice was observed (**Figure 4-16**). Moreover, significant differences were found in males with 8-week HFD-fed males exhibiting significantly higher aortic arch plaque as a percent of total area in both ApoE$^{-/-}$;BRCA2$^{het}$ and ApoE$^{-/-}$;BRCA2$^{endo}$ genotypes vs ApoE$^{-/-}$;BRCA2$^{WT}$ (**Figure 4-17, A**). Significantly higher plaque percent was also observed in the aortic arch of ApoE$^{-/-}$;BRCA2$^{endo}$ vs ApoE$^{-/-}$;BRCA2$^{WT}$ in 12-week HFD-fed male ApoE$^{-/-}$ mice (**Figure 4-17, B**).

**A)**


**B)**

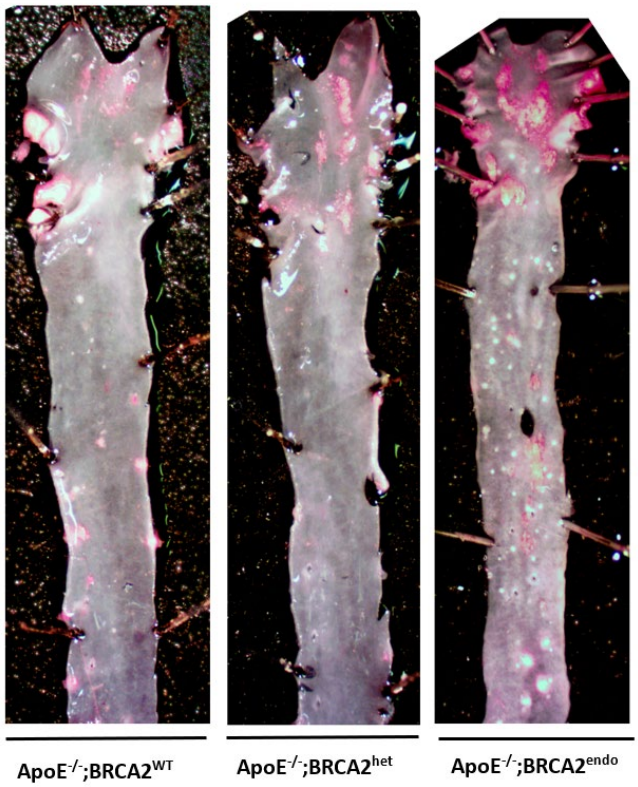



**Figure 4-16. Aortic Plaque Burden of Mice Fed High-Fat Diet.** Representative images of **(A)** Oil Red-O-stained aortas of 12-week HFD-fed male mice and **(B)** *en face* imaged aortas of male mice fed a high fat diet for 8 weeks demonstrating increased atherosclerotic plaque burden in the aortic arch of heterozygote and homozygote endothelial cell-specific BRCA2 knockout mice on an ApoE null background.

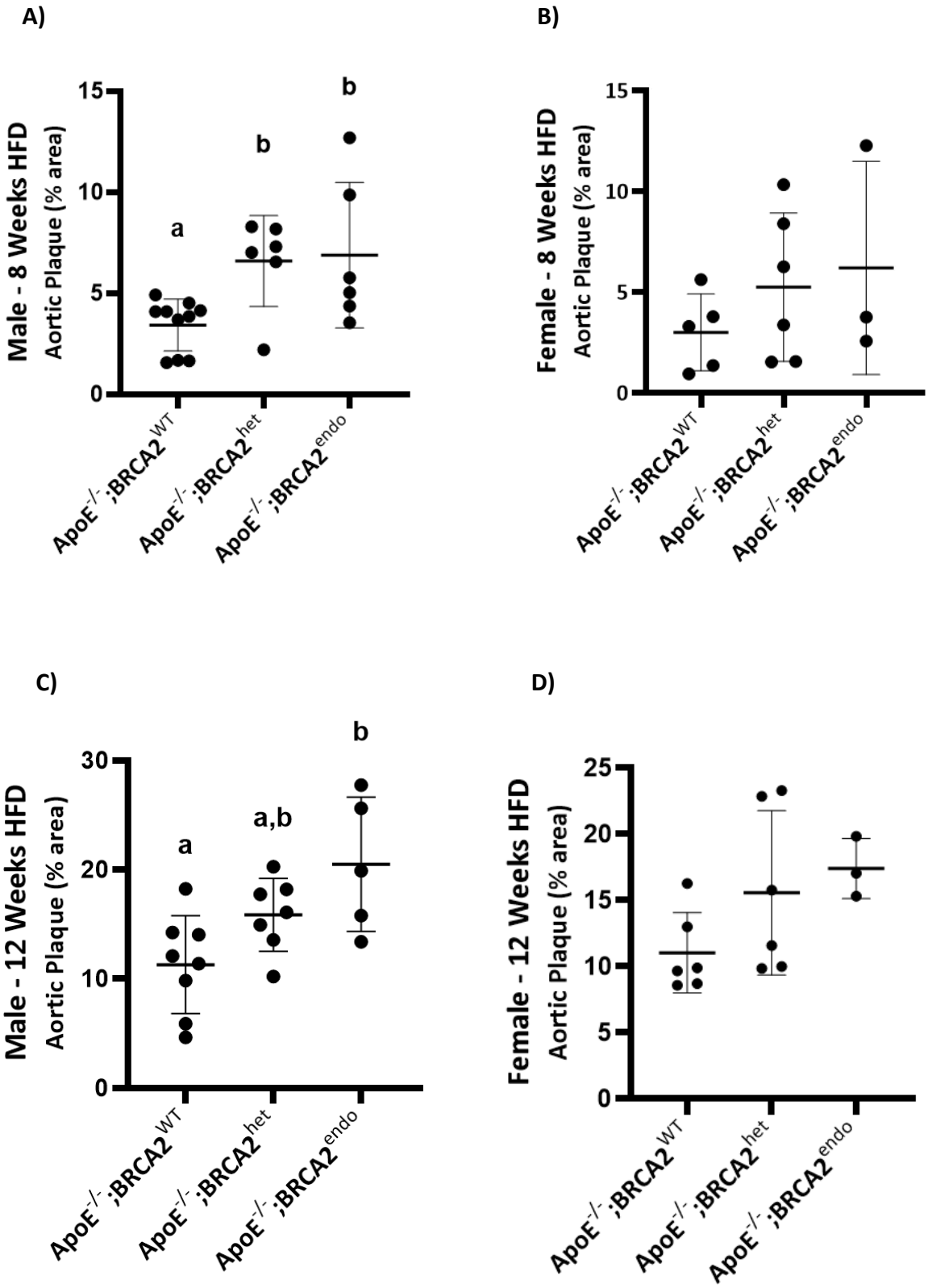

A)
Male - 8 Weeks HFD
Aortic Plaque (% area)
15
10
5
0
a
b
b
ApoE-/- ;BRCA2WT
ApoE-/- ;BRCA2het
ApoE-/- ;BRCA2endo
B)
Female - 8 Weeks HFD
Aortic Plaque (% area)
15
10
5
0
ApoE-/- ;BRCA2WT
ApoE-/- ;BRCA2het
ApoE-/- ;BRCA2endo
C)
Male - 12 Weeks HFD
Aortic Plaque (% area)
30
20
10
0
a
a,b
b
ApoE-/- ;BRCA2WT
ApoE-/- ;BRCA2het
ApoE-/- ;BRCA2endo
D)
Female - 12 Weeks HFD
Aortic Plaque (% area)
25
20
15
10
5
0
ApoE-/- ;BRCA2WT
ApoE-/- ;BRCA2het
ApoE-/- ;BRCA2endo

**Figure 4-17. Aortic Arch Plaque Burden as a Percent of Total Area.** Significant difference in aortic arch plaque burden was found in **(A)** males fed HFD for 8 weeks between ApoE$^{-/-}$;BRCA2$^{WT}$ (n=10, 3.4% ± 1.28) and ApoE$^{-/-}$;BRCA2$^{het}$ (n=6, 6.6% ± 2.25) and ApoE$^{-/-}$;BRCA2$^{endo}$ (n=6, 6.9% ± 3.60) mice (p=0.0135). No significant difference was found in the aortic arch in **(B)** females fed HFD for 8 weeks between ApoE$^{-/-}$;BRCA2$^{WT}$ (n=5, 3.0% ± 1.90), ApoE$^{-/-}$;BRCA2$^{het}$ (n=6, 5.3% ± 3.68), or ApoE$^{-/-}$;BRCA2$^{endo}$ (n=3, 6.2% ± 5.29) mice. Aortic plaque percent total area in **(C)** males fed HFD for 12 weeks was significantly different between ApoE$^{-/-}$;BRCA2$^{WT}$ (n=8, 11.29% ± 4.49) and ApoE$^{-/-}$;BRCA2$^{endo}$ (n=5, 20.5% ± 6.16) (p=0.0091) mice, but ApoE$^{-/-}$;BRCA2$^{het}$ (n=7, 15.9% ± 3.33) was not significantly different from either. **(D)** Females fed HFD for 12 weeks did not differ between ApoE$^{-/-}$;BRCA2$^{WT}$ (n=6, 11.0% ± 3.03), ApoE$^{-/-}$;BRCA2$^{het}$ (n=6, 15.6% ± 6.20), or ApoE$^{-/-}$;BRCA2$^{endo}$ (n=3, 17.4% ± 2.28). Data are analyzed by one-way ANOVA with Tukey's post hoc and are presented as mean ± SD.

