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Germline Mutations in CHEK1 and CHEK2 in Women with Ovarian, Peritoneal, or Fallopian Tube Cancer

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Abstract

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by

Maria Isabel Harrell

M.S., University of Washington, 2004

B.S., University of Washington, 1998

Dissertation Submitted in Partial Fulfillment

Of the Requirements for the Degree of

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Abstract

Ovarian cancer is the deadliest gynecological malignancy affecting women. Diagnosis often occurs late due to non-specific symptoms, but if detected early, there is excellent chance for survival. One of the most important risk factors is family history. Up to 24% of cases are due to inherited loss-of-function mutations in genes involved in the DNA damage response. The theory underlying hereditary cancers is Knudson's two-hit theory of cancer causation, where two hits are necessary for cancer to occur in an individual: one in the germline and one in the tissue. The genes, *CHEK1* and *CHEK2*, are modulators of the DNA damage response, and could be susceptible to a first hit. There is little to no evidence about whether loss-of-function mutations in either of these two genes can lead to ovarian cancer. Using a cohort of 587 ovarian cancer cases and 557 controls, this study sought to determine if *CHEK1* and *CHEK2* are associated with ovarian cancer. Applying Fisher's exact test to compare mutation rates and the *t* test to compare age at time of diagnosis, the alternative hypothesis about an association between disease and mutations in *CHEK1* and *CHEK2* was rejected, but an association between younger age at diagnosis in cases and mutations in either gene was confirmed. The association between age and mutations in either of these genes suggests that there is some influence of age on disease, but a clear association between development of disease and mutations cannot yet be established. This research has implications for social change: By recognizing the need to test earlier in women with mutations in *CHEK1* and/or *CHEK2*, they will have a higher chance of survival and better health outcomes, not only for ovarian cancer but for related cancers as well.

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Dedication

To Trace, thanks for your unconditional love and support throughout this journey and always.

To Pablo, my father, who taught me to keep on learning and showed me that you should use your mind and enjoy life to the fullest while you still can.

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Chapter 1: Introduction to the Study

Introduction

Inherited mutations are a strong causative component for ovarian cancer, one of the deadliest cancers affecting women. Genes such as *BRCA1* (BRreast CAncer), *BRCA2*, and other genes involved in DNA repair have been associated with increasing ovarian cancer risk (Pennington & Swisher, 2012). Two genes that are involved in the DNA repair response are the genes *CHEK1* and *CHEK2*. It is unclear whether these genes could be associated with increased risk for ovarian cancer. This study aimed to understand whether there is a possible association between mutations in these genes and ovarian cancer *susceptibility*. If these two genes are identified as such, then they can be included in the genetic testing panels to determine risk of disease in women with known family history of ovarian carcinoma. If women know they are at risk, they can pursue prophylactic strategies, as well as improved surveillance techniques, to either prevent cancer or identify cancer early. Improving cancer survival rates is a positive social change that in turn leads to prevention and improved surveillance.

The current chapter provides background for the current study, information on why this study was pursued, and the questions this study sought to answer. It also gives an introduction to the conceptual framework of the study and presents the limitations, assumptions, scope and delimitations, and significance of the study.

Background of the Study

The term *ovarian cancer* refers to tumors in ovaries, fallopian tubes, and/or peritoneum (Chen & Berek, 2015). It is the deadliest gynecological cancer that affects

women; it represents 3% of cancers that occur in women; and ranks as the ninth most common cancer overall (Surveillance, Epidemiology and End Results, 2012). Five-year survival rates are low (46%) compared to breast cancer (89%) and cervical cancer (70%), as reported by Weissman, Weiss, and Newlin (2012). Survival rate is highly dependent on the stage of the cancer at diagnosis. Early diagnosis corresponds with better outcomes. Yet only 15% of ovarian cancers are diagnosed early because the disease tends to present with non specific and non gynecological symptoms at later stages of the disease (Goff, Mandel, Muntz, & Melancon, 2000; Weissman et al., 2012).

One of the most important risk factors for ovarian cancer is family history (Pennington & Swisher, 2012). Hereditary ovarian cancer is due to inherited mutations in different genes and accounts for 25% of newly diagnosed cases (Pennington & Swisher, 2012). The most common causes of inherited ovarian cancers are *loss-of-function germline mutations* in *BRCA1* and *BRCA2* (BReast CAncer), which account for 48% and 27% of cases, respectively. However, at least 25% of hereditary ovarian cancer cases are due to mutations in other genes (Pennington & Swisher, 2012).

In the early 1990s, researchers identified the genes *BRCA1* and *BRCA2* as genetic elements that are responsible for inherited breast and ovarian cancer (Check, 2006). This led breast cancer researchers to acknowledge that complex traits and diseases can be linked to mutations in certain genes. Genetic mutations are not just confined to diseases that are of Mendelian inheritance, such as cystic fibrosis or Huntington's disease (Check, 2006). Genetic testing for mutations in these two genes has been performed since the mid-90s. Women found to have mutations in *BRCA1* and *BRCA2* have an increased

lifetime risk of breast and ovarian cancer and may elect to undergo preventative measures to avoid disease. Not only were women who had mutations in *BRCA1* and *BRCA2* found to have an increased risk of disease (Check, 2006), but they were also more likely to develop disease earlier than women without mutations in these genes (Malone et al., 2006). This suggests that age of diagnosis could also be correlated with mutations in these *susceptibility* genes.

Other genes found in a DNA repair pathway, named the Fanconi Anemia-BRCA pathway (FA-BRCA), have been associated with hereditary ovarian cancer (Pennington & Swisher, 2012). The FA-BRCA DNA repair pathway is involved in the repair of DNA by homologous recombination (HR, Pennington & Swisher, 2012). *PALB2*, *RAD51C*, and *RAD51D* are genes that function in the FA-BRCA DNA pathway along with *BRCA1* and *BRCA2*. Mutations in the afore mentioned genes have been correlated with ovarian cancer cases (Casadei et al., 2011; Loveday et al., 2011; Vaz et al., 2010). With the advent of new, highly efficient techniques for sequencing, such as *targeted capture* and *massively parallel sequencing*, gene mutations can be investigated for correlation with ovarian cancer and other diseases. These techniques also may identify previously unknown genes that could impact disease (Walsh et al., 2011).

Walsh et al. (2011) performed a study using a massively parallel sequencing approach (called BROCA) to sequence 21 tumor-suppressor genes on DNA from ovarian cancer patients (Walsh et al., 2011). They found mutations in 12 genes, including *BRCA1* and *BRCA2*, and new candidate genes such as *BARD1*, *BRIP1*, and *CHEK2*, all of which had been suspected of conferring risk for ovarian cancer (Pennington & Swisher, 2012).

In addition, Walsh et al. (2011) found that the average age in mutation carriers was lower than in non-carriers. Inherited mutations are present in a larger number of genes than previously thought, so identifying candidate genes involved in inherited ovarian cancer is important (Walsh et al., 2011). *CHEK1* and *CHEK2* are appropriate genes to investigate given the known involvement of other FA pathway genes in ovarian cancer (Chen & Sanchez, 2004; Cybulski et al., 2004; Shaag et al., 2005). They are both part of the FA-BRCA DNA repair pathway and important effectors of the DNA damage response. Identifying new candidate genes and including these genes in genetic testing can improve the outcomes for women with a family history of ovarian and breast cancer. There is support in the literature for including these variables in this study, which is discussed further in Chapter 2.

Problem Statement

Ovarian cancer is among the top five causes of cancer death in American women (SEER, 2012). Survival rates for ovarian cancer patients are low since most women are diagnosed with advanced stages of the disease (Pennington & Swisher, 2012). When detected early, there is an excellent chance for cure, but current methods of early detection are largely ineffective. One of the most important risk factors for the development of ovarian cancer is family history. Inherited mutations in genes (such as *BRCA1, BRCA2, PALB2*, *BARD1*, and *RAD51C*) involved in DNA repair by HR (Pennington & Swisher, 2012) may contribute to the risk of a woman's chance of developing the disease. Genetic testing for mutations in these genes has been helpful in identifying potential mutation carriers. Many women with a family history of the disease

and with loss of function mutations, especially in *BRCA1* and *BRCA2*, have undergone *prophylactic salpingo oophorectomies*, which in turn have led to reduced incidence of disease and mortality (Pennington & Swisher, 2012).

Yet there are many women with family history of ovarian cancer but no loss of function mutations in the known genes. This suggests that there may be other genes that can harbor inherited mutations and lead to susceptibility to ovarian cancer. Two genes that have been largely overlooked to date are *CHEK2* and *CHEK1*. Both genes encode for serine/threonine kinases and are important regulators of the cell cycle and mediators of the DNA damage response (Shaag et al., 2005). Mutations in *CHEK2* have previously been associated with breast cancer and other types of cancer, such as prostate, lung, and thyroid (Cybulski et al., 2004). Mutations within these genes can lead to truncated proteins and promote errors in DNA repair. Since both *CHEK1* and *CHEK2* are important players in the DNA damage response and are putative tumor suppressor genes, it is important to determine whether they are likely candidates for mutations in ovarian cancer patients. To date, only one publication has reported a mutation in *CHEK1* in an ovarian cancer patient (Pennington et al., 2013a), but no other publication has identified *CHEK1* as a candidate gene for ovarian cancer; the research on *CHEK2* and ovarian cancer is not extensive and has not established a clear association. The literature reviewed in Chapter 2 demonstrates that these are appropriate candidate genes to study.

Purpose of the Study

The purpose of this quantitative study was to use data obtained at the University of Washington to compare the rates of mutations in the genes *CHEK1* and *CHEK2* in

ovarian cancer cases and healthy controls. Mutations were ascertained by a next-generation sequencing approach. Once the mutations were identified in the cohort, I determined whether mutations in these genes were correlated with younger age at time of diagnosis in the cases. Determining these rates and comparing cases contributes to the growing evidence in favor of including *CHEK1* and *CHEK2* as candidate genes for ovarian cancer. The results of this study are expected to allow researchers to investigate whether these genes are susceptible to mutations and whether they can be designated as ovarian cancer susceptibility genes.

Research Questions

The following four research questions guided this study:

*H*1: Are *CHEK2* mutated alleles associated with ovarian cancer?

H_0^1: There are no *CHEK2* mutated alleles associated with ovarian cancer.

H_a^1: *CHEK2* mutated alleles are associated with ovarian cancer.

*H*2: Are *CHEK1* mutated alleles associated with ovarian cancer?

H_0^2: There are no *CHEK1* mutated alleles associated with ovarian cancer.

H_a^2: *CHEK1* mutated alleles are associated with ovarian cancer.

*H*3: Are *CHEK1* mutated alleles associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases?

H_0^3: There is no association between younger age at diagnosis and *CHEK1* mutations in ovarian cancer cases.

H_a^3: Mutated alleles in *CHEK1* are associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases.

*H*4: Are *CHEK2* mutated alleles associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases?

H_0^4: There is no association between younger age at diagnosis and *CHEK2* mutations in ovarian cancer cases.

H_a^4: Mutated alleles in *CHEK2* are associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases.

Conceptual Framework

In 1971, during his work on retinoblastoma, Alfred G. Knudson proposed that germline mutations can lead to cancer (Knudson, 1971). This theory is referred to as the two mutation theory of cancer causation. Knudson also proposed that all cancers were either hereditary or sporadic (Knudson, 1971; Ormiston, 1996). In the case of hereditary cancers, a germline mutation occurs pre-fertilization and is replicated in all cells created post-fertilization (Ormiston, 1996). Knudson also proposed that, for cancer to occur in these individuals, there needs to be another mutation that occurs as an error within one of the many mitotic divisions that occur over a lifetime (Ormiston, 1996). He also postulated that hereditary cancers occur at a younger age and that there is a higher likelihood of recurrence as well as the occurrence of tumors at multiple sites (Knudson, 2002; Ormiston, 1996). In comparison, sporadic tumors occur much later in life and, usually, there is only a single tumor site with very low likelihood of recurrence (Knudson, 2002; Ormiston, 1996).

Individuals with germline mutations in *BRCA1* and *BRCA2* are diagnosed with cancer at a younger age and often have multiple tumor sites (breast, ovaries, fallopian

tubes, peritoneum, pancreas, etc.) compared with women who are diagnosed with breast cancer due to somatic mutations but not germline mutations (Check, 2006; Ormiston, 1996). Additionally, in accordance with Knudson's hypothesis, many tumors from cancer patients with inherited breast and ovarian cancer syndrome have been found to have mutations in genes such as *TP53*, a tumor suppressor protein involved in a number of cellular processes (Greenblatt, Chappuis, Bond, Hamel, & Foulkes, 2001). Based on this evidence, Knudson's two mutation theory of cancer causation is an appropriate framework for this study.

Definition of Terms

Mutation or variant: Change in the DNA sequence of a gene, it can range from a single base to a large segment of a chromosome (Mutation, 2013).

Wildtype allele: The natural state of the DNA as it occurs in the majority of the population; the opposite of being mutant (Wildtype allele, 2013)

Susceptibility genes: genes that if they contain mutations can predispose to a particular disease such as cancer (Genetic susceptibility, 2015)

Salpingo-oophorectomy: surgery to remove fallopian tubes and ovaries (Mayo Clinic, 2015)

Debulking sugery: complete or near complete removal of the tumor by surgical means (American Cancer Society, 2015a)

Germline mutation: a mutation that is present in every cell of the body, because it originated in a germ cell (Germline mutation, 2015)

Loss-of-function mutation: mutation that results in the loss of function of a protein (Loss of function, 2015)

Sequencing: Process by which the order of the nucleotides in a given DNA fragment is determined (Sequencing, 2013).

Sanger Sequencing: Method developed by Fred Sanger to determine the order of specific bases in a DNA fragment (Obenrader, n.d.).

Massive parallel sequencing approach: Also known as next-generation sequencing (NGS) is a high throughput approach to DNA sequencing where several sequencing reactions are occurring in parallel (Tucker, Marra, & Friedman, 2009).

Proband: A subject that is enrolled in genetic testing and affected by a condition that is being studied. Often the first person tested within a family (Proband, 2015).

Occult cancer: Cancers that are hidden, and not clinically apparent. Most often they are found through serial sectioning of ovaries and fallopian tubes in women who underwent risk reducing salpingo-oopherectomy (Brown & Palmer, 2009).

Limitations

This study used secondary data (collected by the Swisher laboratory at the University of Washington in Seattle), which limited my ability of to control the data gathering process (Sorensen, Sabroe, & Olsen, 1996). Therefore, I was not able to validate the data to confirm that the mutations were reported accurately nor to confirm whether there were errors in the reporting of cancer status or age.

Assumptions

This research was based on five assumptions: (a) the controls were cancer free at the time of blood sample collection; (b) all proper protocols for DNA extraction and analysis were performed according to standards set forth by the Clinical Laboratory Improvement Amendments (CLIA); (c) measures were taken to prevent contamination and mishandling of samples (Centers for Medicaid and Medicare Services, 2014); (d) age was reported accurately at time of sample collection; (e) *CHEK1* and *CHEK2* influence the mechanisms of ovarian cancer development in the same way due to their similarity of function within the DNA repair pathway.

Scope and Delimitations

This work is part of a genetic association study in which two candidate genes were evaluated regarding their association with ovarian cancer. This study was a case-control study with available sequence data to determine whether the genes *CHEK1* and *CHEK2*, which functioned as the variables, harbored mutations more often in cases than in controls. Cases were defined as women undergoing primary surgery for ovarian cancer at the University of Washington (UW) between 2001 and present. Controls were defined as healthy women over the age of 50. The age of the controls was a limitation of the dataset given to me. It was assumed that the women treated at the University of Washington were residents of the Pacific Northwest and that they represented the population of that area, a population that may differ in race and ethnicity from women living in other areas of the country. Information on where the participants lived was not available; thus, I could not ascertain whether they represent only one area of the country.