# Chapter 5. Discussion

## 5.1 Discussion Introduction

The nascent field of cardio-oncology has been established in light of an appreciation for the ever-growing understanding of existing overlaps and interactions between cardiovascular diseases and cancer. Links between breast cancer and atherosclerosis are well established,(Mai et al., 2009)(Anna Öfverholm, Zakaria Einbeigi, Antonia Wigermo, 2019)(Kat et al., 2017)(Sajjad et al., 2017)(Geoffrey et al., 2017)(Miao et al., 2017)(Asselbergs et al., 2012) however, there is currently a need for a greater level of scrutiny into genetic and molecular pathophysiological factors shared by these diseases. BRCA1 and BRCA2 mutations disproportionately affect women and predispose them to a high risk of breast and ovarian cancer.(Rebbeck et al., 2015)(Thompson & Easton, 2002)(Roy et al., 2012) In these patients, cardiovascular disease is a common comorbidity and is present at rates higher than that of the general population; partly due to cancer treatment-induced CVD.(Kat et al., 2017)(Fairweather, 2014)(Anna Öfverholm, Zakaria Einbeigi, Antonia Wigermo, 2019) Yet even when controlling for common associated cancers, BRCA mutation carriers also present with an earlier mortality when compared to non-carriers(Mai et al., 2009) – but the precise reason for these excess deaths is unknown. BRCA mutations hinder DNA damage repair allowing for accumulated DNA damage leading to cancer, cellular dysfunction, or apoptosis. Endothelial injury and dysfunction are also early presentations of the inflammatory disease, atherosclerosis.(Suzuki et al., 2013)(M. Li et al., 2018) Endothelial dysfunction emerges as a result of DNA damage, oxidative stress, and apoptosis;(Ballinger et al., 2002)(Lord & Bobryshev, 2002)(Marrocco

et al., 2017)(Suzuki et al., 2013) all of which are also contributors to carcinogenesis.(Negrini et al., 2010)(Heemst et al., 2007) Progression of atherosclerosis in turn promotes increased rates of endothelial cell DNA damage(Botto et al., 2001)(Shah et al., 2018)(S. Singh et al., 2020)(Gray et al., 2015)(Botto et al., 2001)(E. P. K. Yu & Bennett, 2014)(Andreassi, 2008) and BRCA1&2 are crucial factors in cellular homeostasis(Zhao et al., 2017)(Rocca et al., 2015)(Deng, 2006) and the virtually error-free repair of double stranded breaks mechanized by homologous recombination repair.(Rocca et al., 2015)(Roy et al., 2012) Unification of these factors coalesces into a rationale for a comprehensive evaluation of endothelial BRCA1 and BRCA2 in loss-of-function models of atherosclerosis.

Previous work by our group has demonstrated a protective role played by BRCA2 when cells are exposed to oxidative stress induced by oxidized low-density lipoproteins.(S. Singh et al., 2020) This fundamental study revealed an urgent need for further assessment of the role of endothelial BRCA2 in atherosclerosis. Human germline mutations in BRCA2 are systemic and generally result in haploinsufficiency due to mutated copy inheritance leaving only one non-mutant copy which is inadequate for normal function. Impaired function of a single BRCA allele may also result in the second copy being compromised(Salmena & Narod, 2012) and the loss of heterozygosity (**LOH**) as can be seen in many tumors that exhibit LOH.(Sedic et al., 2015) Emulating a systemic knockout/knockdown of BRCA2 in mice is challenging as the gene is developmentally critical and systemic ablation of BRCA2's exon 10/11 causing embryonic lethality prior to day 9.5 of development with these mice demonstrating increased p21 expression and

hindered proliferation – demonstrating a critical role of BRCA2 in embryogenic cellular proliferation.(Hakem et al., 1996) Exon 11 is the largest segment of BRCA2 and shares 58% protein homology between mouse and humans and contains the Rad51-interacting BRC repeats that are highly conserved.(Bignell et al., 1997) Thus, a BRCA2 Exon 11 knockout is translationally relevant, but embryonically lethal. To bypass this limitation and demonstrate the effect of loss of endothelial BRCA2 on atherosclerosis, we developed two novel mouse strains utilizing the Cre-LoxP system to selectively expunge BRCA2 from only the endothelium. The first is a single knockout of endothelial BRCA2 and the second compounds this strain on an ApoE mutation background, creating a relevant *in vivo* model of atherosclerosis.

Our team has extensive experience with successful tissue specific Cre-mediated BRCA knockouts in mice and previous work has shown these models to exhibit no baseline phenotype,(K. K. Singh et al., 2009)(K. K. Singh et al., 2012)(Shukla et al., 2011) yet, under stress conditions, a protective role emerged for BRCA1 as a limiter of inflammation(Teoh et al., 2013) and oxidative stress;(Chessex et al., 2013)(Lovren et al., 2014) and a safeguard against atherogenesis(K. K. Singh et al., 2009). Moreover, both BRCA1 and BRCA2 are evidenced to defend against cardiac dysfunction.(Shukla et al., 2011)(K. K. Singh et al., 2012) Given the developmental challenges posed to an organism lacking BRCA2 in a systemically significant cell type such as the endothelium, the $BRCA2^{endo}$ mouse was evaluated against wildtype and heterozygote knockout littermates for baseline phenotyping congruent with previous *Cre-LoxP* knockout models, including gross

observations, metabolic analysis, and cardiac and vascular assessment as relevant functional metrics of cardiovascular dysfunction.

**5.2 Discussion of Results Part I**

Identification and genotyping of each animal were performed *via* ear clipping and tissue DNA extraction. Further, in a subset of these animals, endothelial BRCA2 loss was substantiated. Whole lung tissue extract was used to confirm the knockout status of animals at the transcriptional/RNA and translational/protein levels given the tissue composition of approximately 30-40% endothelial cells. Under PCR parameters limiting the amplification size to ~1000-1200 bp by reducing the amplification time to 1 min given the expected nucleotide addition rate by *Taq Polymerase* in PCR being ~20 nucleotide/sec, primers spanning BRCA2's exon10 through exon14 produced the shortened RNA product in $BRCA2^{endo}$ mice after the removal of the gene's extensive exon 11 *via* Cre-loxP-mediated deletion. While the presence of this truncated mRNA product indicated transcription, the product is likely unstable, transient, and subject to degradation. Furthermore, the removal of the BRC repeat-containing exon 11 would render recruitment of Rad51 by BRCA2 and ensuing DNA damage repair impossible should the protein be synthesized. However, we did not observe a shortened protein in our knockout mice as previously reported in cardiomyocyte specific BRCA2 knockout mice.(K. K. Singh et al., 2012) This deletion was further validated via PCR amplification and agarose electrophoresis visualization using exon 11 specific primers which revealed the expected considerable reduction of the presence of this exon in both $BRCA2^{het}$ and $BRCA2^{endo}$ mice. Lastly, exon11 F/R primers were again used to corroborate these results with qPCR amplification and fold change analysis revealing a genotype-dependent significant reduction of BRCA2 transcript in $BRCA2^{endo}$ mice.

Final confirmation of BRCA2 knockout was performed *via* immunoblotting and immunohistochemistry for the BRCA2 protein . This is critical as cells lacking the BRCA2 protein are unable to effectuate an efficient DNA damage repair response and the existence, or lack thereof, of functional endothelial BRCA2 protein is the lynchpin of the study. Whole lung tissue protein extract and immunoblot for BRCA2 and β-Tubulin demonstrated a genotype-associated reduction in the amount of BRCA2 protein. These results were further substantiated with immunohistochemistry for BRCA2 in both aortic and lung tissues wherein the endothelial layer is clearly visible and shows extensive positivity for BRCA2 in $BRCA2^{WT}$ mice while being greatly reduced in $BRCA2^{het}$ and $BRCA2^{endo}$ mice.

Studies of young single knockout mice include the analysis of metrics used to reveal the existence of any aberrant or adverse endothelial cell phenotype in the knockout as compared to its WT control littermates. The data generated herein are critical for comparison and interpretation of results of further studies utilizing this novel strain; including that of any accelerated or increased atherosclerotic burden in $ApoE^{-/-}$;$BRCA2^{het}$ and $ApoE^{-/-}$;$BRCA2^{endo}$ mice.

Evaluating the Mendelian ratio of litters born as a result of crossing these two transgenic strains is an early and necessary step that serves to identify embryonic lethality. Since systemic knockout of BRCA2 is known to carry this complication due to early developmental necessity, the expected mendelian ratio observed in males and females, as well as when sexes are aggregated, indicated a lack of developmental perturbance due

to BRCA2 loss in the endothelium. VE-Cadherin only becomes detectable at trace levels of activity at day 7.5 of embryonic development(Giannotta et al., 2013) and by this point, the mouse embryo has progressed significantly into embryogenesis and reached late gastrulation(Kojima et al., 2014) – indicating litter viability was not impacted by loss of endothelial BRCA2 excised by *Cre* expressed under the control of the VE-Cadherin promoter. Moreover, no discernable difference in weight was observed between genotypes in males or females at 8 weeks of age or at any other timepoint measured.

DNA damage is a physiological event that requires repair for immediate cellular homeostasis as well as long term organismal survival. Without BRCA2 functioning in the endothelium, we expected an accumulation of DNA damage in these cells. As discussed, the phosphorylated modification of H2AX to γH2AX is an early sensor and initiator of the homologous recombination repair pathway of double stranded breaks. Therefore, an increased presence of γH2AX indicates an excess of double stranded breaks. Within the HRR pathway, Rad51 functions in conjunction with BRCA2 to effectuate the localized repair of these breaks in the DNA via foci formation. Both molecules may be used as proxies for DNA damage and repair. Representative images of the aorta, lung, and liver were chosen to visually evaluate the effect of DNA damage in mice with endothelial cell specific deletion of BRCA2. The aorta and lung were chosen for endothelial layer identification and the liver as a DNA damage positive control. Immunohistochemical DAB staining for each of these molecules demonstrate no observable genotype-driven effect of DNA damage or response in $BRCA2^{het}$ or $BRCA2^{endo}$ mice compared to their WT control littermates – a result that may be due to a low rate of DNA damage in these mice given

their low stress hermetic environment and their young age. These findings suggest that the setting and time point are insufficient to allow for DNA damage accumulation to emerge at 26 weeks of age. This finding is in line with other data generated by our group in endothelial- or cardiomyocyte-specific BRCA1 or BRCA2 knockout mice respectively wherein DNA damage was not observed to be increased at baseline.(K. K. Singh et al., 2009)(K. K. Singh et al., 2012)

Increased rates of apoptosis are a predictable consequence of accumulated DNA damage which may overwhelm a cells ability to mitigate it through repair mechanisms such as via BRCA2's integration in the homologous repair pathway. Apoptosis was detected by TUNEL staining of histological sections and with this method we examined sections of the aorta and lung to determine the rate of apoptosis in the endothelium of WT, $BRCA2^{het}$ and $BRCA2^{endo}$ mice, and in liver sections used for each of these genotypes as a positive control. At 26 weeks of age, these mice did not exhibit any signs of endothelial apoptosis in any group which is consistent with the lack of DNA damage and repair observed via γH2AX and Rad51 staining in mice of this age group.