Data to determine *CHEK1* and *CHEK2* mutation was generated by next-generation sequencing and provided in form of an Excel database. Statistical association analysis was performed by chi-square (χ^2) tests or Fisher's exact tests, as appropriate, to determine probability of disease association with mutations (Casadei et al., 2011).

This study was delimited to determining the type of mutation within each sample that showed a mutation in *CHEK1* and *CHEK2*. It did not (a) determine the mutation rates in other genes (b) establish how mutations in *CHEK1* and *CHEK2* may or may not contribute to ovarian cancer nor whether mutations in these genes were causative. The data were correlated only to the study population .

Significance of the Study

The role of genes in addition to *BRCA1* and *BRCA2* in inherited ovarian cancer have been investigated and confirmed, especially for genes involved in DNA repair pathways (Pennington & Swisher, 2012; Walsh et al., 2011). Identifying genes in the DNA repair pathway that may be susceptible to mutations associated with ovarian cancer may help improve prevention and could lead to the development of new therapeutic agents. Women at risk for hereditary ovarian cancer who carry mutations in certain genes may choose a proactive approach to surveillance and treatment (Weissman et al., 2012). Genetic counseling and testing may also include these genes to help improve disease prevention, reduce incidence, and increase survival rates.

The improved medical knowledge from this research includes identification of *CHEK1* and *CHEK2* as candidates for association with ovarian cancer and their inclusion on genetic testing panels for hereditary ovarian cancer. It brings about social change

because testing for these genes in addition to *BRCA1* and *BRCA2* and other genes may result in reduced ovarian cancer incidence and increased survival rates. It may also be cost-effective, because prophylactic efforts, such as surveillance and prophylactic surgery, incur less cost than current medical treatments and tumor removal and *debulking surgery* (Grann, Panageas, Whang, Antman, & Neugut, 1998). Additionally, genetic testing can be expanded to include various family members and improve outcomes within that family unit.

Summary

One of the most important risk factors for ovarian cancer is family history and several genes can acquire mutations that increase an individual's lifetime risk of disease (Check, 2006; Pennington & Swisher, 2012). Many of these genes have been found to be important players in the DNA damage response pathway (D'Andrea, 2013). *CHEK1* and *CHEK2* are important mediators for the DNA damage response pathway and, if mutated, could contribute to the development of ovarian cancer (Cybulski et al., 2004; Huang et al., 2008; Kumar et al., 2013). Little is known about their involvement in ovarian cancer risk. Novel sequencing techniques allow researchers to identify mutations in several genes in tandem at lower costs and much more quickly, too (Walsh et al., 2011). These techniques can provide sequencing information to determine whether *CHEK1* and *CHEK2* can harbor mutations and become ovarian cancer susceptibility genes. This study is a case control study that sought to determine whether there is an association between mutations in those genes and disease and whether disease occurs earlier if mutations are present.

Chapter 2 reviews the literature on ovarian cancer genetics, current knowledge of hereditary cancer syndromes, and how mutations in certain genes can lead to an increase in cancer risk. Chapter 2 also discusses why *CHEK1* and *CHEK2* are suitable as potential candidates for association with ovarian cancer. Chapter 3 presents the research methods and research questions. Chapter 4 provides the results of this study and Chapter 5 discusses them. It also offers recommendations for future study.

Chapter 2: Literature Review

Introduction

Ovarian cancer is the deadliest of all gynecological cancers and among the top five causes of cancer-related death among American women (Surveillance, Epidemiology and End Results, 2013). The strongest risk factor for development of disease is family history correlated with the presence of mutations in different genes of the HR DNA repair pathway, such as *BRCA1* and *BRCA2* (Pennington & Swisher, 2012). The purpose of this study was to determine whether two genes involved in the HR repair pathway, *CHEK1* and *CHEK2*, are associated with the development of ovarian cancer and whether they are suitable candidates for ovarian cancer susceptibility.

In this chapter, the literature on the role of genetics in ovarian cancer, and the known ovarian cancer susceptibility genes within the DNA repair pathway was reviewed.It provides a basic introduction to the function of *CHEK1* and *CHEK2*, the known mutations in these genes, which are linked to different cancers, and the likelihood that they could be candidate genes for ovarian cancer susceptibility.

To identify peer-reviewed articles, the following databases—PubMed, CINAHL, Web of Science, SCOPUS, and Embase—were searched, without regard to year, using these keywords: *ovarian cancer, ovarian cancer genetics, hereditary breast and ovarian cancer syndrome, hereditary cancer syndromes, BRCA1, BRCA2, Fanconi Anemia-BRCA pathway, massively parallel sequencing, Next-Generation sequencing, BROCA, two hit theory of cancer causation, Knudson's two hit theory, CHEK1*, and *CHEK2*. The Boolean operators, AND and OR, were applied to optimize the results. . No limits other than

language—English, Spanish or German—were set on the searches. Abstracts were used to judge an article's relevancy to the research questions.

Hereditary Ovarian Cancer

Ovarian cancer is the deadliest of all gynecological cancers and is among the top five causes of cancer-related death in American women, with an estimated 14,030 deaths in 2013 (National Cancer Institute, 2013). It is the ninth most common occurring cancer with an estimated 22,240 new cases in 2013 in the U.S. (NCI, 2013). The survival rates for ovarian cancer patients are low since most women present with symptoms at advanced stages of the disease (Hunn & Rodriguez, 2012). The overall survival rate for all types of ovarian cancer is 44%. When women are diagnosed at stage one of the disease, their survival rate is 98%. However, those diagnosed at stage four have a survival rate of only 18% (American Cancer Society, 2014). Only 15% of ovarian cancers are identified early but when detected early, women have better recovery odds. Attempts to improve early detection have been ineffective (Hunn & Rodriguez, 2012). Most patients present symptoms late in the disease's lifecycle and therefore identification of molecular biomarkers that appear early in cancer development has been a challenge (Hunn & Rodriguez, 2012). In addition, changes in tissue organization are hard to determine due to the lack of accessibility to the ovaries (Hunn & Rodriguez, 2012). This makes ovarian cancer risk very difficult to identify. Identification is, therefore, highly dependent on epidemiological factors.

One of the most important risk factors for ovarian cancer is family history. According to Jervis et al. (2015) the risk for first degree relatives of ovarian cancer

patients is three fold higher than that of women without any known breast or ovarian cancer in their families. Initial evidence for a familial link for ovarian cancer was provided by case control studies performed in the late 1980s and 1990s. In a case control study performed by Koch et al. (1989), there was a higher number of first and second degree relatives with ovarian cancer in the families of cases compared to families of controls.

Schildkraut et al. (1989) also investigated the genetic relationship between breast, ovarian, and endometrial cancer and found that there was a strong genetic component to these cancers. These researchers were part of the Cancer and Steroid Hormone Study (CASH) led by the Centers for Disease Control and Prevention (CDC). The study included information from multiple centers, and the investigators found elevated relative risks for mothers and sisters of ovarian cancer cases (RR = 2.8) as well as for breast cancer cases (RR = 1.6) (Schildkraut, Risch, & Thompson, 1989). They also found an elevated risk for ovarian and breast cancer among other relatives of cases with these cancers (RR = 2.1) (Schildkraut et al., 1989). They applied a multivariate polygenic threshold model to establish that there was a strong genetic component of ovarian cancer. Estimated hereditability of ovarian cancer was approximately 40% and 56% for breast cancer (Schildkraut et al., 1989). With these results, the researchers established the first link between ovarian cancer as a possible hereditary syndrome.

Using the same data from the CASH study, Claus et al. (1993) determined that there was an increased lifetime risk (13–31%) for a woman to develop breast cancer when a first or second degree relative had ovarian cancer, suggesting a genetic link not

only for ovarian cancer itself but also with breast cancer. The likelihood of developing one of these cancers within a family with a member suffering from either breast or ovarian cancer is quite high (Claus, Risch, & Thompson, 1993). These studies therefore support the notion that ovarian cancer is part of a number of cancers that can be dubbed "hereditary cancer syndromes," where mutations in specific genes are passed on from one family member to another and can lead to cancer.

Hereditary Cancer Syndromes

Approximately 5-10% of all cancers are due to hereditary cancer syndromes, where individuals in the family may pass on mutations in specific genes (Banks, Moline, Marvin, Newlin, & Vogel, 2013). One important example for inherited cancer syndrome is hereditary breast and ovarian cancer due to mutations in the *BRCA1* and *BRCA2* genes (Banks et al., 2013; Garber & Offit, 2005). Men and women with inherited mutations in either of these genes have increased risks of ovarian and breast cancer; the estimated risk for breast cancer and mutations in *BRCA1* is between 50% to 80% and in *BRCA2* is between 40% and 70%, while the lifetime risk for ovarian cancer with *BRCA1* mutations is about 40%. With mutations in *BRCA2* it is about 20% (Garber & Offit, 2005). Mutations in *BRCA2* can also lead to higher incidences of prostate cancer, pancreatic cancer, and melanoma (Garber & Offit, 2005). These genes are not the only ones that contribute to a hereditary cancer syndrome.

Just like the *BRCA* genes have an influence on developing hereditary breast and ovarian cancer, other genes can be involved in predisposition to cancer syndromes. One such syndrome, hereditary non-polyposis colorectal cancer (HNPCC) or Lynch

syndrome, also increases the lifetime risk of ovarian and other cancers (Hunn & Rodriguez, 2012; Pennington & Swisher, 2012). Lynch syndrome is an autosomal dominant disorder, which can increase the risk of developing colon cancer, endometrial cancer, gastric cancer, skin, and nervous system cancers (Hunn & Rodriguez, 2012; Pennington & Swisher, 2012). In women it most often increases the risk of ovarian cancer by 12% and patients are usually diagnosed at an early age (Pennington & Swisher, 2012). According to Watson et al. (2001), the mean age of 80 cases with HNPCC that developed ovarian cancer was 42 years of age and, interestingly, they are more often of epithelial histology, quite different from those found in *BRCA1/2* mutation carriers. The genes that are affected in people with Lynch syndrome are *MLH1, MSH2, MSH6, PMS2* and *EPCAM*. The first four genes are involved in the mismatch repair system, which is tasked with repairing errors that occur during DNA replication (Pennington & Swisher, 2012). About 70% of families with Lynch syndrome have mutations in *MSH2* or *MLH1*, with the rest of families having mutations in *PMS2* or *MSH6* (Pennington & Swisher, 2012). This syndrome is an example of the important role DNA repair mechanisms can have on predisposing to cancer.

There are a number of other hereditary cancer syndromes that feature mutations in different genes. Families with these syndromes can benefit from genetic screening to identify their risk of cancer, and physicians can be aided by genetic screening to help them diagnose these syndromes. Examples of such hereditary cancer syndromes include Cowden syndrome, Li-Fraumeni syndrome, Ataxia telangiectasia, Puetz-Juergens syndrome, and several others. A table of common hereditary cancer syndromes adapted

from Garber and Offit (2005), with permission of the American Society of Clinical Oncology, License 3673710531158, can be found in Appendix A. Briefly, Cowden syndrome can lead to breast cancer, thyroid cancer, and endometrial cancer among others and is associated with mutations in the *PTEN* gene (Garber & Offit, 2005). Li-Fraumeni syndrome is associated with soft tissue sarcoma, brain tumors, leukemia, and breast cancer. It is most often correlated with mutations in *TP53*, *BRCA2,* and *CHEK2* (Garber & Offit, 2005). Ataxia Telangiectasia is a syndrome that can result in leukemia and lymphoma and the gene identified to have mutations in these cases is *ATM* (Garber & Offit, 2005). There are a number of other hereditary cancer syndromes that support the framework for this study in that there is a genetic component to cancer.

Conceptual Framework

The first known example of a hereditary cancer syndrome where mutations in a gene were correlated with cancer susceptibility was hereditary retinoblastoma. Retinoblastoma is a rare pediatric primary malignant tumor of the eye and accounts for about 1% of cancers in children (Knudson, 1997). In hereditary retinoblastoma, the tumors are usually bilateral and if caught early eyesight can be saved in affected children. Knudson (1971), observed that about 40% of cases occurred in younger children who often reported a family history of the disease. He suggested that the mutations were to be found on a gene now known as *RB1,* for retinoblastoma (Knudson, 1971). Once the gene was cloned, it was identified that people affected with the disease had germline mutations in *RB1* and many had additional *RB1* mutations present in the tumor (Knudson, 1997). Many people were tested for the presence of mutations in the *RB1* gene and treatment

was undertaken so as to preserve vision. His findings on retinoblastoma led him to derive the two hit model for cancer causation and presented a paradigm shift in the theory that mutations in genes can lead to cancer.

The conceptual framework this research is based on is Alfred G. Knudson's two mutation theory of cancer causation, which was developed during his work on retinoblastoma (Knudson, 1971). This theory postulates that there are two types of cancers, either somatic or hereditary (Knudson, 2002; Ormiston, 1996). In somatic cancers, or those that arise spontaneously, the mutations occur later in life after some of the many mitotic divisions introduce errors into the genetic material of cells (Ormiston, 1996). He believed that two mutations would lead to cancer causation and that both mutations would be a consequence of errors during cell cycle divisions (Knudson, 1971, 2002; Ormiston, 1996). In hereditary cancers, one of the mutations occurs at the time of fertilization and so every cell in the new fetus will have this mutation. These are referred to as germline mutations. Once one mutation is present, it is easy for another one to occur somatically during cell division, leading to cancer (Knudson, 1971, 2002; Ormiston, 1996). The presence of a germline mutation and a somatic mutation represent the two hits Knudson refers to in his theory.

Most cancers are sporadic and occur due to the influence of environmental factors (Ormiston, 1996). But hereditary cancers have become an important factor in current cancer syndromes. It is important to identify individuals who may harbor germline mutations so that prophylaxis can be implemented for the second mutation to possibly be prevented.

The two-mutation theory of cancer causation is a valid conceptual framework for this study. Many ovarian cancer cases arise from germline mutations in genes such as *BRCA1, BRCA2, BRIP1, RAD51C, RAD51D,* and so on (Pennington & Swisher, 2012). Many of the patients identified with these mutations are diagnosed with ovarian cancer at younger ages than their sporadic counterparts and have tumors at different sites (Ormiston, 1996). Many women with germline mutations who develop ovarian cancer have a high likelihood of developing tumors in breasts, the pancreas, and other sites, or develop recurrent tumors (Pennington & Swisher, 2012). Additionally, when analyzing the tumors for somatic mutations, many tumors appear to have mutations in other genes such as *TP53*, a tumor suppressor gene (Greenblatt, Chappuis, Bond, Hamel, & Foulkes, 2001; Pennington et al., 2013b). Many ovarian tumors have mutations in the same gene as their germline mutations and they have been found to have loss of heterozygosity (LOH) where the wildtype allele is no longer present and only two mutant alleles are left (Walsh et al., 2011; Pennington et al., 2013b). This conceptual framework is the basis for this search for mutations in *CHEK1* and *CHEK2* in ovarian cancer cases since possible germline mutations in these activators of DNA damage responses could represent the first hit of two for cancer causation.

BRCA1* and *BRCA2

In the 1990s, two genes were sequenced and identified as breast cancer susceptibility genes: *BRCA1* in 1990 located on chromosome 17q and *BRCA2* in 1994 located on chromosome 13q (Ormiston, 1996). These two genes were further identified as tumor suppressor genes. It was determined that mutations in these genes were responsible

for about 5% of all yearly breast cancer cases in the United States and for approximately two thirds of breast cancer cases with a family history of the disease (O'Donovan & Livingston, 2010; Ormiston, 1996). Additional studies have shown that these genes have not only been associated with breast cancer but also ovarian cancer. It is thought that women with mutations in these genes have an increased life time risk of acquiring ovarian cancer;40% risk for *BRCA1* mutation carriers and 20% increased risk for *BRCA2* (Ormiston, 1996). *BRCA1* and *BRCA2* are responsible for most of the cases of hereditary ovarian carcinoma (Pennington & Swisher, 2012) and according to Jervis et al. (2015), current estimates for the burden of *BRCA1* and *BRCA2* mutations in ovarian cancer patients is 27%.