Left ventricle ejection fraction (**LVEF**) represents a percent of the total amount of blood squeezed from the heart and is an indication of heart health and assessing the risk of coronary artery disease,(Qian-qian Guo et al., 2020) compromised LVEF can be caused by cardiomyopathy,(Agstam et al., 2020)(Zou et al., 2014) previous heart attack causing muscle damage,(Zeb et al., 2013) mitral-valve malfunction,(Grayburn & Smith, 2014) and chronic hypertension.(Little, 2008) Left ventricle fractional shortening (**LVFS**) may be used as a potential predictor for coronary artery disease outcomes in some individuals.(Qian-

qian Guo et al., 2020) Heart function may be affected by endothelial function via altered cardiac remodelling and misfiring of paracrine signalling by endothelial cells to cardiomyocytes and impairing their contractile function(Noireaud & Andriantsitohaina, 2014)(Segers et al., 2018) and demonstrating the value of assessing cardiac function of $BRCA2^{endo}$ knockout mice. Cardiac health of the BRCA2 endothelial knockouts alongside wildtype littermates was evaluated via ultrasound measurements for LVEF and LVFS (**Section 3.12**). Male mice between 8 and 16 weeks of age showed a slight trend towards increased LVEF and LVFS in heterozygous and homozygous BRCA2 knockouts vs wildtype. In females of 16-18 weeks of age, a trend emerged in the opposite direction for both LVEF and LVFS. As neither demonstrated significant differences, it is likely that we are observing variation in otherwise healthy individual mice. However, these trends are worth further exploring by repeating this experiment in older male and female mice as well as assessing DNA damage and repair, apoptosis, ischemia, and hypoxia in the hearts of these animals.

Myography performed on aortic segments was used to assess vascular function at 8-10 weeks of age. Acetylcholine (**ACh**) was used to stimulate an endothelial-dependent vasodilation response by triggering endothelial synthesis of nitric oxide. Similarly, 2-furoyl-LIGRLO-NH2 (**2FLY**) causes vasorelaxation via activity as a proteinase-activated receptor 2 (**PAR2**) agonist resulting in an increase in intracellular calcium and the synthesis of nitric oxide. Lastly, sodium nitroprusside (**SNP**) tests the vasorelaxation response independent of endothelial function by directly breaking down into nitric oxide to stimulate the relaxation of vascular smooth muscle cells. No discernable difference was observed in the dose response curves generated by increasing amounts of the vasoactive

drugs ACh, 2FLY, or SNP. Furthermore, analysis of $\log EC_{50}$, $E_{max}$, and hill slope of the curves generated by four parameter sigmoidal dose response curve for each agonist revealed no significant difference between the aortic response of $BRCA2^{endo}$ and WT mice (**Table 4-4**). Vasorelaxation response between genotypes did not differ with respect to endothelium-dependency when challenged by these three chemicals, thus, it was determined under these parameters that vascular function was maintained in a genotype-dependent fashion at baseline.

Metabolic cages provide quantification data of respiration, activity, and food and water intake. Similar metabolic function was observed between genotypes across all metabolic parameters. $BRCA2^{endo}$ and $BRCA2^{WT}$ mice were monitored for these criteria and no significant difference was found in respiration, energy expenditure, food or water intake, activity, or time asleep, demonstrating a lack of metabolic abnormalities in young mice due to endothelial loss of BRCA2.

The aggregation of these data demonstrated that endothelial BRCA2 is dispensable for cardiovascular function at baseline and represents the essential foundation for comparison in determining the effect of loss of endothelial BRCA2 under stress conditions such as atherosclerosis.

**5.3 Discussion of Results Part II**

There are clear benefits to using mouse models of disease as a translational bridge between cell and human studies. However, there are some limitations in their use as an atherosclerotic model.(von Scheidt et al., 2017) Mice don't naturally develop atherosclerosis due to bile composition and reverse cholesterol transport differences compared to humans that results in an anti-atherogenic lipid profile that needs to be overcome.(Oppi et al., 2019) Given these limitations, rather than feeding the $BRCA2^{endo}$ mice a HFD, induction of atherosclerosis was facilitated by integrating an ApoE mutation transgene on the EC-specific BRCA2 knockout mice. ApoE mutant mice are well described for their propensity towards atherosclerotic development due to reduced hepatic and intestinal uptake of ApoB-containing lipoproteins including VLDL and LDL – and the consequently increased cholesterol-containing molecules circulating within the blood stream resulting in hypercholesterolemia.(Bruffearts et al., 2017)(Ishibashi et al., 1994)(S. H. Zhang et al., 1992) Studies using $ApoE^{-/-}$ mice are extensive and atherosclerosis under various experimental conditions has shown $ApoE^{-/-}$ mice demonstrate modifications to the inflammatory response,(Xue-Mei et al., 2017)(Han et al., 2017)(Di Bartolo et al., 2011), heavy metal exposure,(Oliveira et al., 2019) comorbidities such as diabetes(Han et al., 2017)(Di Bartolo et al., 2011) or cancer,(Tanaka et al., 2016) irradiation,(Mitchel et al., 2011)(Gabriels et al., 2014) the composition of the gut microbiota,(Zhu et al., 2018)(F. Wang et al., 2020) alterations in lipid regulation/metabolism,(Di Bartolo et al., 2011)(Mahmood Hussain & Goldberg, 2018)(Gui et al., 2016) and by changes in reverse cholesterol transport.(Y. Xu et al., 2014) Therefore, creating an atherosclerotic mouse

model in our studies involved implementing an additional ApoE transgene mutation on the BRCA2$^{endo}$ background.

ApoE$^{-/-}$ mice are known to exhibit monocyte-endothelial attachment after 6-8 weeks and develop atherosclerotic lesions similar to human atherosclerosis within 8-10 weeks on a standard chow diet.(Meyrelles et al., 2011) However, as the effects of DNA damage are magnified under increased cellular stress, we selected a controlled mouse diet which performs as an analog for the human Western-type diet (**Section 3.1**). This diet is established as used in conjunction with ApoE$^{-/-}$ strains and is reported accelerate atherogenesis such that these mice exhibit an early increase in cholesterol,(S. H. Zhang et al., 1992)(Nakashima et al., 1994) lesions and foam cells at 6-10 weeks,(Nakashima et al., 1994)(Nakashima et al., 1998) and fibrous plaque and subsequent cap formation at 15 and 20 weeks respectively.(Nakashima et al., 1994) This diet was chosen not only as a Western diet mimic, but also as it contains relatively low cholesterol (0.2%) as compared against many atherogenic diets (1.25%).(Hedrick et al., 2000)(Oppi et al., 2019) This ideally mitigates any extreme lesion formation and limits plaque saturation allowing for the identification of more subtle differences in plaque formation and lesion development.

Initial crossbreeding of BRCA2$^{endo}$ mice with ApoE$^{-/-}$ mice produced ApoE$^{-/-}$;BRCA2$^{het}$ mice, with 50% of individuals expressing VE-Cre. Further crossing these mice bred out any non-mutant ApoE genes and established the atherosclerotic (ApoE$^{-/-}$), BRCA2 endothelial knockout mouse line.

As with the single endothelial cell-specific BRCA2 knockout mice, ApoE$^{-/-}$;BRCA2 mice were born in expected Mendelian ratio. The increased background/transgenic or functional stress as a result of ApoE mutation introduction does not appear negatively impact embryonic survival. However, of note is the relatively low female to male ratio of pups born of 0.42 vs the expected equal birth rates of male and female mice. Supporting this skewed ratio is research showing higher rates of males born to mothers fed high-fat diet compared to standard chow or low-fat diet-fed mice.(Rosenfeld et al., 2003) Lipid dysregulation and early hypercholesterolemia in ApoE$^{-/-}$ mice may induce a compounding physiological effect and explain the altered ratio in these mice, but it does not preclude the possibility of an inbreeding/background effect or early developmental issues.

Weight at 10 weeks of age was measured at the initiation of HFD. At this point, weight did not differ between the BRCA2 genotypes of ApoE$^{-/-}$ mice for either males or females and were of overall healthy in appearance and of average weight. The weight of these mice was also measured at every 2-week interval post HFD initiation. At the relevant timepoints of 8 and 12 weeks for aortic/tissue extraction and plaque quantification, weight did not appear significantly different between genotypes or in either sex. Somewhat more interesting is the weight gain of these mice as illustrated by net weight at each measured interval and as cumulative percent body weight change. Net weight of ApoE$^{-/-}$;BRCA2$^{endo}$ mice is consistently higher in females and males with the exception of males at 12 weeks. A decline in net weight and weight gain of ApoE$^{-/-}$;BRCA2$^{endo}$ males appears to begin after 10 weeks of HFD. These data are consistent with previous studies on mouse weight gain under standard chow and high-fat diet wherein mouse weight tends to plateau and

percent weight gain drops over time.(N. S. Jones et al., 2019)(Timon et al., 1970)(Timon & Eisen, 1969)

Upon examination, ApoE$^{-/-}$ mice on HFD exhibited notable splenomegaly. The decision was made to weigh the organ given the obvious phenotype. Spleen enlargement and weight gain is a an established genotype driven effect in ApoE$^{-/-}$ mice.(Y. Wang et al., 2012) Enlarged spleens are primarily asymptomatic and may result from various underlying conditions. Metabolic disorders such as Gaucher or Neimann-Pick disease which hinders lipid metabolism breakdown may cause spleen and liver fat retention causing enlargement.(Razek et al., 2019)(Parra et al., 2011) The spleen is partly responsible for regulating the immune response to systemic inflammation as seen in atherosclerosis. Inflammation exacerbated by an unhealthy diet and in turn associated with spleen enlargement.(Barrea et al., 2018) Under the stress of obesity, there exists a connected axis of endothelial dysfunction and inflammation of the heart and spleen(Tourki et al., 2020) and endothelial dysfunction has also been shown to play a direct role in promoting splenomegaly.(Jiang et al., 2015) While a more rare cause of enlargement, splenic infarction occurs when the spleen's blood flow is interrupted and may be induced by atherosclerotic clots and is another mechanism by which spleen enlargement manifests.(Frippiat et al., 1996)(Gascon et al., 1999)

Interestingly, upon weight analysis, there was a significantly higher spleen weight in 8-week HFD-fed male ApoE$^{-/-}$;BRCA2$^{endo}$ mice and an otherwise observable trend towards the lowest spleen weights being in ApoE$^{-/-}$;BRCA2$^{WT}$ for each sex and at both 8 and 12 weeks HFD. In all cases except for 8-week HFD-fed female where ApoE$^{-/-}$;BRCA2$^{het}$ spleen

weight was the highest – increased spleen weight trended in BRCA2 heterozygote then homozygote endothelial knockout, ApoE$^{-/-}$ mice.