In a study by Pal et al. (2005), the authors performed genetic testing on 209 women with ovarian cancer and found that 32 women (15.3%) had mutations in either *BRCA1* (20) or *BRCA2* (12). Most of the women with *BRCA1* or *BRCA2* mutations had previous family history of ovarian or breast cancer, but a small percentage had no known family history, suggesting another mechanism for mutation.

Another study by Zhang et al. (2011) showed a relatively high frequency of *BRCA1* and *BRCA2* mutations similar to those found by Pal et al. (2005). The researchers screened 1342 women with invasive ovarian cancer for *BRCA1* and *BRCA2* mutations. They found that 176 of the samples had mutations in *BRCA1* and *BRCA2,* with 107 women harboring mutations in *BRCA1,* 67 with mutations in *BRCA2,* and 2 women with mutations in both genes (Zhang et al., 2011). The researchers also suggested that different populations vary in susceptibility to mutations in these genes, and the proportions of

hereditary ovarian cancer depend on the presence of founder mutations in these genes in people of a specific ancestry (Zhang et al., 2011). They showed a very high prevalence of mutations in these two genes in women of Ashkenazi Jewish descent.

The three specific founder mutations in the Ashkenazi Jewish population that are associated with breast and ovarian cancer are: *BRCA1.187del AG, BRCA1.5382insC,* and *BRCA2.6174delT,* and were previously reported by Roa et al. (1996). These studies indicate that the prevalence of mutations in these two genes account for a large number of ovarian cancer cases and that ancestry is a factor given the hereditary nature of these cancers.

Additionally, besides elevated risk of disease, there are other characteristics within the disease that are affected by mutations in these genes such as survival, sensitivity to chemotherapy, and the clinical phenotype of the carcinoma. In a review of ovarian tumors from 178 *BRCA1* mutation carriers and 29 *BRCA2* mutation carriers compared to 235 controls, researchers found that the predominant histology for *BRCA1/2* mutation carriers was high grade serous adenocarcinomas, which are most often associated with poorer outcomes (Lakhani et al., 2004). They also found higher solid components, which is correlated with necrosis, and also strong staining for P53 protein, a mediator of apoptosis (Lakhani et al., 2004). Overall they found that these characteristics were a hallmark of *BRCA1* and *BRCA2* mutation carriers and resulted in poorer prognoses.

On the other hand, despite the poorer prognosis when treated early and identified early, the overall survival of *BRCA1/2* mutation carriers is longer than in non-carriers

because they have better responses to platinum-based chemotherapy and their tumors are more sensitive to poly-ADP-ribose polymerase (PARP) inhibitors (Yang, Khan, Sun, & et al., 2011). Yang et al. (2011) found a higher number of cases who had higher chemotherapy sensitivity rates (100% for mutation carriers and 80% for wildtype) and higher progression-free, 5-year survival rates (61% vs 25%) than wildtype cases (Yang et al., 2011). A study by Pennington et al. (2013a) concluded that women with germline mutations in *BRCA1* and *BRCA2* as well as other homologous repair pathway genes had improved survival and response to platinum-based chemotherapy. These data show better outcomes for mutation carriers.

Many *BRCA1/2* mutation carriers can undergo risk-reducing salpingo-oophorectomy (RRSO) which has proven to reduce mortality in high risk women and has become the standard of care for women with high risk mutations. A study by Domchek et al. (2010), found that in women who underwent RRSO compared to women who did not, the all-cause mortality was reduced from 10% to 3%, the breast cancer specific mortality was reduced from 6% to 2%, and the ovarian cancer specific mortality was reduced from 3% to 0.4%. Most recently a study by the Gynecologic Oncology Group (GOG) from a trial in which women with *BRCA1* and *BRCA2* mutations underwent RRSO, found that occult cancers were present in 2.6% of high risk women (Sherman et al., 2014). This emphasizes the importance of knowing your mutation status to pursue risk reduction efforts. These studies, and the contribution of *BRCA1* and *BRCA2* mutations to ovarian cancer, highlight the strong genetic component and familial risk of this disease as well as the opportunities that genetic testing may give patients and their families.

Age at Diagnosis

Researchers have demonstrated that ovarian carcinoma diagnosis in mutation carriers occurs at a younger age than those who are not mutation carriers. Pal et al. (2005) found that mutation carriers present with disease at a younger age than wildtype cases. The findings of Pal et al. (2005) regarding age reiterated the results of a previous study by Risch et al. (2001). In that study, researchers studied *BRCA1* and *BRCA2* mutation rates in 515 unselected cases and found a mutation rate of 11.7% (Risch et al., 2001). For those women with *BRCA1* mutations, 80% were younger than 50 at time of diagnosis (Risch et al., 2001). For *BRCA2* only, about 40% of the women with mutations were younger than 50 at time of diagnosis (Risch et al., 2001). These studies suggest women are younger when diagnosed with the disease if they are *BRCA1* mutation carriers.

Age at diagnosis was also a distinction found when researching specific populations such as the Ashkenazi Jewish population. Boyd et al., (2000) reported that women of Jewish origin with ovarian cancer who participated in a retrospective cohort for *BRCA1/2* mutations were significantly younger than women without mutations. The authors suggested that age at diagnosis could be a predictor for carrying a mutation in the aforementioned genes.

More recently Alsop et al. (2012) supported Boyd's suggestion with their population-based study to determine *BRCA1/2* mutation frequencies in ovarian carcinoma patients. They too found that age of ovarian cancer onset is a strong predictor for carrying a *BRCA1* or *BRCA2* mutation. In this study, 22.2% women who carried a mutation in either gene were 50 years or younger, while only 12.1% were older than 50 (Alsop et al.,

2012). These results add to the evidence that mutation carriers are usually younger when they are diagnosed with the disease.

These findings not only apply to mutations in *BRCA1* and *BRCA2*. In a very recent study by Cunningham et al. (2014), the authors looked at the rate of germline mutations, somatic mutations, and methylation status in *BRCA1* and *RAD51C*. Hypermethylation of the promoter region genes resulted in abrogation of their expression in a similar manner as a loss of function mutation would result in a lack of expression of a functional gene. These alterations result in a HR deficient (HRD) phenotype, where the DNA repair by HR is defective. Those women who had an HRD phenotype were of younger age at time of diagnosis than those without abnormality, demonstrating that defects in HR repair can result in disease earlier in life.

In addition, women with mutations in mismatch repair genes are generally younger when they develop ovarian cancer than women without mutations. Pal et al. (2012) presented their population-based study to determine the frequency of mutations in mismatch repair genes. Among other findings, they identified a difference in age at diagnosis of ovarian cancer in three subsets: women with clearly pathogenic mutations, those with mutations that are predicted to be pathogenic, and those with no mutations in these genes. The average age of diagnosis of women with pathogenic mutations was of 47.1 years, the age for those with predicted pathogenic mutations was of 53.2 years, and age of diagnosis for those women with no mutations was 56.1 years (Pal et al., 2012). Since they identified a range of age at time of diagnosis between 40 and 59 years, they

recommended that women with mutations and susceptible to HNPCC associated ovarian cancer should undergo prophylactic salpingo oophorectomy prior to the age of 40.

Overall, these results show that mutation carriers are more likely to be younger at age of diagnosis regardless of what gene and that determining if mutations in *CHEK1* and *CHEK2* are associated with younger age can lead to recommendations for testing at earlier ages rather than later ages.

Fanconi Anemia-BRCA Pathway and Ovarian Cancer

Fanconi anemia (FA) is a rare inherited disorder that can lead to bone marrow failure, developmental abnormalities, and childhood cancers such as leukemia (D'Andrea & Grompe, 2003). It is characterized by increased chromosomal breakage in the presence of DNA interstrand crosslinking reagents such as diepoxybutane (DEB) (D'Andrea & Grompe, 2003). Research in the field of FA identified 14 genes that may be responsible in the inheritance of this disease, and the characteristic of increased chromosomal breakage, increased sensitivity to interstrand crosslinking reagents, and susceptibility to cancers suggested that the proteins encoded by these genes may be involved in DNA repair (Mathew, 2006). Overall, there are 13 genes that have been identified as being part of the FA pathway that are involved in the disease; among them is the Breast Cancer Susceptibility gene *BRCA2* (D'Andrea & Grompe, 2003; Mathew, 2006). In fact, the first big correlation between DNA repair and the FA proteins came when the gene known as *FANCD1* was identified to be identical to the tumor suppressor gene *BRCA2* (Howlett et al., 2002). Since that study *FANCD1* is mostly referred to as *BRCA2* and hardly ever as *FANCD1* (Mathew, 2006). This also led to renaming the pathway from FA pathway to

FA-BRCA pathway to emphasize the relationship between Fanconi anemia and breast cancer.

In 2002, scientists in Dr. D'Andrea's laboratory were able to show that cells derived from patients with two different types of FA groups, FA-B and FA-D1, had biallelic mutations in *BRCA2,* which led to shortened BRCA2 proteins and faulty DNA repair as determined by Mytomycin C (MMC) sensitivity (Howlett et al., 2002). They complemented the FA-D1 cells with an unmutated copy of *BRCA2* and were able to restore the wild-type phenotype in those cells (Howlett et al., 2002). Additionally they were able to determine that the *BRCA2* mutations they found in those cells were present in the patients from which the cells originated and also segregated well within the family of the patient leading to the conclusion that *BRCA2* was responsible for the FA in these patients (Howlett et al., 2002). Howlett et al. (2002) therefore established that biallelic mutations in *BRCA2* cause FA and monoallelic mutations can lead to breast and ovarian cancer.

Because biallelic mutations had not been found in *BRCA1* that would lead to FA, it is not considered a FA gene, but it is thought to be a very important player within the FA-BRCA pathway (D'Andrea & Grompe, 2003). This pathway is involved in DNA repair through HR, one of the main mechanisms to repair double stranded breaks (DSB) in DNA with very few errors (D'Andrea & Grompe, 2003; Mathew, 2006). When DNA becomes damaged, the proteins ATR and ATM kinases activate the FA core proteins to form a complex of 8 proteins which then monoubiquitinates FANCD2 and FANCI (D'Andrea & Grompe, 2003; Mathew, 2006). This complex is then recruited to the site of

DNA damage and recruits 3 other FA proteins (FANCD1, aka BRCA2, FANCN, and FANCJ). These proteins then form a complex with BRCA1 and interact with a number of proteins that have been identified as DNA repair associates and repair the damaged DNA via HR (Mathew, 2006). In HR, the DNA is repaired by aligning homologous sequences of DNA (D'Andrea & Grompe, 2003). The figure in Appendix B shows a schematic of the FA-BRCA pathway and the genes associated with this pathway in DNA repair. More recently it has been shown that the FA pathway interacts with other DNA repair pathways since there is formation of complexes with numerous other proteins such as NBN, BLM, and ATR (Thompson, Hinz, Yamada, & Jones, 2005).

As mentioned previously, *BRCA1* was not considered a FA pathway gene per se, since no biallelic mutations in the gene were found in any FA patients. But a recent case report by Domcheck et al. (2013), identified a woman with ovarian cancer who had biallelic mutations in *BRCA1*. The mutations were a known deleterious mutation, *BRCA1.c2457delC*, and a variant of unknown significance (VUS), *BRCA1.V1736A*, which the authors suggested to be deleterious (Domchek et al., 2013). They showed that this suspected VUS resulted in a BRCA1 protein with a mutated BRCT (BRCA1 C-terminus) domain, which had a lower affinity of binding to DNA damage response associated proteins and reduced localization to damaged DNA (Domchek et al., 2013). This patient was found to have a FA-like syndrome in addition to ovarian cancer, but passed away before the researchers could do specific FA testing (Domchek et al., 2013). These findings show the importance of *BRCA1* in the FA pathway and also that biallelic

mutations could lead to a genetic syndrome that encompasses hereditary ovarian cancer with developmental defects and FA like symptoms.

A number of genes other than *BRCA1* and *BRCA2* involved in the FA-BRCA pathway or are known associates of this pathway have been associated with increased susceptibility to breast cancer and ovarian cancer. *ATM* encodes for a protein kinase, involved in the DNA damage response, and it activates checkpoint signaling in response to damage. Just like for *BRCA2* and FA, people with biallelic mutations in *ATM* suffer from ataxia telangiectasia, a disease characterized by ataxias in the brain, immunodeficiency, and an increased risk of leukemia and lymphoma (Pennington & Swisher, 2012). In addition, these patients are also at increased risk of ovarian, breast, and gastric cancers (Pennington & Swisher, 2012).

Most recently, a group led by Dr. Rahman found that *ATM* was a breast cancer susceptibility gene in people who carry one mutation in the gene (Renwick et al., 2006). They sequenced 443 familial breast cancer cases and 521 controls and they found 2.04% of cases carrying deleterious mutations that could result in premature shortening of the protein or in exon skipping (Renwick et al., 2006). The rate of *ATM* mutations in controls was only 0.4% (Renwick et al., 2006). This provided strong evidence for a role of *ATM* in breast cancer.

Other genes identified as breast cancer susceptibility genes include *CHEK2, BARD1, MRE11A, NBN, RAD50, RAD51C, BRIP1,* and *PALB2* (Pennington & Swisher, 2012). It was thought that only breast cancer would be affected by mutations in non *BRCA* genes and that increased risk of ovarian cancer was only due to mutation in the

two *BRCA* genes. But this notion has changed and several other genes in the DNA damage response pathway or associated with it have been identified as increasing the susceptibility to ovarian cancer.

PALB2 was recently identified as harboring mutations in ovarian cancer patients. This gene is also known as *FANCN* and is an important contributor of the FA pathway. A study by Casadei et al. (2011), which initially just investigated breast cancer cases, showed that in 18 families of the 33 studied, family members with ovarian cancer had *PALB2* mutations. They found that people with a *PALB2* mutation had a 1.3 fold higher chance of having a relative with ovarian cancer (Casadei et al., 2011). While the number was not statistically significant due to the small sample size, it still showed that *PALB2* could be a susceptibility gene for ovarian cancer.

Further support for *PALB2* as an ovarian cancer susceptibility gene was provided by Dansonka-Mieszkowska et al. (2010)in Poland, who found that truncating mutations in *PALB2* were present 7 times more often in ovarian cancer cases (2/339) than in controls (1/1310). These studies present a strong case for *PALB2* mutations as possible hits for ovarian cancer.

Another FA main complex gene, *BRIP1* (also known as *FANCJ*), was also identified as a susceptibility gene for ovarian cancer. Rafnar et al. (2011) performed two studies in two different populations. They started with a whole genome sequencing project in Iceland, where they assessed 457 Icelanders (Rafnar et al., 2011). They found a rare mutation in *BRIP1, c.2040_2041insTT*, that was associated with an increased risk of ovarian cancer, with an OR = 8.13 (Rafnar et al., 2011). They performed further research

and sequenced *BRIP1* in a Spanish cohort of 144 cases and 1,780 controls (Rafnar et al., 2011). They found another rare frameshift in *BRIP1* (*BRIP.c1702_1703del*) in two of the cases and in only one control, resulting in an OR = 25 (Rafnar et al., 2011). These results suggest that *BRIP1* can play a role in susceptibility to ovarian cancer.

More recently, *RAD51C* and *RAD51D*, two important genes in DNA damage repair and HR, have been implicated in contributing to ovarian cancer. Meindl et al. (2010), initially described a link between *RAD51C* and ovarian cancer; they found mutations in *RAD51C* in 6 families out of 480 that had breast and ovarian cancer and were screened for that gene, while in other 620 families with only breast cancer and 2,912 healthy controls there were no mutations in *RAD51C* (Meindl et al., 2010). This made for a strong point for this gene's role in ovarian cancer.