While generally not considered a direct corollary to atherosclerotic magnitude, the genotype dependency of increasingly larger spleens in the knockout mice provide promising avenues for further work. Given the discussed links between spleen weight and such factors as diet, lipid metabolic regulation, endothelial dysfunction, inflammation, and atherosclerosis, as well as the existence of a known cardiac-spleen-liver axis, there is compelling reason to further investigate the precise reasons for the observed splenic phenotypes. A possible culprit of the observed splenomegaly is lipid retention, which is of primary interest due to evidence for BRCA2's role in lipid regulation,(Genetic et al., 2015)(Oliverio et al., 2020) yet further quantitative histological and molecular analysis of these tissues is required and underway to reveal any underlying mechanisms.

The primary goal of this study is to evaluate the extent of atherosclerosis of ApoE$^{-/-}$;Brca2$^{endo}$ mice after high-fat diet. Atherosclerosis occurs primarily in branched regions and areas of that vasculature that experience turbulence or low shear stress.(Foteinos et al., 2008)(Tricot et al., 2000) The aortic arch encompasses each of these factors and is routinely used as a standard for atherosclerotic measurements and the selected ApoE$^{-/-}$ strain consistently develops plaque in this location due to its susceptibility. Endothelial injury and plaque accumulation occurs preferentially here, and the arch and thoracic aorta may be used to evaluate atherosclerotic burden.

Evaluation of the aortic arch plaque as a percentage of total area revealed that *at 8 weeks of HFD, male mice exhibited a significantly increasing area of plaque in ApoE$^{-/-}$;Brca2$^{het}$ and ApoE$^{-/-}$;Brca2$^{endo}$, and after 12 weeks HFD, male mice exhibited significantly higher aortic arch plaque burden in ApoE$^{-/-}$;Brca2$^{endo}$ mice vs ApoE$^{-/-}$;*BRCA2$^{WT}$. These results are distinctly visible in representative whole stained, and *en face* minutien pin mounted aortas. Moreover, in females after 8 and 12 weeks HFD, a compelling trend in the same direction is emergent. It is possible that the significant difference is only visible in males at 8 and 12 weeks due to an early onset of plaque formation in male mice given their relatively higher vulnerability to CVD (ischemic heart disease in particular) compared to their female counterparts.(Kaplan et al., 1996) Men have a statistically earlier onset across all manifestations of CVD and tend towards coronary artery disease while women are at a higher risk of cerebrovascular disease and heart failure.(Leening et al., 2014) These differences are thought to be due to differential expression of endogenous hormones between the sexes(Rexrode, 2017) and while menopause appears to exacerbate atherogenesis as communicated,(Kat et al., 2017)(Fairweather, 2014) the result a later onset of severe cardiovascular disease. Moreover, in the stained aortas of BRCA2$^{WT/het/endo}$;ApoE$^{-/-}$ mice, the great majority of branches along the descending thoracic aorta to the iliac branch were heavily burdened by plaque past the 12-week mark with notably high burden at 8 weeks – suggesting peripheral artery disease.

This evidence presents a strong correlation between greater atherosclerotic burden and loss of endothelial BRCA2. Should the results of this study be proven to translate to human studies, there may be a paradigm shift specifically in how both males with BRCA2

mutation are screened and evaluated, but also in females receiving BRCA-related cancer treatments which, as previously iterated, contain their own cardiovascular risks including cardiotoxicity(Schmidt et al., 2016)(Aleman et al., 2007)(Belt-dusebout et al., 2021) and metabolic syndrome.(Dørum et al., 2008) These patients and those with CVD risk factors may also stand to benefit from BRCA2 targeted gene therapy, providing the impetus for development of novel treatments and opening the door for future studies into the roles and effects of BRCA2.

# Chapter 6. Conclusions and Future Directions

## 6.1 Conclusions

Extensive phenotyping of the single knockout mouse revealed endothelial BRCA2 as being dispensable at baseline (8-16 weeks of age). This is congruent with how impaired DNA damage repair results in a progressive accumulation of DNA damage over time. At a young age, these effects may not produce any clinically relevant differences. However, with time or stress, accumulation may overwhelm the capacity to mitigate the deleterious effects of chronic unrepaired DNA and result in the emergence or exacerbation of pathologies such as cancer or cardiovascular disease. In this study, the single BRCA2 knockouts at a young age were under no acute stress and were not phenotypically different than their control littermates.

Creating a double knockout with endothelial BRCA2 and ApoE functional loss and inducing atherosclerosis on a high-fat diet provided the parameters for the evaluation of these mice under atherogenic-stress conditions. After 8 weeks of HFD, male heterozygote and homozygote endothelial BRCA2 knockout mice showed significantly greater atherosclerotic burden in their aortic arches as compared to $ApoE^{-/-}$;$BRCA2^{WT}$ controls and significantly more plaque was also observed in 12-week HFD-fed $ApoE^{-/-}$;$BRCA2^{endo}$ male mice. Females after 8 and 12 weeks HFD also demonstrated a compelling trend towards an increased atherosclerotic magnitude in heterozygote and homozygote knockout mice. The addition of more mice to these groups as well as additional intervals of HFD are ongoing and may serve as statistical reinforcement to delineate genotype-dependent atherogenesis.

With the knowledge gained in this study, we have identified BRCA2 as an integral protector against atherosclerosis and a novel therapeutic target for overexpression in ECs by adenoviral or lentiviral vectors carrying the BRCA2 gene as controlled by EC-specific promoters or by stimulating BRCA2 endothelial expression in atherosclerotic patients. Further, we believe this work provides justification for the consideration of expanded screening for the early onset of cardiovascular-related complications in BRCA2 mutation carriers.

### 6.2 Study Limitations

Our primary limitation is the number of mice at each HFD interval for certain genotypes. Where a strong trend exists, further mice should be added to determine the veracity of these trends.

Our model only lacks BRCA2 in the endothelium. While excellent for understanding the role of endothelial BRCA2, atherosclerosis is a disease of the complete vasculature and involves extensive participation and remodelling by vascular smooth muscle cells, amongst other complex cellular interactions. As this is not a systemic knockout/knockdown, the effect of BRCA2 loss in other participating cell types is not being addressed and cannot be evaluated within the scope of our model. Moreover, as frequently whole lung tissue was used in experiments, isolating lung endothelial cells for validation of these results would be prudent. Moreover, as isolation of aortic endothelial cells may have resulted in VSMC contamination given the followed protocol, flow sorting will be used for definitive isolation.

Molecular work is currently lacking in the atherosclerotic model and experiments for immunohistochemistry, qPCR, and immunoblotting for established for markers of DNA damage and repair, apoptosis, and proliferation are underway.

Cardiovascular functional data may also be generated in atherosclerotic mice to evaluate the relative effect on the double knockout on HFD vs baseline.

Our earliest timeframe is at 8 weeks HFD and the data indicates that these mice already exhibit a potentially earlier initiation of atherosclerosis. The addition of a 4-week HFD

cohort would allow for the identification of the onset of atherosclerotic plaque and possibly delineate differences in plaque emergence between genotypes.

Finally, the limitations of COVID including delayed collaborative efforts as well as deliveries of lab supplies and difficulties in scheduling and obtaining access to necessary equipment/machines for experiments. Thus, said delays resulted in a wider age range than desired in our baseline mouse studies ($BRCA2^{endo}$ phenotyping).

### 6.3 Future directions

We have demonstrated that atherosclerosis is exacerbated in mice oh a high-fat, Western-type diet. With this knowledge, it is imperative that follow-up studies on human samples of BRCA2 mutation carriers be performed to identify any translation into human patients.

As cardiovascular diseases are age-associated, further studies on aged mice of the single knockout genotype are ongoing and may include the non-endpoint experiments performed at baseline, as well as the creation of a survival curve may reveal an impaired long-term viability in endothelial BRCA2-deficient mice.

Given the single knockout mouse's baseline phenotype has already established the model as embryonically viable and without aberration, these mice may be used for further studies to assess the effect of endothelial cell specific BRCA2 loss under a variety of stress conditions.

In light of the results of this study, we are obliged to expand the use of this mouse model to other diseases of the endothelium and DNA damage. These include studies on doxorubicin-induced cardio- and neurotoxicity, radiation-induced cardiotoxicity, sepsis, STZ-induced diabetes, and Angiotensin II-induced hypertension.

Such future studies may similarly reveal an indispensable role played by endothelial BRCA2, thus providing the scaffolding and justification for further investigation into BRCA2 as a performer in other tissue types and pathologies; potentially influencing the

direction of screening and treatment of BRCA2 mutation and other DNA damage and repair molecules.

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## Appendix: Animal Use Protocol

| | |
|---|---|
| **PI :** | Singh, Krishna |
| **Protocol #** | 2018-163 |
| **Status :** | Approved (w/o Stipulation) |
| **Approved :** | 02/01/2019 |
| **Expires :** | 02/01/2023 |
| **Title :** | Role of Endothelial Breast Cancer Gene 2 in Cardiovascular Pathobiology |

### Table of Contents



Protocol Introduction

The questions on this page activate specific sections within the AUP form.

Note that species selection is part of this introductory page

**Does this AUP involve teaching?**

Yes ○ No ◉

**Is the animal work on this project shared by another Animal Care Committee?**

Yes ○ No ◉

**Will you be using hazards?**

Yes ◉ No ○

**Will live animals be moved outside of their housing facility?**

Yes ○ No ◉

**Will field studies be conducted?**

Yes ○ No ◉

**Add/Update/Remove Species Used on this Protocol**

| Species | Agents | Drugs | Restraint | Breeding |
|---|---|---|---|---|
| Mouse | No | Yes | No | No |

# Animal Use Protocol Overview

**Animal Use Protocol Title**

Role of Endothelial Breast Cancer Gene 2 in Cardiovascular Pathobiology

**Application Type. If this is a post-pilot project, please attach the Pilot Report to this section, below.**

New

**Provide Associated Previous Protocol Number**

**Please provide a report detailing the previous AUP's use of Animals**

# Lay Summary

In terms that a lay reader can understand—using language appropriate to an intelligent but non-specialist audience—and using a minimum of technical jargon, complete the following three questions:

What is the purpose of your study?