In a Finnish study where they screened breast and/or ovarian cancer patients and families for *RAD51C*, they found that mutations in *RAD51C* were most often associated with "an increased risk of familial breast and ovarian cancer (OR 13.59, 95% CI 1.89–97.6, P =0.026 compared with controls), but especially with familial ovarian cancer in the absence of breast cancer (OR 213, 95% CI 25.6–1769, P = 0.0002) and also with unselected ovarian cancer (OR 6.31, 95% CI 1.15–34.6, P = 0.033), with a significantly higher mutation rate among the familial cases (two out of eight, 25%) than the unselected ovarian cancer cases (4 out of 409, 1%) (OR 33.8, 95% CI 5.15–221, P = 0.005)" (Pelttari et al., 2011, p. 3278). They also found that all ovarian cancer cases occurred in those who were less than 60 years old, suggesting that ovarian cancer cases with mutations in this gene are younger than patients with ovarian cancer that do not have

mutations (Pelttari et al., 2011). These results presented further evidence for *RAD51C*'s involvement in ovarian cancer.

Loveday et al. (2012) also reported a study in which they sequenced *RAD51C* in 272 ovarian cancer cases and 1,156 population controls and found that about 1% of ovarian cancer cases harbored germline mutations in that gene. In addition they also found that ovarian cancer cases with mutations in RAD51C were diagnosed at younger ages, one of the patients as young as 43 (Loveday et al., 2012). *RAD51C* is currently considered a gene for ovarian cancer susceptibility.

RAD51D was also implicated as an ovarian cancer gene by the same group that investigated the relationship of ovarian cancer with *RAD51C*. Loveday and colleagues (2011) investigated *RAD51D*, another paralog for *RAD51*, prior to their findings with *RAD51C*. They found 8 damaging mutations in 911 individuals from breast and ovarian cancer families, and only 1 in 1,060 controls (Loveday et al., 2011). They found the association to be stronger in ovarian cancer since 3 of the mutations were found in the 59 families that had 3 or more individuals with ovarian cancer most of which were under 60 years of age (Loveday et al., 2011). The study led by Loveday (2011) established a relative risk for ovarian cancer for those people with *RAD51D* mutations to be 6.3, while for breast cancer the relative risk was only 1.32, leading them to the conclusion that *RAD51D* is predominantly an ovarian cancer risk gene.

Another study led by Wickramanyake, found that out of 360 ovarian, fallopian tube, and peritoneal cancer patients, three carried loss of function (LOF) mutations in *RAD51D* (Wickramanyake et al., 2012). When the researchers sequenced 449 women and

10 men with breast cancer for *RAD51D* mutations, they were not able to find any, leading to the conclusion that *RAD51D* is a gene that, if mutated, can confer risk for ovarian cancer (Wickramanyake et al., 2012).

Taken together, this research shows that *BRCA1* and *BRCA2* are not the only genes conferring risk for ovarian cancer but other genes involved within the FA-BRCA pathway or associated with it or with DNA repair can confer risk for ovarian cancer. Jervis et al. (2015) reported that approximately 10% of ovarian cancer cases can be attributed to rare variants in other genes such as the MMR genes, *RAD51C*, *RAD51D*, and *BRIP1*. In addition, like it is the case for people with mutations in *BRCA1* and *BRCA2,* the age of diagnosis for ovarian cancer cases with mutations in some FA genes are younger than patients without mutations (Loveday et al, 2012, Norquist et al., 2013, Pelttari et al., 2011). Continued research in genes associated with DNA repair is of great importance to determine their possible association with ovarian cancer.

CHEK1 **and** ***CHEK2***

The following discussion will include mention of genes *CHEK1* and *CHEK2* and the proteins CHEK1 and CHEK2. Per HGNC (Human Genome OrganizationGene Nomenclature Committee) guidelines, the gene names will be italicized and protein names will be set in the standard font (Gray, Gordon, Seal, Wright & Bruford, 2013).

The genes *CHEK1* and *CHEK2* encode the proteins CHEK1 and CHEK2, two serine threonine kinases that are required for cell cycle arrest in response to DNA damage and modulate the activation of different DNA repair pathways (National Center for Biotechnology Information , 2014a, 2014b; Reinhardt & Yaffe, 2009). They are

structurally unrelated but perform similar functions. These kinases receive signals from two other kinases, ATM and ATR, which are activated in response to DNA damage (Reinhardt & Yaffe, 2009). It is thought that for the most part the protein ATM activates CHEK2 in response to double-stranded breaks (DBS) and ATR activates CHEK1 in response to single stranded breaks (Reinhardt & Yaffe, 2009), but ATM can also interact with CHEK1, and CHEK1 can also be involved in DBS repairs. The activation of these checkpoint kinases represents a DNA surveillance program, which ensures that there is faithful transmission of the DNA and that the integrity of the DNA is conserved during different cell cycles (Reinhardt & Yaffe, 2009). These checkpoint kinases prevent the progression of damaged DNA further into the cell cycle and even activate apoptosis or programed cell death if the damage is beyond repair (Reinhardt & Yaffe, 2009).

CHEK2

CHEK2 is activated by ATM, which phosphorylates CHEK2 at threonine residue 68 (Dai & Grant, 2010). Upon phosphorylation by ATM, CHEK2 homodimerizes and finally fully activates by the "trans-phosphorylation" of two of its threonine residues, 383 and 387 (Dai & Grant, 2010). It then phosphorylates the protein Cdc25C and confines it to the cytoplasm where it cannot activate other proteins to move ahead through the different phases of mitosis, in this case from M (mitotic) phase to G2 (interphase second gap) phase (Stolz, Ertych, & Bastians, 2011). Hence, CHEK2 leads to cell cycle arrest prior to G2. It is also able to promote cell cycle arrest at G1 (interphase first gap) phase by interacting with *p53*, a tumor suppressor gene (NCBI, 2014b). It also interacts with p53 to initiate apoptosis when the DNA is beyond repair (Stolz et al., 2011). CHEK2 also

phosphorylates BRCA1 on serine residue 988, which in turn allows BRCA1 to become soluble and proceed to become involved in the FA-BRCA pathway mediated HR repair (Stolz et al., 2011).

The implication of *CHEK2* as a cancer susceptibility gene has been reported by several groups especially in the field of breast cancer research. Several specific alleles have been associated with specific populations and with specific cancers. In a study by Cybulski et al. (2004), the authors reported an analysis they performed on 4,000 controls and 4,008 cancer cases, for which many different cancer sites were represented (Cybulski et al., 2004). They looked for three specific founder alleles for *CHEK2*: *CHEK2.1100delC, CHEK2.IVS2+1G->A* and *CHEK2.I157T* (Cybulski et al., 2004). They established positive associations with thyroid (OR = 4.9), breast (OR = 2.2), and prostate cancer (OR = 2.2) and the two truncating alleles (*CHEK2.1100delC* and *CHEK2.IVS2+1G->A*) (Cybulski et al., 2004). For the specific missense, they found strong associations for increased risks of breast (OR = 1.4), colon (OR = 2.0), kidney (OR = 2.1), prostrate (OR = 1.7), and thyroid cancer (OR = 1.9) (Cybulski et al., 2004). The odds ratio for that specific allele in ovarian cancer was 1 (Cybulski et al., 2004), which doesn't support a possible connection to ovarian cancer. The authors believe that this gene is a multiorgan susceptibility gene, but because they only tested three alleles their study is not robust in regard to a possible association to ovarian cancer.

Further research from this group analyzed the presence of these alleles in Polish ovarian cancer patients compared to controls. What they found was that the same missense, *CHEK2.I157T*, resulted in a positive association with ovarian cancer patients

that had low grade invasive tumors (OR = 2.5) (Szymanska-Pasternak et al., 2006). They followed up with a small sample of Russian patients and had similar findings, (OR = 2.7), for that specific missense in patients with borderline ovarian tumors, which are non-invasive ovarian tumors (Szymanska-Pasternak et al., 2006). This study shows a small association between *CHEK2* and borderline ovarian tumors but they only focused on one specific mutation in the gene as opposed to looking at mutations within the whole gene.

One particular allele that has been reproducibly associated with breast cancer is *CHEK2.1100delC*. It was positively associated with breast cancer in the study by Cybulski et al. (2004). It was also the focus of a meta-analysis performed by a group in Denmark that reviewed the literature to identify 26,000 patients and 27,000 controls analyzed for this allele. They found that there was an "aggregated odds ratios of 2.7 (95% CI, 2.1 to 3.4) for unselected breast cancer, 2.6 (95% CI, 1.3 to 5.5) for early-onset breast cancer, and 4.8 (95% CI, 3.3 to 7.2) for familial breast cancer" (Weischer, Bojesen, Ellervik, Tybjærg-Hansen, & Nordestgaard, 2008, p.542). This led them to calculate a cumulative risk for breast cancer for people with this mutation to be 37%, with a 95% CI of 26% to 56% (Weischer et al., 2008). Furthermore, individuals who carry this specific mutation, *CHEK2.1100delC*, are at higher risk for bilateral breast cancer and male breast cancer (Mellemkjær et al., 2008). A similar risk for ovarian cancer has not been calculated.

There are other alleles that have been found to confer susceptibility to breast cancer, such as a *CHEK2.S428F* variant found to increase breast cancer risk by 2 fold in women of Ashkenazi Jewish descent, two other variants in women of Italian descent, and

one new variant in a high risk breast cancer family from France (Escudie et al., 2010; Manoukian et al., 2011; Shaag et al., 2005). One study analyzed whether the variant *CHEK2.1100delC* was associated with ovarian cancer in 486 cases and 323 controls, but found no correlation, possibly because they only looked at this specific mutation and not at the entire gene (Baysal et al., 2004). Based on the evidence from these previous studies, it is important to find out if *CHEK2* is an ovarian cancer susceptibility gene like it is for breast cancer.

Ovarian cancer susceptibility has not been clearly established, but there have been reports of rare variants in *CHEK2* in ovarian cancer patients. In a Next-Generation sequencing study, Minion et al. (2015), sequenced 19 genes in women with a personal history of breast cancer (353 women), ovarian cancer (466 women), and breast and ovarian cancer (92 women). Mutations in *CHEK2* were found in 7% of women with a history of ovarian cancer and in 5% of women with both a history of ovarian and breast cancer (Minion et al, 2015). The authors only reported rates and did not compare cases versus controls. This study supports the necessity to clearly determine whether there is an association between *CHEK2* mutations and ovarian cancer.

Of especial interest is research by Ow et al. (2014), who performed analysis on clinical data from The Cancer Genome Atlas (TCGA), and found that in patients diagnosed with High Grade Serous Ovarian Cancer (HG-SOC) with *CHEK2* mutations, the survival prognosis was poor. These mutations may be associated with resistance to existing chemotherapy (Ow et al., 2014). This research contrasts the Pennington et al. (2013a) research that in 3 out of 367 women with ovarian cancer with mutations in

CHEK2, there was an association with improved survival and response to platinum-based chemotherapy in women with mutations in homologous repair (HR) genes (Pennington et al., 2013a). These differences may be related to the grade and histology of the ovarian cancer, but it certainly supports the notion that in order to appropriately manage treatment for ovarian cancer it is important to know if mutations in *CHEK2* are present.

CHEK2 is activated by ATM and interacts directly with BRCA1. It is an integral part of activating the DNA damage repair pathway and therefore the lack of a functioning CHEK2 protein due to mutations in the gene would result in a lagging DNA damage response. The research in this study may lead to the analysis of the entire gene in ovarian cancer patients, rather than just looking at specific mutations as in previous published papers. Such a global view of possible candidate variants would give a clearer picture of whether *CHEK2* is truly an ovarian cancer susceptibility gene or not.

CHEK1

The protein CHEK1 is activated by ATR and to a lesser extent by ATM (Dai & Grant, 2010). ATR phosphorylates CHEK1 at either the serine residue 317 or 345, activating CHEK1 and allowing it to phosphorylate Cdc25A/C, leading to cell cycle arrest in S or G2 phases of the cell cycle (Dai & Grant, 2010). CHEK1 plays a dominant role in replication initiation during S phase and is an amplifier of the DSB response signaling mediated by ATM and CHEK2 (Dai & Grant, 2010). CHEK1 is also an important protein in the delaying of anaphase in cells with spindle defects, and during G2/M phase it helps with stabilization and proteasomal degradation (Dai & Grant, 2010). In addition, during DNA damage and repair, CHEK1 targets kinases important for DNA

repair. CHEK1 dependent phosphorylation of RAD51 induces HR and its phosphorylation of FANCE (one of the FA proteins) is also critical for FA-BRCA mediated repair (Dai & Grant, 2010).

In order to show that the protein CHEK1, encoded by *CHEK1*, is required for HR repair, Sorensen et al. (2005), were able to inhibit the function of the CHEK1 protein in Chinese Hamster cells (CHO cells), by way of using small interfering RNAs that inhibit the translation of *CHEK1* message into protein. They then induced replication associated Double Strand Breaks (DSB), which normally initiates HR repair, by adding hydroxyurea and camptothecin to the media (Sorensen et al., 2005). They determined the survival of cells after DNA damage and replication arrest. They saw that cells proficient in HR repair had poor survival when CHEK1 was inhibited and they also found and increase in DSB (Sorensen et al., 2005). They were also able to show that CHEK1 interacts with RAD51 in order to induce HR repair. Cells where the threonine residue 309 was mutated on RAD51, the site of CHEK1 phosphorylation, had increased sensitivity to hydroxyurea due to the inability to initiate HR repair (Sorensen et al., 2005). This paper highlights the importance of CHEK1 protein in the pathway for DNA repair, because cell survivability was reduced and there was an increase in double strand breaks when CHEK1 function was abrogated.

Another study by Guervilly et al. (2008) showed that inhibition of CHEK1 can lead to reduced monubiquitination of FANCD2, an important step within the FA-BRCA damage repair pathway (Guervilly, Macé-Aimé, & Rosselli, 2008). They demonstrated that mitomycin C (a DNA crosslinker) sensitivity was reduced in cells where the

ATR/CHEK1 activation was inhibited, suggestive of a central role of CHEK1 in the arrest of the G2 cell cycle (Guervilly et al., 2008). These functions of the protein encoded by the gene *CHEK1* show the gene's importance within the DNA repair pathways and that any mutations could lead to a shortened protein with loss of function.

Finally, there have also been suggestions that the protein BRCA1, one of the important modulators of the FA-BRCA pathway, interacts with CHEK1 protein. A report by Yarden et al. (2002), showed that activation of CHEK1 in response to ionizing radiation was only possible when the BRCA1 protein was being expressed by measuring CHEK1 kinase activity in cells that were expressing BRCA1 or not. They were also able to show that BRCA1 interactions with CHEK1 affect G2/M cell cycle arrest by showing that cells expressing BRCA1 and CHEK1 proteins move from G2 to M after radiation damage, compared to cells that have these proteins inhibited (Yarden, Pardo-Reoyo, Sgagias, Cowan, & Brody, 2002). Their results suggested that BRCA1 involvement in cell cycle arrest is mediated by its interaction with CHEK1 (Yarden et al., 2002). The research above shows clearly the importance of CHEK1 to the DNA damage response in cells; cells lacking a proper functioning CHEK1 protein are deficient in fixing double strand breaks and don't survive well those insults. Clearly CHEK1 plays an important role in damage repair and interacts with proteins in the FA-BRCA pathway, therefore *CHEK1* gene is a good candidate as a cancer susceptibility gene, like some of the members involved in that pathway such as *ATM*, *RAD51D*, *RAD51C*, *PALB2*, and *BRIP1* (see Appendix B), many of which interact directly with *BRCA1* as *CHEK1* does.

There is little evidence to associate *CHEK1* with cancer because not too many studies have looked at *CHEK1* specifically. A few studies have used genome wide association studies (GWAS) where they looked at common single nucleotide polymorphisms (SNPs) over a span of several genes including *CHEK1* in breast cancer cases but these were inconclusive (Haiman et al., 2008; Pooley et al., 2008). Because GWAS only identify common SNPs and not the whole gene, they cannot detect rare mutations or deletions that are potentially associated with disease.

Another study by Lin et al. (2013) also investigated common SNPs in *ATR* and *CHEK1* in breast cancer patients and found that these common alleles were not implicated in conferring risk to breast cancer, but since they looked at only common alleles they could not determine whether rare alleles could be involved (Lin et al., 2013). This underscores the need for an approach that targets the whole gene rather than common SNPs that are found in many people.

A Finnish study investigating national breast cancer families took the approach of looking at large genomic rearrangements in the genes *BRIP1* and *CHEK1* and were unable to uncover any large insertions or deletions (Solyom, Pylkäs, & Winqvist, 2010). The lack of findings could be due to the fact that they had a small cohort of only 111 cases.

These reports are suggestive of a low chance of the *CHEK1* gene being a possible cancer associated gene at least in breast cancer, but there is some evidence that mutations in this gene are present at least in colorectal and endometrial cancers. Researchers in Italy performed a small study in which they analyzed colon cancers and endometrial cancers

for the presence of *CHEK1* mutations and found frameshift mutations in 1 out of 10 colon cancers and in 2 of 17 endometrial cancers (Bertoni et al., 1999). They did not go any further than that and unfortunately did not analyze whether these mutations were also present in the germline or whether they were only somatic mutations. Yet this data presents initial evidence for a possible implication of *CHEK1* in cancer.

Pennington et al. (2013a) reported that they found a germline mutation in *CHEK1* in 1 of 367 subjects, in addition to 87 other mutations in HR genes tested by Next-Generation sequencing. They did not compare the rates to controls and therefore where not able to associate this rare variant in *CHEK1* with ovarian cancer within their cohort (Pennington et al, 2013a). Women with mutations in HR genes had significantly better survival than those without mutations in and had better response to platinum-based chemotherapy (Pennington et al., 2013a). Therefore, establishing whether mutations in *CHEK1* are associated with ovarian cancer still remains to be elucidated. Knowing whether mutations are present in *CHEK1* or any of the HR genes can not only inform for risk reduction but also help manage therapy.

Kumar et al. (2013), performed a "Boolean logic framework" to rank genes for association with ovarian cancer (Kumar, Breen, & Ranganathan, 2013). They mined the literature and relied on functional characteristics of other cancer susceptibility genes and *CHEK1* is among the genes they found to possibly have an important role in ovarian cancer (Kumar et al., 2013).

Most recent studies have looked at CHEK1 protein inhibitors as potential adjuvants and single agents for cancer chemotherapy (Kim, Min, Wright, Goldlust &

Annunziata, 2014; Kim, James & Annunziata, 2015). The addition of CHEK1 protein inhibitor increases the response of *BRCA1/2* or *TP53* mutation positive ovarian carcinoma cells to chemotherapeutic compounds, such as Topotecan and others (Kim, Min, Wright, Goldlust & Annunziata, 2014; Kim, James & Annunziata, 2015). Results like this have led to the implementation of a phase 2 clinical trial to determine the effects of CHEK1 protein inhibitors in women with ovarian cancer (Kim, James & Annunziata, 2015). These results add to the question of the role of genetic mutations in *CHEK1* and the development of ovarian cancer. A non-functioning CHEK1 protein, resulting from a mutation in the *CHEK1* gene could be protective if indeed CHEK1 protein inhibitors result in a better response to chemotherapeutic agents. A better understanding of the association between a mutated *CHEK1* and ovarian cancer, whether positive or negative, can not only have implications for reduction of disease risk but also management of chemotherapy during disease. Further research with a whole gene approach to determine whether *CHEK1* is an ovarian cancer susceptibility gene still needs to be undertaken. The research proposed will elucidate the effect of mutations in *CHEK1* (with a whole gene approach) on ovarian cancer.

Next-Generation Sequencing and BROCA

Most of the studies that have identified susceptibility genes to date were done by Sanger sequencing specific genes or by identifying susceptibility loci by genome wide association studies (Manoukian et al., 2011; Walsh et al., 2006). But with the advent of new, highly efficient technologies, the trend has shifted toward next-generation sequencing approaches involving targeted capture and massively parallel sequencing

approaches of several genes at once (Walsh et al., 2011). These techniques allow the users to sequence a large number of different genes at once in a large cohort of people in a very quick time period (Walsh et al., 2011). It also allows for a lower cost method that enables people to get a deep coverage within the genome and accurate mutation calls.

Walsh et al. (2010), developed a genomic capture and massively parallel sequencing approach, they called BROCA, that allowed them to sequence 21 genes in tandem in 20 women that were diagnosed with breast and ovarian cancer and who had previously been identified as mutation carriers by Sanger sequencing (Walsh et al., 2010). The genomic DNA is hybridized to capture oligonucleotides that span the genes or regions of interest and then sequenced on a next-generation sequence analyzer (Walsh et al., 2010). They were able to find all mutations that were present in the samples and demonstrate that this method can be applied to comprehensively test for mutations in several genes and several patients at once (Walsh et al., 2010).

In a follow up study, the group used BROCA to analyze germline DNA from 360 ovarian cancer cases and sequenced 21 genes concurrently (Walsh et al., 2011). They found that about 24% of these ovarian cancer patients had mutations in different genes (Walsh et al., 2011). The mutations identified were in *BRCA1* and *BRCA2*, about 18% of cases had mutations in these two genes (Walsh et al., 2011). But, interestingly, 6% of cases carried mutations in other genes including *BARD1, BRIP1, CHEK2, MRE11A, MSH6, NBN, PALB2, RAD50, RAD51C,* and *TP53* (Walsh et al., 2011). While *BRIP1, PALB2, MSH6,* and *RAD51C* had previously been associated as risk conferring genes for

ovarian cancer, the other 6 had not (Walsh et al., 2011). Most of these genes are involved in the DNA damage response.

Most recently Harrell et al. (2013) reported at the 63rd annual Meeting of The American Society of Human Genetics in Boston that researchers in Dr. Elizabeth Swisher's lab at the University of Washington expanded the study initially undertaken by Walsh et al. in 2011. The BROCA gene panel was extended to include 52 genes, some within the FA-BRCA pathway or associated genes and genes in related damage response pathways (Harrell et al., 2013). The researchers tested 1,418 cases with ovarian, fallopian tube, or peritoneal carcinoma. They found that 15.7% of patients carried mutations in *BRCA1* and *BRCA2*, 10.3% in *BRCA1*, and 5.4% in *BRCA2*. 6.3% of cases were harboring mutations in other DNA repair genes which are known or suspected to cause ovarian cancer (Harrell et al., 2013). Overall, they found 313 mutations in 1,418 individuals, which represented 22% of the cohort (Harrell et al., 2013). *BRCA1* and *BRCA2* accounted for about 73% of all mutations while 27% of mutations were found in other genes (Harrell et al., 2013). The next most commonly mutated gene was *BRIP1* with 6% of mutations, followed by *RAD51C, RAD51D*, *PALB2*, and *ATM*, each gene harboring almost 3% of mutations within the cohort (Harrell et al., 2013). Mutations in the Lynch syndrome genes (*MSH6, MSH2, MLH1, PMS2*), all taken together accounted for 1.6% of mutations (Harrell et al., 2013). There were also a number of truncating mutations in some genes that had not been previously associated with breast or ovarian cancer, which may be good candidates for association with ovarian cancer. These included *ATR* and *RBBP8,* also known as *CTIP* (Harrell et al., 2013). These genes are

attractive candidates for ovarian cancer susceptibility because of their central roles within the FA-BRCA pathway and the minimal number of loss of function mutations reported in public databases (Harrell et al., 2013).

This research was also supported by a recent study by Minion et al., (2015) where they performed next-generation sequencing on 19 genes on DNA from ovarian cancer patients, breast cancer patients, and patients with a history of both. Their findings looked beyond *BRCA1* and *BRCA2* at rates in genes that have previously been identified as ovarian and breast cancer susceptibility genes such as *BRIP1* (15%), *NBN* (6%), *PALB2* (6%), *BARD1* (3%) and others (Minion et al., 2015). This research brings to light how novel sequencing techniques can aid in researching novel cancer susceptibility genes and that identification of hereditary risk for ovarian cancer requires assessment of many genes in all cases, which can be done effectively with cancer gene panels such as BROCA. This new technology was used to assess mutations in *CHEK1* and *CHEK2* and helped identify whether these genes can also be considered candidate genes for ovarian cancer.

Summary

It has been shown that one of the most important risk factors for ovarian cancer is familial risk (Check, 2006; Goff, Mandel, Muntz, & Melancon, 2000; Pennington & Swisher, 2012). Certain genes will acquire mutations that will increase a person's likelihood of developing ovarian cancer (Dansonka-Mieszkowska et al., 2010; Susan M. Domchek et al., 2013; Loveday et al., 2011; C. Loveday et al., 2012; Renwick et al., 2006; Walsh et al., 2011). Many of these genes are found to be important players in DNA repair pathways and are considered tumor suppressor genes (D'Andrea, 2013; D'Andrea

& Grompe, 2003; Pennington & Swisher, 2012). Novel sequencing techniques have made it easier to identify mutations in cases and controls in many genes at once, rather than just one gene at a time (Harrell et al., 2013; Walsh et al., 2011; Walsh et al., 2010). These techniques will allow for the identification of other candidate genes that may increase the risk for ovarian cancer. *CHEK1* and *CHEK2* are two genes that, due to their function and associated with known risk genes and DNA repair pathways, are considered good candidates for ovarian cancer susceptibility (Cybulski et al., 2004; Huang et al., 2008; Kumar et al., 2013). The evidence to date for an association between these two genes and ovarian cancer is sparse and additional research elucidated their roles in cancer risk.

Chapter 3 contains information about the study design, including a description of the variables, the sample size, and data generation techniques, as well as a summary of the chapter and plans for how this research will be disseminated.

Chapter 3: Research Method

Introduction

This research investigated whether mutations in the genes *CHEK1* and *CHEK2* are associated with ovarian cancer and identifiable as candidate genes for this disease within this cohort. It also investigated whether there was an association between age at diagnosis and mutations in these genes. This chapter describes the participants in this study, the tools used to ascertain mutations in the probands and controls, as well as the statistical analysis that was used to establish a correlation between disease and mutations in each gene. Finally, it describes the ethical protections for participants and how the resulting data will be disseminated to the public.

Previous genetic studies have shown that a myriad of genes are associated with cancer when they harbor loss of function mutations (Loveday et al., 2012; O'Donovan & Livingston, 2010; Pelttari et al., 2011; Szymanska-Pasternak et al., 2006; Wickramanyake et al., 2012). There is some published research correlating *CHEK1* and *CHEK2* genes to ovarian cancer, but clear evidence has not been presented to date (Cybulski et al., 2004; Szymanska-Pasternak et al., 2006; Vahteristo et al., 2001; Walsh et al., 2011;Pennington et al., 2013a; Minion et al. 2015). This research helped elucidate the role of *CHEK1* and *CHEK2* in predisposition to cancer and whether this phenomenon corroborates Knudson's two hit theory of cancer causation. The framework of this study was based on Knudson's (2002) two-hit theory of cancer causation, which states that for cancer to occur there must be a germline mutation in a gene and then another somatic

mutation within the tumor cells. This research exposed *CHEK1* and *CHEK2* as potential first-hit mutation bearers.

This research constituted a secondary data analysis using data provided by Dr. Elizabeth Swisher at the University of Washington, Seattle, WA as well as publicly available data from the Exome Variant Server and from a paper by Kanchi et al. (2014). No original data were collected for this study. Patients of Dr. Swisher and other gynecologic oncologists at the University of Washington consented to be included in a number of different studies to support the university's gynecologic oncology tissue bank. DNA from these probands underwent BROCA, a massively parallel targeted sequencing approach. Identified mutations by Next-generation sequencing were validated by Sanger sequencing. Data for mutations in controls was also available from previous studies that were part of the Women's Health Initiative and the Exome Variant Server and acquired by whole-exome sequencing. Data from controls have also been published by Kanchi et al. (2014). The data included information on mutations identified in the cohort, which in turn allowed for ascertaining mutation rates in cases versus controls. The comparison of mutation rates helped determine whether there was an association between ovarian cancer and mutations in each gene. This research also determined whether there was any correlation between *CHEK1*or *CHEK2* mutations and ovarian cancer diagnosis as well as age at diagnosis.

Research Questions

The research questions evaluated in this study are as follows:

*H*1: Are *CHEK2* mutated alleles associated with ovarian cancer?

H_0^1: There are no *CHEK2* mutated alleles associated with ovarian cancer.

H_a^1: *CHEK2* mutated alleles are associated with ovarian cancer.

*H*2: Are *CHEK1* mutated alleles associated with ovarian cancer?

H_0^2: There are no *CHEK1* mutated alleles associated with ovarian cancer.

H_a^2: *CHEK1* mutated alleles are associated with ovarian cancer.

*H*3: Are *CHEK1* mutated alleles associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases?

H_0^3: There is no association between younger age at diagnosis and *CHEK1* mutations in ovarian cancer cases.

H_a^3: Mutated alleles in *CHEK1* are associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases.

*H*4: Are *CHEK2* mutated alleles associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases?

H_0^4: There is no association between younger age at diagnosis and *CHEK2* mutations in ovarian cancer cases.

H_a^4: Mutated alleles in *CHEK2* are associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases.

Participants

The cases included 587 prospectively enrolled women who underwent primary surgery for ovarian, fallopian tube, or peritoneal carcinoma at the University of Washington, between 1998 and 2013 and had no known familial risk for ovarian cancer

or had not undergone any genetic testing to date. All cases provided informed consent to participate in the University of Washington institutional gynecologic oncology tissue bank and related genetic study and donated about 9 ml of blood for genetic analysis. Age at diagnosis for these women ranged from 30 through 70 years of age. All cases have been analyzed using a BROCA assay for 52 genes including *CHEK1* and *CHEK2*.

Controls included 557 females older than age 50, with no personal history of breast or ovarian cancer, who gave permission for their genomic DNA to be used anonymously for research. Healthy individuals are part of the Women's Health Initiative and data on controls was previously published by Kanchi et al (2014). The age of the controls was a limitation of the dataset used for this study. The data on mutations in these controls were generated using whole exome sequencing, which expands the genes in the BROCA panel to all genes in the human genome and is also available online (Kanchi el al., 2014). All participants provided informed consent for genetic studies.

Laboratory Component

Data collection in the Swisher laboratory followed laboratory testing procedures as set forth under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) regulations and followed the general CLIA quality systems requirements for non-waived testing and the CLIA personnel requirements for tests of high complexity.

DNA Extraction

DNA from patients was obtained from blood collected at their pre-surgery appointments. About 9 milliliters (mls) of blood was placed in an Acid Citrate Dextrose (ACD) containing BD vacutainer blood collection tube (Becton, Dickinson and

Company, New Jersey) by a phlebotomist or nurse. Blood tubes were then provided to Dr. Swisher's laboratory, free of any personal identifiable information, where the DNA was extracted from blood.

DNA was extracted from blood or lymphoblast cell lines by desalting method (Walsh et al., 2011; Wickramanyake et al., 2012). The blood was centrifuged down to separate the plasma, buffy coat and Red Blood Cells (RBC). The buffy coat was then isolated and placed in RBC lysis buffer at a 1 to 3 ratio, incubated for 30 minutes, then centrifuged. The pellet was resuspended in cell lysis buffer and incubated with 20% SDS and Proteinase K (Promega) at 37^{o}C overnight. Saturated 6M NaCl was added and then the solution centrifuged. The supernatant was collected and mixed with 3 volumes of pure 100% ethanol at which point the DNA precipitated out of solution, was collected, then resuspended in Tris-EDTA solution.