Explain the significance of this study.

Describe what specifically will be done to live animals in this study. Summarize each study procedure in sequential order.

**Lay Summary**

Endothelial cells, which line the innermost layer of blood vessels, play important roles in maintaining blood vessel function. Understanding the role of endothelial cells in abnormal blood vessel function in diseased condition may find novel therapeutic target to treat atherosclerosis (fatty deposit or clogging of arteries) and other associated cardiovascular diseases (example: heart failure).

DNA is the genetic material present in every cell type. BRCA2 (breast cancer gene 2) is a protein molecule, which maintains the DNA integrity. Mutation in BRCA2 gene causes breast and ovarian cancer, and cell death. Our aim is to understand if loss of endothelial BRCA2 results in increased endothelial cell death and if it also promotes atherosclerosis-associated cardiovascular diseases.

In this proposal, we will use an animal model of atherosclerosis. This animal model will be created by using Cre-LoxP method, where a mouse carrying VE-Cadherin-Cre will be crossed with another mouse with floxed BRCA2 gene to give rise an animal model that will lack BRCA2 in their endothelial cells. We want to investigate if there will be increased atherosclerosis and endothelial cell death after feeding these animals with high fat diet (high fat diet induces

atherosclerosis).

Our study will delineate a new role of BRCA2 in atherosclerosis, which may help identifying a potentially new therapeutic target to treat cardiovascular diseases.

**GLOSSARY OF TERMS - Identify each individual scientific term and abbreviation using *CAPITAL LETTERS*, and then briefly define each term to be referenced in any section of this protocol.**
**e.g. ALLELE - The genetic variant of a gene responsible for the different traits of certain characteristics and genetic diseases.**

Atherosclerosis: It is the narrowing of arteries from a buildup of plaque (usually made up of fat substances) inside the arteries.

Cre-LoxP method: This method is one of the most powerful method developed for mouse genetics. This method gives mouse researchers sophisticated control over the location and timing of the particular gene expression. In this proposal, the Cre/loxP method will be used to delete BRCA2 gene in the endothelial cells of mice.

DNA: Deoxyribonucleic Acid

BRCA2: Breast Cancer Gene 2

# IMPORTANT NOTE:

***Before*** an AUP can be reviewed by the ACC, it **must receive a positive scientific merit review** in accordance with Scientific Merit Review Policy (https://www.uwo.ca/research/_docs/animal_ethics/POL-013-Scientific-Merit-Review-Policy.pdf). If scientific merit review *was not* part of the funding process, please follow the Scientific Merit Review Procedures (https://www.uwo.ca/research/_docs/animal_ethics/PROC-013-Procedures-for-Undertaking-Scientific-Merit-Review.pdf) by submitting to your department Chair and/or ADR (as applicable) the SMR Application Form (https://www.uwo.ca/research/_docs/animal_ethics/PROC-013-APP1-Scientifici-Merit-Review-form.docx).

**Has the work outlined in this AUP received favourable scientific peer review?**

Yes (•) No ( )

**Do you wish to provide a funding peer review assessment, which may be considered in lieu of internal scientific peer review? If 'YES', please attach the funding assessment.**

Yes ( ) No (•)

**If this is a RESEARCH AUP, please provide a list of one to three publications relevant to the work outlined in this AUP.**

**This AUP will not be reviewed by the ACC prior to a positive scientific merit review.**

**Using only key words, specify the animal models and procedures described within this AUP.**

Research, genetically modified animals, tissue & organ collection, anesthesia

## Funding Source List

| Fund Source | Grant Title | Funded? | Grant Number | Start Date | Grant Holder |
|---|---|---|---|---|---|
| Canadian Institutes of Health Research | The Emerging Field of Cardiovascular-Oncology | Yes | 0000045276 | 07/01/2018 | |

**Funding Source Name**

Canadian Institutes of Health Research

**Proposal Title**

The Emerging Field of Cardiovascular-Oncology

**Is this award currently funded?**

☑

**Please provide the associated GRANT #**

0000045276

**Funding START date *mm/dd/yy***

07/01/2018

**PI on Grant (if different than PI on Protocol)**

# Purpose of Animal Use

**Identify PRIMARY purpose of animal use**

1-Fundamental Research

# Hazardous Materials

**Microorganism, Biological Agent or Hazardous Species Used?**

Yes ○ No ◉

**Institute Biosafety Committee #**

**Recombinant DNA or Viral Vector Directly into Animals Used?**

Yes ○ No ◉

**Experimental Agents or Veterinary Drug Used?**

Yes ◉ No ○

**Nuclear Substance, Radiation, or Imaging Device Used?**

Yes ○ No ◉

**Radiation Permit #**

# Animal Groups and Experimental Timelines Overview

### 'C', 'D' and 'E'-level AUPs

Using simple diagrams the following must be attached:

- Animal Groups - names the animal groups (with unique identifiers) as well as the number of animals requested

and

- Experimental Timelines - names, in chronological order, ALL procedures that animals undergo (note that the description of each of these procedures is detailed in the *Procedure Narrative* section of the AUP

### 'B'-level AUPs

- Attach Animals Groups and Experimental Timeline diagrams as described above

or

- Attach a document that describes the same information in paragraph format.

**In the textbox below, please list file names of the most recent attachments**

Year 1:
Group1: EC-BRCA2-/- (n= 20 males and 20 females)
Group 2: WT littermate control (n= 20 males and 20 females)
Baseline characterization (n = 5/ group for RNA & protein, n= 5/ group for histology (aorta), n= 10/ group for functional assays)

Year 2&3:
Group 3: EC-BRCA2-/-;ApoE-/- (n= 60 males and 60 females)
Group 4: WT littermate control (n= 60 males and 60 females)

Group 3 and 4 wil be fed high fat diet. Aortas and their branches will be harvested at 4, 8, 12 and 16 weeks post-high fat diet. Collected samples will be used as follows: immunohistochemistry (n= 5/ group), RNA extraction (n= 5/ group), and protein extraction (n= 5/ group). We will require 60 animals per group, which includes 15 animals for each of the 4 time points, to achieve statistical significance in the above experiments.

Please attach your Groups and Timelines documents above.

# Mouse

# Tissue Collection

**Will live animals be used in this study?**

Yes

**Will this species be used exclusively for tissue collection?**

Yes (•) No ( )

# Justification for Choice of Species

**Justify the choice of species by stating why**
**a) this is the most appropriate species, and**
**b) a species lower on the phylogenic scale is not appropriate.**

Mouse represent the ideal model to generate endothelial cell-specific knockout. Apolipoprotein E-deficient mice (Apoe-/-KO) that spontaneously develop atherosclerotic lesions on a standard high fat diet are widely used as an animal model for experimental atherosclerosis research. These models are not possible with any other species.

# the 3Rs: Replace, Reduce, Refine

The Three Rs concept originated from the scientific community and is a widely accepted cornerstone of policies on animal-based science around the world.

Ethical animal use requires consideration of animal welfare needs http://3rs.ccac.ca (http://3rs.ccac.ca)

Prior to any animal-based science, the 3 Rs should be considered.

Replacement refers to methods which avoid or replace the use of animals in an area where animals would otherwise have been used

Please show how you've considered the tenet of Replacement in your AUP.

For more information, please see Western's Alternative Use Guide (https://guides.lib.uwo.ca/animalalternatives).

**Replacement Consideration**

Non-animal alternatives are not available as it is not feasible/possible to use computational methods as an alternative method for the proposed research.

We will use the cell culture technique to understand the role of BRCA2 at cellular level in endothelial cells. However, to understand the role of BRCA2 at tissue level, specially in he endothelial cells, mice represent the best and only feasible/possible knockout model for the proposed study.

Reduction refers to any strategy that will result in fewer animals being used.

Please show how you've considered the tenet of Reduction in your AUP.

For more information, please see Western's Alternative Use Guide (https://guides.lib.uwo.ca/animalalternatives).

**Reduction Consideration**

Breeding strategy will maximize the tissue-specific knockout and control wild-type littermates generation, as required for the experiments.

Samples will be collected and analyzed from the lowest possible number of animals to reach the statistical significance.

Refinement refers to the modification of husbandry or experimental procedures to minimize pain and distress in your animals.

Please show how you've considered the tenet of Refinement in your AUP.

For more information, please see Western's Alternative Use Guide (https://guides.lib.uwo.ca/animalalternatives).

**Refinement Consideration**

-Animals will be anesthetized before the end procedure.

-This protocol is associated with very small amount of discomfort (if any) and does not involve any surgery.

-Post-high fat diet, the animals will be monitored frequently to look for any signs of discomfort.

# Species Strains

| Species Strain | Age/Weight | Vendor Stock# |
|---|---|---|
| B6.Cg-Tg(Cdh5-cre)7Mlia Brca2^tm1Brn/Nci | 8-10 weeks | In-house breeding 2018-159 |
| B6.Cg-Tg(Cdh5-cre)7Mlia Brca2^tm1Brn/Nci Apoe^tm1Unc | 8- 10 weeks | In-house breeding 2018-159 |

**Strain Name**

B6.Cg-Tg(Cdh5-cre)7Mlia Brca2^tm1Brn/Nci

**Is this strain acquired commercially?**

Yes ○ No ◉

**Are the animals coming from a non-commercial source or another AUP?**

Other AUP

**Provide 'supplier name' and stock #, if available**

In-house breeding 2018-159

**Age or weight at procurement**

8-10 weeks

**Provide phenotype detail for non-genetically altered strains**

No basal phenotype expected.

**Is this strain genetically altered?**

Yes ◉ No ○

**If Genetically Altered Animals are COMMERCIALLY AVAILABLE, insert VENDOR STRAIN INFO URL.**

**If Genetically Altered Animals are from a NON-COMMERCIAL SOURCE, PROVIDE the Original Source of Animal(s)**

**Describe the NATURE of the genetic modification in heterozygous and homozygous animals. Identify the SYSTEMS AFFECTED and SPECIAL CARE required.**

These mice will do not possess gene BRCA2 only in the endothelial cells.

**Strain Name**

B6.Cg-Tg(Cdh5-cre)7Mlia Brca2^tm1Brn/Nci Apoe^tm1Unc

**Is this strain acquired commercially?**

Yes ○ No ◉

**Are the animals coming from a non-commercial source or another AUP?**

Other AUP

**Provide 'supplier name' and stock #, if available**

In-house breeding 2018-159

**Age or weight at procurement**

8- 10 weeks

**Provide phenotype detail for non-genetically altered strains**

NO basal phenotype expected.

**Is this strain genetically altered?**

Yes (•) No ( )

**If Genetically Altered Animals are COMMERCIALLY AVAILABLE, insert VENDOR STRAIN INFO URL.**

**If Genetically Altered Animals are from a NON-COMMERCIAL SOURCE, PROVIDE the Original Source of Animal(s)**

**Describe the NATURE of the genetic modification in heterozygous and homozygous animals. Identify the SYSTEMS AFFECTED and SPECIAL CARE required.**

These mice will not have gene ApoE systemically.

# Animal Transfers

**Will animals originate from a DIFFERENT CITYWIDE PROTOCOL NUMBER?**

Yes (•) No ( )

**Are any animals being transferred from another AUP that have previous use?**

Yes ( ) No (•)

**List AUP number and PI name from which animals will be transferred**

2018-159 Singh

**Describe the previous use of animals sourced from different citywide AUPs.**

No previous use.

# Environmental Enrichment

**Will all animals be group housed?**

Yes (•) No ( )

**Justify why group housing is not planned and specify which experimental animals will be singely housed**

**May Animal Care staff provide ENVIRONMENTAL ENRICHMENT to all animals of this species, as per its facility-specific Environmental Enrichment SOPs?**

Yes (•) No ( )

**May FOOD TREATS be given to all animals of this species by animal care staff as per its facility-specific Environmental Enrichment SOPs?**

Yes ( ) No (•)

**Explain why additional enrichment and/or food treats may not be provided by Animal Care staff**

The double knockouts and the respective littemates will be fed high-diet (Cat No; D12079B, ResearchDiets). To avoid the variability in the diet, these mice will not be given food treats.

**Will any animals of this species undergo fasting at any point in the project?**

Yes ( ) No (•)

**Provide justification and duration of fasting.**

**If this species has other specialized caging, dietary or environmental requirements that you wish the animal facility manager(s) to be aware of, please identify them here.**

The double knockouts and the respective littemates will be fed high-diet (Cat No; D12079B, ResearchDiets). To avoid the variability in the diet, these mice will not be given food treats.

# Animal Holding/Housing and Use Location Information

| Location/Building | Room | Type |
|---|---|---|
| *Health Sciences Animal Care Facility | 6026 | BOTH |
| *West Valley Barrier | 118 | USE |
| *West Valley Barrier | *Housing Room | HOUSING |

Procedures within Animal Holding Rooms is governed by *POL-11 Procedures within Animal Holding Rooms,* found at https://www.uwo.ca/research/ethics/animal/animal_care_and_use_policies.html. (https://www.uwo.ca/research/ethics/animal/animal_care_and_use_policies.html)

**ANIMAL LOCATION**

*Health Sciences Animal Care Facility 6026

**Location Type**

BOTH

**Identify the Procedure Location PURPOSE**

Anesthesia;Euthanasia;Holding Beyond 12 Hours

Procedures within Animal Holding Rooms is governed by *POL-11 Procedures within Animal Holding Rooms,* found at https://www.uwo.ca/research/ethics/animal/animal_care_and_use_policies.html. (https://www.uwo.ca/research/ethics/animal/animal_care_and_use_policies.html)

**ANIMAL LOCATION**

*West Valley Barrier 118

**Location Type**

USE

**Identify the Procedure Location PURPOSE**

Anesthesia;Blood Collection;Euthanasia

Procedures within Animal Holding Rooms is governed by *POL-11 Procedures within Animal Holding Rooms,* found at https://www.uwo.ca/research/ethics/animal/animal_care_and_use_policies.html. (https://www.uwo.ca/research/ethics/animal/animal_care_and_use_policies.html)

**ANIMAL LOCATION**

*West Valley Barrier *Housing Room

**Location Type**

HOUSING

**Identify the Procedure Location PURPOSE**

# Animal Holding within Extra Vivarial Spaces (EVSs)

Holding in extra-vivarial spaces is governed by *POL-16 Animal Holding and Use Within Extra-Vivarial Spaces and PROC-16 Procedure for Requesting Animal Holding or Use Within Extra-Vivarial Spaces,* found at https://www.uwo.ca/research/ethics/animal/animal_care_and_use_policies.html. (https://www.uwo.ca/research/ethics/animal/animal_care_and_use_policies.html)

**Will animals be held outside a laboratory animal facility for more than 12 hours and/or overnight?**

Yes ○ No ◉

**Per EVS location, list the room #, the specific procedures to be performed per room, and justify why animals must be held outside an Animal Facility for more than 12 hours and/or overnight.**

**Per area, provide the maximum duration and timeframe that live animals will be held, and estimate the number of cohorts anticipated per year.**

# Acclimatization Period & Quarantine

**Will this species be held for the species-appropriate holding period prior to any form of USE, as per SOP 310?**

Yes ○ No ◉

**Provide Justification for this exemption**

Not Applied.

**Will this species require quarantine?**

Yes ○ No ◉

**If quarantine requirements differ from the animal holding/housing facility's standard practice, please outline the requested QUARANTINE DETAIL.**

# Veterinary Drugs

Add all veterinary drugs to be used for therapeutic purposes in this AUP - planned veterinary treatments, e.g. anaesthesia, analgesia, post-op care, and euthanasia.

Note: Agents, materials, drugs and devices that are included in the experimental design of this AUP for this species should be added to the next **Experimental Agents** web page.

| Drug | Dosage | Route of Administration | Frequency | Justification of Divergence | Pharma Grade |
|---|---|---|---|---|---|
| Isoflurane | 5% induction 2% maintenance | Inhalation | once per session | | |

**Drug Generic Name**

Isoflurane

**Drug Type**

Anesthetic, Sedative

**Drug Dosage**

5% induction 2% maintenance

**Frequency of Administration**

once per session

**Route of Administration**

Inhalation

**Please justify any divergence from the standard dosage**

**Is this a Pharmaceutical Grade Drug?**

Yes ○ No ○

**Please justify the use of this drug and indicate how it is sterilized or determined to be pathogen-free.**

# SOP List

Add all Standard Operating Procedures that will be followed within this AUP.

Go to the ACVS SOPs web page for SOP details - http://uwo.ca/animal-research/sops/index.html (http://uwo.ca/animal-research/sops/index.html)

| SOP Name | Divergences |
|---|---|
| 354 - Rodent Anesthesia/isoflurane In A Static Apparatus | bell jar isoflurane exposure |
| Cln-320 - Methods Of Euthanasia | |
| Cln-321 - Criteria For Early Euthanasia In Rodents | |
| Cln-361 - Blood Collection In Mice | |

**Select an SOP**

354 - Rodent Anesthesia/isoflurane In A Static Apparatus

**Are you following the SOP exactly?**

Yes ○ No ◉

**If you are not following the SOP exactly, please list and justify all divergences from the SOP**

bell jar isoflurane exposure

**Select an SOP**

Cln-320 - Methods Of Euthanasia

**Are you following the SOP exactly?**

Yes ◉ No ○

**If you are not following the SOP exactly, please list and justify all divergences from the SOP**

---

**Select an SOP**

Cln-321 - Criteria For Early Euthanasia In Rodents

**Are you following the SOP exactly?**

Yes ◉ No ○

**If you are not following the SOP exactly, please list and justify all divergences from the SOP**

---

**Select an SOP**

Cln-361 - Blood Collection In Mice

**Are you following the SOP exactly?**

Yes ◉ No ○

**If you are not following the SOP exactly, please list and justify all divergences from the SOP**

# Procedures Checklist for Reporting and Training

Use the checklist below to identify all AUP elements to be used **with this species**. If none of the listed AUP elements pertain to this species, select *Not Applicable.

Entries selected here will be linked to other AUP pages, including Personnel Training Requirements and the eSirius Training Module where animal user training records are maintained. Therefore, please ensure that this list is complete.

| Procedure Name |
| --- |
| 01. Blood Collection - Intracardiac |

12. Cervical Dislocation - Under Anesthesia

16. Anesthesia - Gas

# Procedures Narrative

In view of the live animal activities identified within this AUP and listed below, provide a concise description of the procedural events identified within the **Groups and Timelines** page associated with this specific species.

The intent is to name and briefly describe the procedural events and associate them with each experimental group within this species.

Specific detail pertaining to drug dosage, monitoring, euthanasia/endpoint method, breeding, and physical restraint methods have been captured within other AUP sections, so they do not need to be described in detail here.

| Species | Description |
|---|---|

Use the following formatting method to complete **each procedure listed** within this section:

1. **Bold Font for Procedure Name - e.g. Anesthesia**
2. *Italicized Font for Group Identifiers - e.g. Groups 1, 2, and 6*
3. Regular Font for Procedure Description - e.g. Animals will be placed in a clean cage for transport to the OR

Please note that the AUP will be returned for updates if this section does not align with the above formatting method.

**Procedures Narrative**

**Intracardiac Blood Collection:** mice will be anaesthetized with isuflurane (2-5 %), then the blood will be collected by cardiac puncture followed by cervical dislocation. 10 animals from each group (1-4) will undergo this process.

**Anesthesia & Cervical Dislocation:** mice will be anaesthetized with isuflurane (2-5 %) before cervical dislocation. All the animals from each group will undergo this process.

**High Fat Diet:** Mice will be fed high fat diet for 4, 8, 12 and 16 weeks.

# Procedural Consequences & Monitoring

**From both the project overview & detail perspectives, identify and describe specific procedural or other/combined elements of this AUP that may produce pain, distress, or impairment - and identify all possible consequences - Behavioural, Physical, Biochemical, Physiological, and Reproductive - for this species.**

Following high fat diet for 4 to 16 weeks abnormality is not expected. However, if at any time mice exhibit dehydration, lethargy or aggressive behaviour, failure to groom, eat or ambulate, they will be promptly and humanely euthanized in accordance with the guidelines.