Sequencing

Sequencing data for cases has been obtained from samples by undergoing BROCA, a massively parallel sequencing approach, which allows for the sequencing of multiple genes. To prepare the DNA for BROCA, 3ug of DNA, paired-end libraries with 150 base pair inserts were prepared and hybridized to a custom pool of oligonucleotides targeting 52 exomic regions (Walsh et al., 2011), using SureSelectXT^{TM} (Agilent, Santa Clara, CA) enrichment system on a Bravo liquid handling instrument (Agilent, Santa Clara, CA). Following capture, samples were barcoded with 96 different indexed primers, pooled 96 per lane, and sequenced on a 2500HiSeq (Illumina, San Diego, CA) (Walsh et al., 2011). Sequence alignment and variant calling were done against the reference human

genome (UCSC hg19) (Walsh et al., 2010). All suspected deleterious mutations were verified by Sanger sequencing. Polymerase chain reaction (PCR) was performed using specific PCR primers to amplify the region where the mutations were found. PCR amplicons were sequenced bidirectionally using the Applied Biosystems BigDye Terminator v3.1 sequencing kit (Applied Biosystems, and analyzed on an ABI 3130xl genetic analyzer (Wickramanyake et al., 2012). Trace sequences were analyzed using Sequencher 4.9 software. Information on validated mutations and which samples have the mutation will be provided to me in the form of an excel database. Controls were sequenced by whole exome sequencing as previously described (Kanchi et al., 2014).

Research Approach

Mutations

The data provided for this study was in the form of an excel spreadsheet that contained the code for the patient, age at diagnosis, which mutation a certain patient was found to have (*CHEK1* or *CHEK2*, if any), the coordinates of the mutation in the genome as well as in the coding sequence and effect in the coding region or protein, and whether this is a frameshift, a premature truncation (e.g. a base pair change results in a stop codon), a splice site variation or a copy number variation, or a missense. For example, patient X, with age at diagnosis Y, may have a *CHEK2.c1100delC*, which is a frameshift deletion, which in turn results in a premature stop codon and with it a loss of a functional protein. The BROCA approach identifies many types of mutations. Specific mutation identification was a part of this study for any mutations within the cases or controls. This

research only included clear loss of function mutations: frameshifts, splice site variations, premature truncations, and copy number variations.

Missenses in *CHEK2* only were counted if there was clear evidence of loss of function of the protein. This was determined via literature review and whether functional studies had been performed to determine if that mutation results in a loss of gene function. Several *CHEK2* missense mutations have already been identified in breast cancer (Le Calvez-Kelm et al, 2011; Roeb, Higgings, & King, 2012; Shaag et al., 2005). Roeb et al. (2012) published a seminal paper where functional studies have been done to identify whether known missense mutations led to loss of function. Therefore, the missenses reported as damaging in previously published studies and the Roeb et al. (2012) study were included as clearly damaging mutations if present. Appendix C includes a list of *CHEK2* missenses that were considered as loss of function mutations based on the literature. *CHEK1* missense mutations were not included since none have been reported in the literature to date.

Statistical Analysis

Data for mutations in *CHEK1* and *CHEK2* included how many mutations there were in this data set and which specific mutations were identified. Cancer status was set as the dependent variable. Independent variables included *CHEK1* and *CHEK2* variants and age at diagnosis.

In order to determine the strength of the association in this study, a power analysis was performed with PS Power and Sample Size Calculations, a freely available program on the web (Dupont & Plummer, 1998). For this study with 587 cases and 557 controls,

and prior research supporting a 0.5 probability of *CHEK2* mutations among controls (Cybulski et al.,2004), a true odds ratio for mutations of 0.704 or 1.420 in cases relative to controls with probability (power) of 0.8 should be obtainable. The type I error probability associated with an OR=1 for this hypothesis testing was 0.05 and was based on using χ^2-squared statistic or Fisher's exact test to evaluate this null hypothesis. This calculation was limited to *CHEK2* since previous research has already been conducted by other researchers to estimate percentage of mutations in controls. No such research is available for *CHEK1* and so power was not predicted prior to the analysis for *CHEK1*. I also performed post-hoc power analysis to determine the level of power for my analysis. This was calculated using PS Power and Sample Size Calculations as above and also using the website ClinCalc.com and using their Post-hoc power calculator (http://clincalc.com/Stats/Power.aspx).

Initial analysis determined how many mutations were identified in the cohort. I compared the proportions of prevalence of all the alleles in *CHEK1* and *CHEK2* in cases versus controls. Each gene was analyzed separately. Any mutations that resulted in a truncated protein, which may include frameshifts, copy number variations, splice alterations and stop gains, and functionally tested missenses in *CHEK2* (see Appendix C), were counted as a damaging mutation for each gene. Then, the sum total of damaging mutations was compared between cases and controls, for each gene independently, to answer the research questions as to whether *CHEK1* is associated with disease or whether *CHEK2* is associated with disease. Odds ratios for mutations in either *CHEK1* and/or *CHEK2* were generated using two by two tables and statistical significance was

determined between cases and controls using Fisher's exact test. Odds ratios were calculated at 95% confidence intervals. Overall, this analysis investigated whether there was a correlation between variants in these genes and disease, and presents evidence as to whether CHEK1 and/or *CHEK2* can be nominated as candidate genes for ovarian cancer.

Age at Time of Diagnosis

Age at diagnosis was asked for each study participant. For the purposes of this research, age was analyzed within cases in ten-year increments: under 40, 40-49, 50-59, 60-69, 70-79, and 80 and over. This is supported in similar studies where the age variable was grouped using the same increments (Shaag et al., 2005; Walsh et al., 2011) comparing non-mutation versus mutation cases. Since controls do not have an age of diagnosis, due to their cancer free status, it is not feasible to compare their age at time of diagnosis with cases. Therefore, for this question I determined whether any mutation found in either of the genes under investigation correlated with age at diagnosis. Age at diagnosis in mutation carriers versus non-mutation cases for each gene was listed in columns and statistical significance was determined by *t* test.

Protection of Human Participants

Cases provided informed consent for genetic analysis to participate in the institutional gynecologic oncology tissue bank as approved by the human subjects division of the institutional review board of the University of Washington (University of Washington Protocol 34173). Data from controls was from Kanchi et al., 2014 (Lic#3695490410900). No original data was collected on either cases or controls. No personal identifiers are connected with any of the existing data I obtained from cases and

controls. This study obtained approval and sought out protection from Walden University's Institutional Review Board, IRB approval #12-09-14-0059711.

Dissemination of Findings

Findings from this study will be presented at professional conferences and submitted for publication to a peer-reviewed journal.

Summary

This study is a case-control quantitative study that aimed to determine whether mutations in *CHEK1* and *CHEK2*, two genes involved in promoting DNA repair, are associated with development of ovarian cancer and whether age at diagnosis is different for those people with mutations in these genes.

Chapter 4 presents the results for this study.

Chapter 4: Results

Introduction

The purpose of this study was to compare the rates of mutations in *CHEK1* and *CHEK2* for ovarian cancer cases to healthy controls.If mutations were identified in the cases and/or controls, the plan was to determine whether there was an association between mutations in these genes and ovarian cancer. The plan also sought to establish whether age at diagnosis was lower in cases with mutations than in those without mutations, since it has previously been shown that women with mutations have a lower age of diagnosis than those without mutations (Boyd et al., 2000; Rish et al., 2001; Pal et al., 2005; Alsop et al., 2012).

This chapter introduces descriptive statistics for the study population and provides inferential statistics for each research hypothesis. It concludes with a summary and interpretation of the data for each hypothesis.

Study Population

Cases included in this study (n = 587) were women with fallopian tube, primary peritoneal, and/or ovarian cancer whose DNA were sequenced by Dr. Swisher's laboratory cases. Controls (n = 557) were healthy women with detailed sequencing information . The total population size for this study was N = 1144.

Descriptive Statistics

Demographic information available for this cohort included sequencing information as well as gene and chromosomal coordinates. More specifically for controls, there was also age at time of enrollment. More detailed information was available for

cases, which included age at diagnosis and type of cancer patient was diagnosed with, and whether it was primary peritoneal, fallopian tube, or ovarian cancer. The following table summarizes the age at diagnosis for all cases and type of cancer they were diagnosed with. Table 2 shows the age of controls at time of enrollment. All are above 50 years of age and assumed to be cancer free at time of enrollment. No other demographic information was available for this study.

Table 1

Age at Diagnosis and Cancer Site for Cases

	Number of cases (%)
Age at diagnosis	
<40	31 (5.3)
40-49	84 (14.3)
50-59	153 (26.1)
60-69	172 (29.3)
70-79	81 (13.8)
80 and up	29 (4.9)
No age available	37 (6.3)
Mean age	59.45
Cancer site	
Fallopian tube	93 (15.9)
Primary peritoneal	57 (9.7)
Ovarian	437 (74.4)

Table 2

Age at Enrollment for Controls

Age at baseline	Number of controls (%)
<40	0
40-49	0
50-59	209 (37.5)
60-69	206 (36.9)
70-79	142 (25.4)
80 and up	0
Mean age	63.3

Mutation Status Summary

Sequencing for mutations in a large number of genes was performed for cases and controls, and all available information was provided for this study. The genes that were queried and where mutations were found were *ATM*, *ATR*, *BARD1*, *BRCA1*, *BRCA2*, *BRIP1*, *CHEK1*, *CHEK2*, *FAM175A*, *MSH2*, *MSH6*, *NBN*, *PALB2*, *RAD50*, *RAD51C*, and *RAD51D*. Deleterious loss of function mutations were found in 141 cases, which represented 24.02% of all cases. Deleterious loss of function mutations were found in 23 controls, which represented 4.28% of all controls. Fisher's exact test was used to determine the association between cases, controls, and mutations. There was statistical significance with mutations being more strongly associated with cases than controls ($p < 0.0001$). The odds ratio for this association was found to be OR = 8.5 (95% CI = 5.3 to 13.8). Table 3 shows the distribution of mutations in cases and controls and in which genes these mutations were found. Of those cases with mutations, 24 (17%) had been

diagnosed with fallopian tube carcinoma, 103 (73%) with ovarian carcinoma, and 14 (10%) with primary peritoneal carcinoma.

Table 3

Distribution of Mutations in Cases and Controls

Genes in which mutations were identified	Cases	Controls	
ATM	3	1	
ATR	1	1	
BARD1	1	0	
BRCA1	67	6	
BRCA2	32	4	
BRIP1	6	1	
CHEK1	2	1	
CHEK2	7	2	
FAM175A	1	2	
MSH2	1	2	
MSH6	2	2	
NBN	3	0	
PALB2	4	0	
RAD50	1	1	
RAD51C	6	0	
RAD51D	4	0	
Total individuals with mutations	141	23	$p<0.0001$ (Fisher's exact test)
No mutations	446	534	OR=8.5 (95%CI=5.2-13.8)

There were 2 cases and 1 control with *CHEK1* mutations as well as 7 cases and 2 controls with *CHEK2* mutations. The description of the *CHEK1* and *CHEK2* mutations are listed in Table 4 (*CHEK2* mutations) and Table 6 (*CHEK1* mutations). All cases with *CHEK1* and *CHEK2* mutations were diagnosed with Stage 3 ovarian cancer, rather than primary peritoneal or fallopian tube carcinoma.

Analysis of Hypothesis 1

All analyses were completed using Graphpad PRISM software v6.05 (San Diego, CA). The following hypothesis was analyzed:

*H*1: Are *CHEK2* mutated alleles associated with ovarian cancer?

H_0^1: There are no *CHEK2* mutated alleles associated with ovarian cancer.

H_a^1: *CHEK2* mutated alleles are associated with ovarian cancer.

The following Table 4 lists the *CHEK2* case and control mutations, which is written to describe the base pair in the cDNA coordinate that has been altered. This is standard nomenclature for reporting mutations. Also included in the table are information of type of mutation, effect at the protein level, and chromosomal coordinates, e.g. where they are located in the genome. For instance *CHEK2.c1100delC* refers to the deletion of base C (cytosine) at the 1100 base pair of the cDNA sequence. This mutation is located at chromosome 22, position 29091857. The effect at the protein level is a stop at codon 381. Finally, the type refers to whether it is a deletion, a missense, an insertion, or a nonsense. In the case of *CHEK2.c1100delC* it is a deletion.

The effect at protein level for missenses (if identified as a damaging alteration) is based on functional test and previous reports (Shaag et al., 2005; Le Calvez-Kelm et al, 2011; Roeb, Higgings, & King, 2012). For a list of damaging missense in *CHEK2*, see Appendix C. All mutations result in a truncated protein that will result in a loss of function.

Table 4

CHEK2 Mutations in Cases and Controls

CHEK2 mutations	Genomic coordinates	Effect at protein level	Type
Cases			
CHEK2.c1100delC	chr22:29091857	381 stop	deletion
CHEK2.c1100delC	chr22:29091857	381 stop	deletion
CHEK2.c1100delC	chr22:29091857	381 stop	deletion
CHEK2.c758_761delACTG	chr22:29107931	252 stop	deletion
CHEK2.c428A>G	chr22:29121247	H143R,damaging alteration	missense
CHEK2.c1283C>T	chr22:29091207	S428F, damaging alteration	missense
CHEK2.c1283C>T	chr22:29091207	S428F, damaging alteration	missense
Controls			
CHEK2.c1229delG	chr22:29091857	367 stop	deletion
CHEK2.c499G>A	chr22:29121057	G167R, damaging alteration	missense

For this hypothesis, *CHEK2* mutation rates were first established. For the cases, the *CHEK2* mutation rate was 1.1%, whereas for controls it was 0.35%. In order to test the hypothesis and determine an association between mutation carrier and cancer status, a contingency table was built (Table 5) with the number of mutations in *CHEK2* in cases and controls and those participants without mutations. There were seven cases found to have deleterious *CHEK2* mutations as outlined in Table 4, and two controls with *CHEK2*

mutations. This is in contrast to 446 cases and 537 controls that did not have any mutations, either in the *CHEK* genes or any other genes queried by the next-generation sequencing approach.

Table 5

Contingency Table for CHEK2 Mutations

Data analyzed	Cases	Controls	Total
CHEK2	7	2	9
No mutations	446	535	981
Total	453	537	990

The resulting odds ratio was 4.191 (95% CI = 0.87 to 20.28). The statistical significance as calculated by a Fisher's exact test is p = 0.0884. Based on these results, an individual with *CHEK2* mutations has a 4-fold higher likelihood of developing ovarian cancer, but the association is weak since this analysis is not statistically significant at the 0.05 level. The confidence interval for this odds ratio crosses 1, and therefore the association is not significant at the 0.05 level. Therefore, the alternative hypothesis is rejected.

I performed a post-hoc power analysis and found the power to be at 30.1%, which indicated that the alternative hypothesis was rejected 70% of the time. In this case, I reject the alternative hypothesis.

Analysis of Hypothesis 2

The following hypothesis was analyzed:

*H*2: Are *CHEK1* mutated alleles associated with ovarian cancer?

H_0^2: There are no *CHEK1* mutated alleles associated with ovarian cancer.

H_a^2: *CHEK1* mutated alleles are associated with ovarian cancer.

The table that follows indicates *CHEK1* mutations identified, their location, and effect as explained above for Table 4. All of these mutations are loss of function mutations that result in a truncated protein.

Table 6

CHEK1 Mutation in Cases and Controls

CHEK1 mutations	genomic coordinates	effect at protein level	type
Cases			
CHEK1.c1036C>T	chr11:125,513,598	Q346 stop	stop gained
CHEK1.c1036C>T	chr11:125,513,598	Q346 stop	stop gained
Controls			
CHEK1.c1044_1045delAT	chr11:125,513,598	C349fs	deletion

The rate of mutations for *CHEK1* in cases was 0.34% and in controls was 0.18%. Table 7 shows the contingency table used to calculate the odds ratio and p-value for this analysis. *CHEK1* mutations were found in 2 cases and 1 control, whereas 446 cases and 535 controls had no mutations in either *CHEK1*, *CHEK2*, or any other genes queried.