**Detail relief to be provided for each of the above-stated potential consequences, and, if relief is not planned, offer scientific justification for not doing so.**

In case of aggressive behaviour, the mice will be separated.

The CCAC and OMAFRA require that all AUPs include:

- a 'Monitoring Plan' to minimize animal pain, distress, or discomfort, and
- a plan for 'Early Euthanasia' for the purpose of emergency intervention in advance of the experimental endpoint.

As per Western's *Animal Care and Use Records Policy*, (found at https://www.uwo.ca/research/ethics/animal/animal_care_and_use_policies.html (https://www.uwo.ca/research/ethics/animal/animal_care_and_use_policies.html)) Animal Records, e.g. scoring sheets, procedure logs, anaesthetic and surgery records (except those involved in Field Studies) must be kept with the animals at all times.

---

**Please see the ACC OWL site for all monitoring and surgery sheet templates:**
https://owl.uwo.ca/portal/directtool/58e67139-c859-4b64-b6cb-54bf866d392a/ (https://owl.uwo.ca/portal/directtool/58e67139-c859-4b64-b6cb-54bf866d392a/)

**Has a monitoring sheet used for determining interventions and early euthanasia endpoints been developed for this species, e.g. scoring sheets, anaesthetic record, surgery record.**
**If YES, please attach the monitoring sheet(s) below.**

**If NO, please complete the following checklist**

Yes ○ No ◉

**Weight -*When checked, this indicates that weights will be recorded***

☑

**Food/Water Intake**

☐

**Behaviour**

☑

**Fecal/Urine Output**

☐

**Body Condition Score**

☑

**Appearance**

☑

**Other Monitoring**

☐

**Please Specify Other Monitoring Type.**

**For every individual monitoring element checked above:**
**Describe the frequency, Specify the intervention points including criteria for early euthanasia, Provide other relevant detail. If attached monitoring sheets capture this information, then indicate this here.**

Mice will be monitored weekly following high fat diet. In case of any abnormality, the monitoring frequency will increase.

Following high fat diet for 4 to 16 weeks abnormality is not expected. However, if at any time mice exhibit dehydration, lethargy or aggressive behaviour, failure to groom, eat or ambulate, they will be promptly and humanely euthanized in accordance with the guidelines.

Please attach your monitoring sheets.

# Endpoint Method Information

| Endpoint Method |
| --- |
| Cervical Dislocation Under Anesthesia |

**Endpoint Method**

Cervical Dislocation Under Anesthesia

**CCAC Classification**

Acceptable

**This method is conditionally acceptable. Please provide sufficient justification for using this method. Please note that conditionally acceptable methods may require additional training prior to use.**

**Provide Additional experimental endpoint detail, as required**

Not applied.

**Provide endpoint detail for animals not euthanized**

For endpoint methods selected above that use drugs, please list them below, and include the dosage.

| Drug | Dosage |
| --- | --- |
| Isoflurane | 2-5% |

# Animal Numbers Requested

With a view to the animal numbers disclosed on the **Groups and Timelines** web page, please provide your requested total four- and first-year animal numbers by Category of Invasiveness as well as justification for these numbers.

Please consider the activities selected for this species in the list below with a view to their combined impact upon an animal.

| Species | Type | Description |
| --- | --- | --- |

Please select the top Category of Invasiveness for this species and, for AUPs containing breeding colonies, please separate these numbers into the 'Z' category.

**Categories of Invasiveness –** Levels assigned to AUPs in accordance with CCAC policy. Experiments involving:

- **B -** Little or no discomfort or stress
- **C -** Minor stress or pain of short duration
- **D -** Moderate to severe distress or discomfort
- **E -** Procedures causing severe pain at or above the pain tolerance threshold of unanaesthetized conscious animals
- **Z -** Animals used for breeding purposes (internal letter designation to separate out breeding from research numbers - a CCAC requirement)

For more detail go to the CCAC Website:

http://www.ccac.ca/en_/standards/policies/policy-categories_of_invasiveness (http://www.ccac.ca/en_/standards/policies/policy-categories_of_invasiveness)

| CCAC Category | 4 YR # | 1st YR # |
|---|---|---|
| B | 320 | 60 |
| C | 0 | 0 |
| D | 0 | 0 |
| E | 0 | 0 |
| Z | 0 | 0 |

**Justification for Number of Animals Requested**

Group1: EC-BRCA2-/- (n= 20 males and 20 females)
Group 2: WT littermate control (n= 20 males and 20 females)
Baseline characterization (n = 5/ group for RNA & protein, n= 5/ group for histology (aorta), n= 10/ group for functional assays)

Group 3: EC-BRCA2-/-;ApoE-/- (n= 60 males and 60 females)
Group 4: WT littermate control (n= 60 males and 60 females)

Group 3 and 4 wil be fed high fat diet. Aortas and their branches will be harvested at 4, 8, 12 and 16 weeks post-high fat diet.

Collected samples will be used as follows: immunohistochemistry (n= 5/ group), RNA extraction (n= 5/ group), and protein extraction (n= 5/ group). We will require 60 animals per group, which includes 15 animals for each of the 4 time points, to achieve statistical significance in the above experiments.

# Personnel List

Complete the table below to include all individuals directly associated with animal-based science activities for this AUP. In this section personnel must be associated with the specific animal activities they will be involved with.

For Personnel Already Listed Below - Please highlight the table row containing each name, and then select the 'Edit Personnel' button to complete or update information.

| Name | Role | Phone | Primary Email | HANDS ON? |
|---|---|---|---|---|
| Singh, Krishna | Principal Investigator | | | Yes |
| Nguyen, Hien | Researcher Staff Members | | | No |

| | | |
|---|---|---|
| Michels, David | Researcher Staff Members | Yes |
| Wang, Lin | Researcher Staff Members | Yes |

**Name**

Singh, Krishna

**Role**

Principal Investigator

**Organization Department**

Schulich School Of Medicine & Dentistry Medical Biophysics

**Weekday Phone #**

**Emergency Contact #**

**UWO or Lawson Email**

**Other Email**

**Copy this Individual on all Emails**

☐

**CCAC-Mandated Training Requirements** – As per MAPP 7.10, each person working with live animals requires training that aligns with his/her hands-on animal activity.

At minimum, all individuals listed within this AUP must complete the *Basic Care and Use* online 'animal ethics' course.

The requirement for additional online training, hands-on workshops or competency assessments will be determined by the species and animal procedures associated with each individual as well as his/her previous Canadian training and experience.

For further information, please contact

**Will person be handling animal species?**

Yes ◉ No ○

| Species Name | Type | Procedure Description |
|---|---|---|
| Mouse | Procedures | 01. Blood Collection - Intracardiac |
| Mouse | Procedures | 12. Cervical Dislocation - Under Anesthesia |
| Mouse | Procedures | 16. Anesthesia - Gas |

Based upon elements selected in the previous 'Personnel Activities' tab for this individual, below is a list of all required training activities - online OWL modules and/or hands-on workshops. Training activities listed below with dates indicates completion of that specific training element.

Please contact for further details.

**Degrees**

**Experience and Qualifications**

| Training Event | Description | Type | Date Certified | Training ID |
|---|---|---|---|---|
| Assessment, Monitoring and Intervention | Behavioural responses of research animals in pain; how to assess; monitoring procedures | Internet-based Course | | AMI |
| Basic Animal Care & Use Ethics Course | ethics, regulations, 3 Rs, SOPs, safety. Species specific animal care, housing, EE, etc. | Internet-based Course | 01/09/2019 | BACUEC |
| Cervical Dislocation - Under Anesthesia - Mouse | Cervical Dislocation of mice under gas anaesthesia. | Demonstration | | CDANMSE |
| Decapitation Without Anesthesia - Mouse | Decapitation without the use of anesthesia for the mouse | Demonstration | | DECWAMSE |
| Gas Anesthesia - Mouse | Principals of Gas Anesthesia in Mice including set-up, monitoring, record keeping and recovery. | Demonstration | | GASAMSE |
| Handling & Care - Mouse | covers behaviour, moving mice, sexing, euthanasia and some scruffing. Students complete webcts: basic handling of rodents, and assessment, intervention and monitoring | Demonstration | | HCMSE |
| Intracardiac Blood Collection - Mouse | Intra-cardiac blood collection reviewed and practised. with Iso or Co2. | Demonstration | | INCBLMSE |

**Name**

Nguyen, Hien

**Role**

Researcher Staff Members

**Organization Department**

Schulich School Of Medicine & Dentistry Medical Biophysics

**Weekday Phone #**

**Emergency Contact #**

**UWO or Lawson Email**

**Other Email**

**Copy this Individual on all Emails**

☐

**CCAC-Mandated Training Requirements** – As per MAPP 7.10, each person working with live animals requires training that aligns with his/her hands-on animal activity.

At minimum, all individuals listed within this AUP must complete the *Basic Care and Use* online 'animal ethics' course.

The requirement for additional online training, hands-on workshops or competency assessments will be determined by the species and animal procedures associated with each individual as well as his/her previous Canadian training and experience.

For further information, please contact

**Will person be handling animal species?**

Yes ○ No ◉

| Species Name | Type | Procedure Description |
|---|---|---|

Based upon elements selected in the previous 'Personnel Activities' tab for this individual, below is a list of all required training activities - online OWL modules and/or hands-on workshops. Training activities listed below with dates indicates completion of that specific training element.

Please contact for further details.

**Degrees**

**Experience and Qualifications**

| Training Event | Description | Type | Date Certified | Training ID |
|---|---|---|---|---|
| Basic Animal Care & Use Ethics Course | ethics, regulations, 3 Rs, SOPs, safety. Species specific animal care, housing, EE, etc. | Internet-based Course | 02/06/2020 | BACUEC |

**Name**

Michels, David

**Role**

Researcher Staff Members

**Organization Department**

Schulich School Of Medicine & Dentistry Medical Biophysics

**Weekday Phone #**

**Emergency Contact #**

**UWO or Lawson Email**

**Other Email**

**Copy this Individual on all Emails**

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**CCAC-Mandated Training Requirements** – As per MAPP 7.10, each person working with live animals requires training that aligns with his/her hands-on animal activity.