Table 7

Contingency Table for CHEK1

Data analyzed	Cases	Controls	Total
CHEK1	2	1	3
No mutations	446	534	980
Total	448	535	983

The odds ratio for the likelihood of developing ovarian cancer if there is a *CHEK1* mutation present is OR = 2.4 (95% CI = 0.22 to 26.66). The statistical significance as calculated by a Fisher's exact test is p = 0.59, which indicates that this association is not significant. Moreover the confidence interval crosses 1, therefore the association is not significant at the 0.05 level. I also performed a post-hoc power analysis for this question, and found the power to be 7.7%. Therefore, the alternative hypothesis was rejected.

Analysis of Hypothesis 3

Literature indicates that ovarian cancer cases with mutations are diagnosed with the disease at a younger age (Boyd et al., 2000; Rish et al, 2001; Pal et al., 2005; Alsop et al., 2012). When comparing the mean age at diagnosis between those cases with mutations and those without mutations, age is lower for those with mutations (54) than those without (61) [Table 8]. This difference is statistically significant at $p < 0.0001$. The following hypothesis was analyzed:

*H*3: Are *CHEK1* mutated alleles associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases?

H_0^3: There is no association between younger age at diagnosis and *CHEK1* mutations in ovarian cancer cases.

H_a^3: Mutated alleles in *CHEK1* are associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases.

Table 8 shows age for all cases with mutations and without mutations. The first column indicates age at diagnosis. There was no age at time of diagnosis available for 29 wildtype cases and 8 mutation carrier cases, indicated by NA. The second column

indicates the number of cases with no mutations for each age group. The third column shows number of cases with mutations in any of the genes queried for each age group. Genes queried were: *ATM*, *ATR*, *BARD1*, *BRCA1*, *BRCA2*, *BRIP1*, *CHEK1*, *CHEK2*, *FAM175A*, *MSH2*, *MSH6*, *NBN*, *PALB2*, *RAD50*, *RAD51C*, and *RAD51D*. The fourth column shows *CHEK1* mutation carriers at time of diagnosis in cases whereas the fifth column shows *CHEK2* mutation carriers at time of diagnosis. The ages at diagnosis for those with *CHEK1* mutations carriers were 42 and 43. The ages at diagnosis for *CHEK2* mutations carriers were 31, 40, 45, 59, 60, and 68. There was no age at time of diagnosis reported for one of the *CHEK2* mutation carriers. The final column shows the number of cases with either *CHEK1* or *CHEK2* mutations.

Table 8

Age at Time of Diagnosis for all Mutation carriers, CHEK1 only, and CHEK2 only Mutation Carriers Versus non Carriers Among Cases

Age group at time of diagnosis	No mutations	Cases with a mutation in any of genes queried	*CHEK1* only	*CHEK2* only	*CHEK1* and *CHEK2* combined
less than 40	21	10	0	1	1
40-49	52	32	2	2	4
50-59	106	47	0	1	1
60-69	138	34	0	2	2
70-79	72	9	0	0	0
80+	28	1	0	0	0
NA	29	8	0	1	1

Table 9 shows the median age for all cases without mutations, the median age for all cases with mutations, and the p-value comparing median age for cases with mutations versus no mutations. It also shows the median age for *CHEK1* and *CHEK2* mutations determined at diagnosis and the corresponding p-values versus cases without any mutations. Because *CHEK1* and *CHEK2* genes encode for protein kinases with similar function, I also combined the age at time of diagnosis for all *CHEK1* and *CHEK2* mutations carriers, see Table 8, last column, and determined the median age as well as the p-value versus non mutation carriers, as shown on Table 9, last row.

Table 9

Average Median Age and p-Values for Age at Time of Diagnosis in Cases With and Without Mutations

	Median Age	p-value vs. cases with no mutations
No mutations	61	
All mutation carriers	54	<0.0001
CHEK1	42.5	0.0398
CHEK2	50.5	0.0456
CHEK1 and *CHEK2*	48.5	0.0061

In order to determine whether ovarian cancer patients with mutations in *CHEK1* are diagnosed at a younger age than those with no mutations, an unpaired *t* test was performed between the ages of the 2 mutation carriers and the ages of the 417 that did not carry any mutation. A contingency table and χ^2 square analysis was not possible since more than 20% of values were below 5 and there were many values of 0 for *CHEK1* mutation carriers. The unpaired *t* test resulted in a p-value of $p = 0.04$ ($p < 0.05$, 95% CI

= 0.8617-35.96), indicating that there is statistical significance between the ages of the cases with *CHEK1* mutations and those with no mutations. The mean age for cases with *CHEK1* mutations was 42.5 versus 61 for cases without mutations. Therefore, the alternative hypothesis is accepted and the null hypothesis is rejected. I performed a post-hoc power analysis for this question and obtained a power of 82.9%.

Analysis of Hypothesis 4

The following hypothesis was analyzed:

*H*4: Are *CHEK2* mutated alleles associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases?

H_0^4: There is no association between younger age at diagnosis and *CHEK2* mutations in ovarian cancer cases.

H_a^4: Mutated alleles in *CHEK2* are associated with younger age (<60 years of age) at diagnosis in ovarian cancer cases.

Table 8 shows ages for those cases with *CHEK2* mutations compared to those without mutations. Once again an unpaired *t* test was performed to determine statistical significance. Table 9 shows the median age as well as the p-value obtained when comparing the age at diagnosis of *CHEK2* mutations carriers versus those cases with no mutations. This test indicated that the difference between the two groups was statistically significant at a 95% confidence level, with $p = 0.045$ ($p < 0.05$, 95% CI = 0.203-20.61). The mean age for those cases with *CHEK2* mutations was 50.5, while the mean age for those without mutations was 61. Therefore, the alternative hypothesis is accepted and the

null hypothesis is rejected. The post-hoc power calculated for this analysis was at 99%, which allows me to reject the null hypothesis.

In addition to determining the individual significance of the mutations carrier's age at diagnosis for each of the *CHEK* genes, I sought to determine whether mutation carriers in either of both genes combined have a lower age at time of diagnosis than those cases without mutations. There were nine cases with either *CHEK1* or *CHEK2* mutations, but for one of the *CHEK2* mutations carriers there was no age at diagnosis available. The median age for all *CHEK1/2* mutation carriers was 48.5 and the p-value = 0.0061 ($p < 0.05$, 95% CI = 3.564-21.25) (Table 9). Combined, the association of younger age at diagnosis and harboring a mutation is lower than in each gene individually. The post-hoc power analysis provided a power of 100%, showing that there will not be a type II error (known also as a false negative).

Summary

There is no clear association between *CHEK1* and *CHEK2* mutations and ovarian cancer within this cohort. Yet the data supports an association between age at diagnosis and *CHEK1* and *CHEK2* mutations. There is a clear association between diagnosis at a younger age (less than 60) when harboring a mutation in *CHEK1* and *CHEK2* compared to those cases without any mutations found.

Chapter 5 presents an overview and summary of the research, limitations of the study, an interpretation of the findings, implications for social change, recommendations for action, and suggestions for further studies.

Chapter 5: Discussion

Research Overview

Ovarian cancer is the most deadly gynecological cancer, and it is among the top five causes of cancer-related death in American women (Surveillance, Epidemiology and End Results, 2012). Survival rates for ovarian cancer patients are low since most women are diagnosed at advanced stages of the disease (Goff, Mandel, Muntz & Melancon, 2000; Weissman, Weiss, & Newlin, 2012). When detected early, though, there is an excellent chance for survival, but current methods of detection are ineffective (Goff et al, 2000). Among the most important risk factors for ovarian cancer is family history; 25% of all new ovarian cancer cases are due to hereditary breast and ovarian cancer from mutations in cancer-associated genes (Pennington & Swisher, 2012). The genes that are most often affected are genes involved in DNA repair pathways (Pennington & Swisher, 2012).

Two very important genes have been identified as contributors to ovarian cancer: *BRCA1* and *BRCA2* (Check, 2006). Mutations in *BRCA1* contribute to about 48% of cases with inherited ovarian cancer, while *BRCA2* accounts for about 27% (Pennington & Swisher, 2012). Other genes in the DNA repair pathways account for about 25% (Pennington & Swisher, 2012). Yet there are many women with inherited ovarian cancer where no mutations are identified in the known cancer-associated genes. Therefore, other genes in the DNA repair pathway may harbor mutations that could be responsible for the disease .

The purpose of this study was to determine if two genes previously not identified as ovarian cancer-associated genes could be included this grouping. These two genes, *CHEK1* and *CHEK2*, which encode for proteins that function similarly, are both mediators of the DNA damage response (Reinhardt & Yaffe, 2009; NCBI, 2014a; NCBI, 2014b). *CHEK2* has previously been associated with breast cancer susceptibility (Cybulski et al., 2004; Shaag et al., 2005; Reinhardt & Yaffe, 2009). But not much is known about *CHEK1* and its association with cancer. Previous reports found *CHEK1* mutations in an ovarian cancer cohort; the authors reported the rates but did not pursue a case control study (Pennington et al. 2013a). Thus, to date, no association study has been undertaken for mutated *CHEK1* and ovarian cancer to look at cases and controls.

This study aimed to compare the rates of mutations of *CHEK1* and *CHEK2* in an ovarian cancer cohort to rates in controls. It also sought to establish whether women with mutations in these genes were diagnosed at a younger age than cases with no mutations at all. Many studies have reported that women with mutations in cancer-associated genes present with disease at a younger age (Boyd et al., 2000; Risch et al., 2001; Pal et al., 2005; Alsop et al., 2012).

In order to perform this study, sequencing data were obtained from a cohort of 1,144 women: 587 cases with ovarian cancer and 557 controls. This secondary data analysis assessed the mutations identified by next-generation sequencing techniques in known cancer genes, as well as the *CHEK1* and *CHEK2 g*enes of interest. The mutations were compared in the two cohorts and contingency tables were built to ascertain the odds ratio for association of mutation with disease. In addition, *t* tests were used to determine

whether there was an association between the age at time of diagnosis and mutation status in cancer cases. The rates of mutations in *CHEK2* and *CHEK1* were higher in cases than in controls. Once the analysis was completed, H_a^1 and H_a^2 were rejected, indicating there was no clear association between mutation in either *CHEK1* or *CHEK2* and ovarian cancer. *T* tests of age at diagnosis of cases with mutations compared to those without mutations resulted in rejecting the null hypothesis three and null hypothesis four, resulting in an association between age at time of diagnosis in *CHEK1* and *CHEK2* mutations carriers than in cases with no mutations at all.

Interpretations of the Findings

I found that the overall rate of mutation in cases was higher than in controls, with 24% of cases harboring mutations in the genes queried versus only 4.3% of controls with mutations. These results are in agreement with previous studies that have shown that ovarian cancer cases harbor mutations in genes at a rate of 25 - 30% (Walsh et al., 2011; Minion et al., 2015). I also found that those cases with mutations in any related gene analyzed were younger at diagnosis than cases that were wildtype for any mutations (Pal et al., 2012; Cunningham et al., 2014). Overall, the mutation rates and age at diagnosis for mutation found in cases all mirror previous studies.

Regarding the *CHEK1* and *CHEK2* mutation rates, they were higher in cases than controls. But despite those observations the findings of this study do not support an association between mutations in either *CHEK1* or *CHEK2* and development of ovarian cancer due to the lack of statistical significance and lack of power. It was, however, able to support the hypothesis that women with mutations in either gene would present with a

diagnosis of cancer at an earlier age than women without a mutation in either of these genes. Not only are mutations in each individual gene associated with younger age at diagnosis in cases but both genes combined make a stronger point that mutations in those genes are associated with diagnosis at a younger age.

The rate of *CHEK2* mutation in this cohort of cases was 1.1%, which was lower than previously reported (Pennington et al, 2013a; Minion et al., 2015). The odds ratio for women with cancer harboring *CHEK2* mutations versus controls, was OR = 4.191 with a 95% CI between 0.87 and 20.28. While the odds ratio is above 1 and would suggest a relationship between ovarian cancer and *CHEK2* mutations, the confidence interval crosses the null value of 1 and therefore makes this result not statistically significant and therefore I have to reject the alternative hypothesis. In addition to the confidence interval crossing the null value, a post hoc power analysis resulted in a power of 30.1%, suggesting that the possibility for a type II error was large and would deem this analysis not significant. While the statistical significance is not there, it would be inappropriate to conclude completely that there is no association and the interpretation of the OR crossing the null value would suggest that more studies are needed (Young & Lewis, 1997). These results, along with a low power, are most likely due to the small sample size in this cohort and the rarity of the variants identified. A larger sample size may result in a more statistically significant result.

CHEK2 has previously been associated with development of breast cancer, thyroid cancer, and prostate cancer (Cybulski et al., 2004). The allele that Cybulski et al. (2004) analyzed in those three type of cancers as well as in ovarian cancer is

CHEK2.1100delC, and is one of the ones found within this cohort in three cases. Their findings were also negative for an association with ovarian cancer with their odds ratio being OR=1 for that specific allele (Cybulski et al., 2004).

A second allele found among ovarian cancer cases in this dissertation study was *CHEK2.S428F* (*CHEK2.c1283C>T*), a variant found to increase breast cancer risk by two-fold in women of Ashkenazi descent, Italian descent, and a high risk breast cancer family from France (Escudie et al., 2010; Manoukian et al. 2011; Shaag et al., 2005). This allele was the second most common one found among ovarian cancer cases in this study. The lack of association in this study does not allow me to infer that these cases have an increased risk for ovarian cancer but based on other studies it suggests that these women have an increased risk for breast cancer and HBOC in addition to having suffered from ovarian cancer.

This study could not establish a statistically significant association between ovarian cancer and mutations in *CHEK2,* and the alternative hypothesis was rejected due to the fact that the OR crosses the null value and the lack of power. In addition I found an association between development of cancer at younger age when having mutations in this gene and therefore, I believe that there may be some implications for cancer development when harboring mutations in this gene. The inclusion of *CHEK2* in a panel of genes for diagnostic sequencing should not be rejected and it would still be an important asset for ovarian cancer and breast cancer pre-screening.

*CHEK1*mutations showed at a rate of 0.34% in cases and 0.18% in controls. This *CHEK1* mutation rate was lower than the only other time there was an observation of a

CHEK1 mutation in an ovarian cancer cohort (Pennington et al, 2013a). There was also no clear association between disease and mutation. The odds ratio was OR = 2.4, with 95% CI = 0.22 to 26.66. Once again while the odds ratio is higher than 1, which would indicate that there could be a positive association between disease and mutation in *CHEK1*, the confidence interval crosses the null value which makes this OR not statistically significant. I also performed a post-hoc power analysis for these events and found the power to be 7.7%, which suggests a lack of power overall. This is most likely due to the fact that the mutations were rare variants in a relatively small cohort.

Previous research on association with *CHEK1* and cancer is limited. A study by Lin et al. (2013) addressed the association between common alleles in *CHEK1* and breast cancer and found no association. A group of Finnish researchers who looked at genomic rearrangements did not find any large insertions or deletions in *CHEK1* in breast cancer patients (Solyom, Pylkas, & Winqvist, 2010). However, their sample size was also small and these large genomic rearrangements are also very rare events (Solyom, Pylkas, & Winqvist, 2010). It seems therefore that this dissertation research only adds more questions as to whether *CHEK1* could be a gene associated with ovarian cancer. The results were not statistically significant, so further studies with a larger cohort may be needed to clarify an association. Further research into the association of *CHEK1* mutation and ovarian cancer may shed more light on how protein CHEK1 inhibitors can be used for treatment (Kim, Min, Wright, Goldlust & Annunziata, 2014; Kim, James & Annunziata, 2015). This study failed to make an association and therefore did not provide any more insight into that aspect of CHEK1 physiology.