At minimum, all individuals listed within this AUP must complete the *Basic Care and Use* online 'animal ethics' course.

The requirement for additional online training, hands-on workshops or competency assessments will be determined by the species and animal procedures associated with each individual as well as his/her previous Canadian training and experience.

For further information, please contact

**Will person be handling animal species?**

Yes ◉ No ○

| Species Name | Type | Procedure Description |
|---|---|---|
| Mouse | Procedures | 01. Blood Collection - Intracardiac |
| Mouse | Procedures | 12. Cervical Dislocation - Under Anesthesia |
| Mouse | Procedures | 16. Anesthesia - Gas |

Based upon elements selected in the previous 'Personnel Activities' tab for this individual, below is a list of all required training activities - online OWL modules and/or hands-on workshops. Training activities listed below with dates indicates completion of that specific training element.

Please contact for further details.

**Degrees**

**Experience and Qualifications**

| Training Event | Description | Type | Date Certified | Training ID |
|---|---|---|---|---|
| Assessment, Monitoring and Intervention | Behavioural responses of research animals in pain; how to assess; monitoring procedures | Internet-based Course | | AMI |
| Basic Animal Care & Use Ethics | ethics, regulations, 3 Rs, SOPs, safety. Species specific | Internet- | | |

| | | | | |
|---|---|---|---|---|
| Course | animal care, housing, EE, etc. | based Course | 10/01/2019 | BACUEC |
| Cervical Dislocation - Under Anesthesia - Mouse | Cervical Dislocation of mice under gas anaesthesia. | Demonstration | 10/23/2019 | CDANMSE |
| Decapitation Without Anesthesia - Mouse | Decapitation without the use of anesthesia for the mouse | Demonstration | | DECWAMSE |
| Gas Anesthesia - Mouse | Principals of Gas Anesthesia in Mice including set-up, monitoring, record keeping and recovery. | Demonstration | 10/03/2019 | GASAMSE |
| Handling & Care - Mouse | covers behaviour, moving mice, sexing, euthanasia and some scruffing. Students complete webcts: basic handling of rodents, and assessment, intervention and monitoring | Demonstration | 10/01/2019 | HCMSE |
| Intracardiac Blood Collection - Mouse | Intra-cardiac blood collection reviewed and practised. with Iso or Co2. | Demonstration | 10/22/2019 | INCBLMSE |

**Name**

Wang, Lin

**Role**

Researcher Staff Members

**Organization Department**

Schulich School Of Medicine & Dentistry Anatomy & Cell Biology

**Weekday Phone #**

**Emergency Contact #**

**UWO or Lawson Email**

**Other Email**

**Copy this Individual on all Emails**

☑

**CCAC-Mandated Training Requirements** – As per MAPP 7.10, each person working with live animals requires training that aligns with his/her hands-on animal activity.

At minimum, all individuals listed within this AUP must complete the *Basic Care and Use* online 'animal ethics' course.

The requirement for additional online training, hands-on workshops or competency assessments will be determined by the species and animal procedures associated with each individual as well as his/her previous Canadian training and experience.

For further information, please contact

**Will person be handling animal species?**

Yes (•) No ( )

| Species Name | Type | Procedure Description |
|---|---|---|
| Mouse | Procedures | 01. Blood Collection - Intracardiac |
| Mouse | Procedures | 12. Cervical Dislocation - Under Anesthesia |
| Mouse | Procedures | 16. Anesthesia - Gas |

Based upon elements selected in the previous 'Personnel Activities' tab for this individual, below is a list of all required training activities - online OWL modules and/or hands-on workshops. Training activities listed below with dates indicates completion of that specific training element.

Please contact for further details.

**Degrees**

**Experience and Qualifications**

Lin Wang has basic training in handling mice.

| Training Event | Description | Type | Date Certified | Training ID |
|---|---|---|---|---|
| Assessment, Monitoring and Intervention | Behavioural responses of research animals in pain; how to assess; monitoring procedures | Internet-based Course | | AMI |
| Basic Animal Care & Use Ethics Course | ethics, regulations, 3 Rs, SOPs, safety. Species specific animal care, housing, EE, etc. | Internet-based Course | | BACUEC |
| Cervical Dislocation - Under Anesthesia - Mouse | Cervical Dislocation of mice under gas anaesthesia. | Demonstration | 11/29/2019 | CDANMSE |
| Decapitation Without Anesthesia - Mouse | Decapitation without the use of anesthesia for the mouse | Demonstration | | DECWAMSE |
| Gas Anesthesia - Mouse | Principals of Gas Anesthesia in Mice including set-up, monitoring, record keeping and recovery. | Demonstration | | GASAMSE |
| Handling & Care - Mouse | covers behaviour, moving mice, sexing, euthanasia and some scruffing. Students complete webcts: basic handling of rodents, and assessment, intervention and monitoring | Demonstration | | HCMSE |

| | | | | |
|---|---|---|---|---|
| Intracardiac Blood Collection - Mouse | Intra-cardiac blood collection reviewed and practised. with Iso or Co2. | Demonstration | 10/30/2019 | INCBLMSE |

# Protocol Attachments

The following is a list of all attachments listed on this Protocol

| File Name | Description | Original File Name |
|---|---|---|
| 2018-163_1_0001_2018-163_1_0001_Experimental Outline_BRCA2_REVISED.ppt (https://esirius.uwo.ca/esirius3g/attachment/2018-163_1_0001_2018-163_1_0001_Experimental Outline_BRCA2_REVISED.pptx) | | 2018-163_1_0001_Experimental Outline_BRCA2_REVISED.pptx |
| 2018-163_1_0001_2018-163_1_0001_Experimental TimeLine_BRCA2_REVISED.pp (https://esirius.uwo.ca/esirius3g/attachment/2018-163_1_0001_2018-163_1_0001_Experimental TimeLine_BRCA2_REVISED.pptx) | Timeline | 2018-163_1_0001_Experimental TimeLine_BRCA2_REVISED.pptx |
| 2018-163_1_0001_Isoflurane Baxter MSDS 2016.pdf (https://esirius.uwo.ca/esirius3g/attachment/2018-163_1_0001_Isoflurane Baxter MSDS 2016.pdf) | SDS | Isoflurane Baxter MSDS 2016.pdf |
| 2018-163_1_0001_Isoflurane Safety 2018-163.doc (https://esirius.uwo.ca/esirius3g/attachment/2018-163_1_0001_Isoflurane Safety 2018-163.doc) | Isoflurane Safety Form | Isoflurane Safety 2018-163.doc |

# Amendment Reason

**Protocol Number**

2018-163

**Protocol Version**

7

**Protocol Title**

Role of Endothelial Breast Cancer Gene 2 in Cardiovascular Pathobiology

**Approve Date**

02/01/2019

**Expiration Date**

02/01/2023

**Full Name**

Singh, Krishna

**Reason for Change**

| | | | | |
|---|---|---|---|---|
| Intracardiac Blood Collection - Mouse | Intra-cardiac blood collection reviewed and practised. with Iso or Co2. | Demonstration | 10/30/2019 | INCBLMSE |

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| 2018-163_1_0001_Isoflurane Safety 2018-163.doc (https://esirius.uwo.ca/esirius3g/attachment/2018-163_1_0001_Isoflurane Safety 2018-163.doc) | Isoflurane Safety Form | Isoflurane Safety 2018-163.doc |

# Amendment Reason

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2018-163

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Role of Endothelial Breast Cancer Gene 2 in Cardiovascular Pathobiology

**Approve Date**

02/01/2019

**Expiration Date**

02/01/2023

**Full Name**

Singh, Krishna

**Reason for Change**

Adding new location to this AUP.

## Curriculum Vitae

| | |
|---|---|
| **Name:** | David Charles Robert Michels |
| **Title:** | M.Sc. Graduate student and Graduate Research Assistant, Department of Medical Biophysics, University of Western Ontario, Schulich School of Medicine and Dentistry |
| **Post-secondary Education and Degrees:** | BSc, Honors Specialization in Biology, Major in Linguistics, University of Western Ontario, 2013-2019<br><br>MSc Candidate, Medical Biophysics, University of Western Ontario, 2019-present |
| **Honours and Awards:** | Ontario Graduate Scholarship ($15,000), Government of Ontario, 2020-2021<br><br>Bennie and Shirley Bradshaw Award in Science ($2,100), University of Western Ontario, 2015-2016<br><br>JASSO (Japan Student Services Organization) Scholarship ($9,000), Government of Japan, 2015-2016<br><br>Deans Honor List, University of Western Ontario, 2014-2019<br><br>UWO Part-time Scholarship ($1,000), University of Western Ontario, 2013 |
| **Related Work Experience:** | LIDAR Observer, Department of Physics and Astronomy, University of Western Ontario, 2017<br><br>Laboratory Assistant, Department of Biology, University of Western Ontario, 2017-2019 |

**Publications:**

Singh S, Nguyen HC, Ehsan M, Michels DCR, Singh P, Qadura M, Singh KK. (2021). Pravastatin-induced changes in expression of long non-coding and coding RNAs in endothelial cells. Physiological Reports.

Nguyen H, Singh S, Michels D, Bazinet H, Wang L, Singh KK. (2020). Chloroquine Up-regulates
Expression of SARS-CoV-2 receptor Angiotensin Converting Enzyme-2 in Endothelial Cells. PLoS One.

Singh S, Adam M, Matkar PN, Bugyei-Twum A, Desjardins JF, Chen HH, Nguyen H, Bazinet H, Michels D, Liu Z, Mebrahtu E, Esene L, Joseph J, Ehsan M, Qadura M, Connelly KA, Leong-Poi H, Singh KK. (2020). Endothelial-specific Loss of IFT88 Promotes Endothelial-to-Mesenchymal Transition and Exacerbates Bleomycin-induced Pulmonary Fibrosis. Scientific Reports.

Singh S, Nguyen H, Michels D, Bazinet H, Matkar PN, Liu Z, Esene L, Adam M, Bugyei-Twum A, Mebrahtu E, Joseph J, Ehsan M, Chen HH, Qadura M, Singh KK. (2020). BReast CAncer susceptibility gene 2 deficiency exacerbates oxidized LDL-induced DNA damage and endothelial apoptosis. Physiological Reports.