While this study rejects the alternative hypothesis due to the low power and that the OR's 95% CI spans the null value, I cannot say (based on Young and Lewis (1997)) that it provides a lack of evidence of association between mutations in these genes and disease. I see a decrease in the p-value when comparing ages in cases, which indicates that these mutations most likely have an effect on disease development. When there is an association between mutation and disease where the confidence interval is wide and not very precise, this is due to the small sample size (Young & Lewis, 1997). The same is true for the almost non-existent power in this analysis, that it is a result of a small sample size and the rarity of the variants. A larger sample size would narrow the point estimate, increase power, and clarify the results. Overall, this result indicates that the sample size is too small, yet does not rule out an association (Young & Lewis, 1997). This interpretation is supported when I compared my findings for *CHEK1* with a population database readily available on the internet. This publicly available database, the ExAC browser, offered by the Broad Institute reports on variants found in 60,706 unrelated individuals sequenced in population genetic studies (Exome Aggregation Consortium (ExAC), 2015). When I searched for variants in *CHEK1*, they reported only 44 found among 60,706. When I proceeded to calculate the odds ratio comparing my data to the ExAC data, I obtained an odds ratio of 4.7, with a 95% CI of 1.14-19.5. Just looking at the mutation found among the cases in this study, *CHEK1.Q346X*, the ExAC browser only reports 2 individuals having that variant. This comparison resulted in an odds ratio of 103.8 and a 95% CI of 14.59-738.3. My interpretation is that I cannot rule out an

association between *CHEK1* and ovarian cancer and that a larger study is needed due to the complexities of analyzing the association of disease with rare variants and mutations.

Lee, Abecasis, Boehnke & Lin, (2014), highlighted the issues that rare gene variant association studies face statistically. According to Lee et al. (2014), single variant testing to identify associations with low frequency and rare variants are difficult if samples sizes are not large enough, because the power is not quite there. This was evidenced in this study. Sample size was not high enough to provide enough power for such rare events (Walsh et al., 2011; Pennington et al., 2013a; Minion et al., 2015).

This study also found that if cases have a mutation in either *CHEK1* or *CHEK2*, the likelihood of the cancer occurring earlier is higher than in cases without any mutations. The difference in age at diagnosis between the cases harboring mutations in either gene or both genes combined is statistically significant. The average age at time of diagnosis of cases with *CHEK2* mutations from this study is about 10 years younger than those with no mutation. Cases with mutations are usually under age 60 (Risch et al., 2001). The average age at time of diagnosis of cases with *CHEK1* mutation was 42.5 in this study and was also much younger than the average for the cases without mutations, which was 61 in this study. For all instances of *CHEK1* and *CHEK2* mutation combined, the median age at time of diagnosis was 48.5 years versus 61 for those cases without mutations, as shown on Table 9, last row (p.69). This was statistically significant and post-hoc power analysis confirmed that the power was sufficient to avoid a type II error. These results are in line with previously reported studies where women with mutations are usually diagnosed with disease at younger ages than cases without mutations (Risch

et al., 2001; Pal et al., 2005; Alsop et al., 2012). In addition, when looking at all the cases with mutations in this cohort, the median age for diagnosis for women with any mutations in any of the genes was 54, statistically significantly younger ($p<0.0001$) than those with no mutations. These results suggest that for ovarian cancer patients harboring mutations in any of the genes tested, including *CHEK1* and *CHEK2*, the age that the disease will develop is lower than in those patients without any inherited mutations identified. This suggests testing people for inherited mutations in genes such as *CHEK1* and *CHEK2* as well as *BRCA1* and *BRCA2*, may allow them to find out that these alterations may predispose them to developing cancer at a younger age than people without these mutations.

In summary, the study failed to establish a clear association between mutations in the genes *CHEK1* and *CHEK2* most likely due to the small cohort investigated and the rarity of the variants. Yet the age of diagnosis of cases with mutations in either of these genes was found to be statistically significantly younger for those with mutations compared to those without mutations. These results taken together do not rule out a possible role for *CHEK1* and *CHEK2* in ovarian cancer.

Limitations of the Study

During the analysis of the secondary data available it became obvious that this study had a very big limitation. Given the rarity of the variants analyzed, the size of the cohort was too small to establish any significant association regarding the existence of mutations in *CHEK1* and *CHEK2* and ovarian cancer. While the overall rates of mutations were comparable to other studies published, the rates for *CHEK1* and *CHEK2*

were lower than previously reported. Gene sequence analysis may have missed mutations due to incorrect reporting or using the wrong variant of the gene nomenclature. Also there may have been mutations that could have been reported as germline mutations but they could have been somatic mutations of tumor circulating in the blood. If the percentage of the variant reads are low, many times it is due to circulating tumor in the blood and the sequencing will pick it up in the germline DNA. If this is not identified accurately, a somatic mutation could be interpreted as a germline mutation, and with it provide a false information. It is assumed that there was accurate reporting of the sequencing information, but this could not be verified personally as I did not have access to that more specific information and was beyond the scope of this study.

In addition, there was limited data available for the cohort in general, and this limited the analysis to sequencing data, cancer site, and age. Additional information on other cancers was not available either, which would have been helpful to evaluate whether some of these women have had breast cancer prior to their ovarian cancer. Some of these mutations have been found to be present in breast cancer patients in other studies and so it could have informed me of whether this cancer was a recurrence of cancer at another site or a primary event.

It would have been helpful to have familial information on these cancer cases to determine whether these ovarian cancers were inherited or sporadic. Having segregation data on the family as well as family history could have informed me more thoroughly of the effect of these mutations on cancer development.

This sample set represented a relatively small cohort that was enrolled within this region of the Pacific Northwest. I am unsure of how the makeup of this cohort represents the diversity of the U.S. and how these mutations were at all related to ethnicity. We know that in breast cancer certain *CHEK2* mutations are prevalent in women of Ashkenazy Jewish descent or Czechoslovakian descent (Cybulski et al, 2004; Shaag et al, 2005), and so not knowing this aspect of the patients could not allow for an association based on ethnicity or race.

One of the bigger limitations of this study that came to light in the analysis stage was cohort size. While a priori power analysis indicated that the size would be sufficient, the rate of mutations found and the rarity of the alleles resulted in an underpowered study that could not establish a clear association between disease and variants. This could be remedied by proceeding with larger studies.

Recommendations

As an approach to improve this study and to obtain statistically significant values, there is a need to obtain a larger number of cases and many more controls known to be cancer free. As Lee et al., (2014) state, rare variants can be found to have associations with disease if the effect and the sample size is large. They also suggest that research in rare variant analysis may have to evolve from current methods and "will require more methodological development" (Lee, Abecasis, Boehnke, & Lin, 2014, p. 9). In order to have larger sample sizes and more of a representative sample of the U.S. population, several institutions should join together into a multi-center study that will allow testing of many individuals that have ovarian cancer. In addition, the number of controls should be

higher than that of the cases in order to be able to reach strong predictive values for odds ratios (Young & Lewis, 1997). Organizations like the Gynecologic Oncology Group (GOG), the Ovarian Cancer Association Consortium (OCAC), and the Australian Ovarian Cancer Study (AOCS), are beginning to undertake such studies that will sequence a large number of probands and will help answer the question as to whether some candidate genes such as *CHEK1* and *CHEK2* can be upgraded to cancer-associated genes.

In the meantime, women should continue to enroll in genetic testing studies such as the one that these data were obtained from. Ovarian cancer patients and their families should continue to be tested on next-generation sequencing panels, which contain many cancer-associated genes as well as candidate genes such as *CHEK1* and *CHEK2*. This study failed to show a positive association between ovarian cancer and *CHEK1* and *CHEK2* due to the lack of power and the 95% CI crossing the null value, despite the odds ratio being above 1. This leads to no definite conclusions, but being that the odds ratios obtained are above 1 and the fact that there was an association between mutations in these genes and younger age at time of diagnosis, the results suggest the possibility that these genes may be associated with ovarian cancer. Continued testing will increase the number of individuals tested for mutations in those genes and the accumulation of results from the sequencing studies will add to the knowledge regarding these genes and others.

In addition, in order to have a better understanding as to whether these genes can be upgraded to ovarian cancer associated genes, I need to determine the segregation among affected families. Establishing whether this mutation is inherited in cancer cases

within a family is a hallmark of establishing whether a gene can be associated with the cancer in that family (Newman, Millikan, & King, 1997). This information was not available for this study and was beyond the scope of the IRB approval, but another study which provides information on familial segregation and pedigrees could be undertaken.

Further information on the cases could also provide insight into their risk for breast cancer. Several of the alleles in *CHEK2* had previously been associated with breast cancer by Cybulski (2004) and others (Mellemkjaer et al., 2008; Weischer, Bojesen, Ellervik, Tybjaerg-Hansen, & Nordestgaard, 2008). Research on whether these ovarian cancer patients have a history of breast cancer or have taken precautionary methods to prevent breast cancer or are under surveillance for developing breast cancer is needed.

Additional information can also be gleaned from functional studies to determine how mutations in these genes affect the proper functioning of the protein. If functionality of the protein is affected, this can be established experimentally in cells and other model systems, such as yeast, mammalian cells, and mice. Segregation in families, functional studies, and larger case control studies will help me determine whether candidate genes can be considered cancer associated genes.

Implications for Social Change

Recognizing potential causes for ovarian cancer is an important tool for early detection. Ovarian cancer is many times referred to as the silent killer, because many patients go undiagnosed until late in the stage of disease when treatment is often too late and survival is marginal (Goff et al, 2000). Since inherited ovarian cancer is responsible for about 25% of cases, a better knowledge of genes associated with a diagnosis would

help in identifying potential cases. Genetic testing is becoming more available and affordable for the general public. This is a helpful tool to pre-screen for potential ovarian cancer or allow for early detection. Currently researchers and many doctors know that *BRCA1*, *BRCA2*, *PALB2*, *BRIP1*, *RAD51C*, and *RAD51D* are genes that contribute to cases of inherited ovarian cancer (Pennington & Swisher, 2012). All of these genes are in the DNA repair pathway. But there are other inherited ovarian cancer cases where these genes are not mutated. Other mutated genes in this pathway may, instead, be contributing to the cancer development. Therefore research identifying these genes could provide information for family members with a strong history of familial cancers.

This knowledge would allow women to undergo increased surveillance and prophylactic efforts to prevent cancer. Most recently a paper by Easton et al. (2015), called out for the need of well-designed population- and family-based studies in populations that are highly diverse so that practitioners can provide accurate counseling of disease risks. Not only will it help patients understand their risk but will also help to inform researchers with this broad and systematic collection of data that would link clinical and epidemiological data to outcomes and risk (Easton et al., 2015). Access to such data and new level of understanding not only contributes to a positive outcome for ovarian and breast cancer patients or those at risk but also for people facing other complex inherited diseases as prostate cancer, colon cancer, etc.

Doctors who are dealing with patients with a family history of ovarian cancer need to be aware of the hereditary aspect of this disease. It is important they also understand that testing is important and to approach their patients about genetic testing so

that they can work on a prophylactic plan not only for the patients but also for the rest of the family, especially if the patient has children (American Cancer Society, 2015).

Insurance companies are coming around to paying for these services when the family history warrants a test (American Cancer Society, 2015). It is in their own interest to invest in knowledge and prophylaxis rather than having to pay for treatment. The cost of treatment for the disease is much larger than that for prophylactic approaches and risk reducing surgery than for prolonged therapy and treatments (Grann, Panageas, Whang, Antman, & Neugut, 1998).

The more genes researchers and doctors as well as patients are aware of with a role in ovarian cancer development, the more information all have in the arsenal against ovarian cancer. While this study did not establish a clear association between *CHEK1* and *CHEK2* mutations with ovarian cancer, it showed that women with mutations in these genes are diagnosed at younger age. Knowing this provides information that will help people take action earlier. Patients with known mutations can then develop a plan with their physician to undergo half yearly exams with transvaginal ultrasounds and CA-125 testing that will hopefully help with early detection (ACOG, 2002). Following this the plan can be expanded to include risk reducing surgery when the patient is ready (ACOG, 2002). These approaches that are based on knowing your family history and mutational status can lead to reduced mortality rates.

Conclusion

The aim of this study was to determine whether mutations in *CHEK1* and *CHEK2*, two genes within the DNA repair pathway, were associated with ovarian cancer

and whether mutations in these genes were associated with a younger age at diagnosis. While the odds ratios for association between ovarian cancer and mutations in both genes were above the null value of 1, the 95% confidence interval crossed the null value in both cases indicating a lack of statistical significance. This statistical outcome resulted in the alternative hypothesis being rejected. Results suggest that the sample size was too small to establish a clear association for such rare events. This, paired with the statistical significance for younger age at diagnosis in cases with mutations in *CHEK1* and *CHEK2* makes a strong point against completely dismissing a lack of association between mutations in these genes and ovarian cancer.

Larger studies with more cases and more controls and cooperation between multiple centers would be needed to further study these potential genes as candidate genes. Also, studies that include segregation in cancer families as well as functional studies into the effect of these mutations on the protein function would support conclusions gleaned from case-control studies. What is certain is that with the advent of next-Generation sequencing, the lower cost and the increasing availability of panel testing will allow patients to get diagnostic testing that may help with earlier detection or preventative measures. Such testing supports social change by allowing patients to take an active role in prevention, including prophylaxis and surveillance, and reducing mortality from ovarian cancer due to delayed detection.

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Appendix A: Syndromes of Inherited Cancer Predisposition

Syndrome (OMIM entry)	Component Tumors	Mode of Inheritance	Genes
Hereditary breast cancer syndromes			
Hereditary breast cancer and ovarian cancer syndrome (113705, 600185)	Breast cancer	Dominant	*BRCA1*
	Ovarian cancer		*BRCA2*
	Prostate cancer		
	Pancreatic cancer		
	Fanconi anemia/medulloblastoma	Recessive	*BRCA2*
Li-Fraumeni Syndrome (151623)	Soft tissue sarcoma	Dominant	*p53*
	Breast cancer		*CHEK2*
	Osteosarcoma		
	Leukemia		
	Brain tumors		
	Adrenocortical carcinoma		
Cowden Syndrome (158350)	Breast cancer	Dominant	*PTEN*
	Thyroid cancer		
	Endometrial and other cancers		
Bannayan-Riley-Ruvalcaba syndrome (153480)	Breast cancer	Dominant	*PTEN*
	Meningioma		
	Thyroid follicular cell tumors		
Ataxia telangiectasia (208900)	Leukemia	Recessive	*ATM*
	Lymphoma		
Hereditary gastrointestinal malignancies			
HNPCC, including "Lynch II" syndrome (120435, 120436, 114500, 114400)	Colon cancer	Dominant	*MLH1*
	Endometrial cancer		*MSH2*
	Ovarian cancer		*MSH6*
	Renal pelvis cancers		

	Ureteral cancers		
	Pancreatic cancer		
	Stomach and small bowel cancers		
	Hepatobiliary cancers		
Familial polyposis, including attenuated phenotype (175100)		Dominant	*APC*
Familial attenuated polyposis (175100)	Colon cancer	Dominant	*APC*
Hereditary gastric cancer (137215)	Stomach cancers	Dominant	*CDH1*
Juvenile polyposis (174900)	Gastrointestinal cancers	Dominant	*SMAD4/DPC4*
	Pancreatic cancer		*BMPR1A*
Peutz-Jeghers syndrome (175200)	Colon cancer	Dominant	*STK11*
	Small bowel cancer		
	Breast cancer		
	Ovarian cancer		
	Pancreatic cancer		
Hereditary melanoma pancreatic cancer syndrome (606719)	Pancreatic cancer	Dominant	*CDKN2A/p16*
	Melanoma		
Hereditary pancreatitis (167800)	Pancreatic cancer	Dominant	*PRSS1*
Turcot Syndrome (276300)	Colon cancer	Dominant	*APC*
	Basal cell carcinoma		*MLH1*
	Ependymoma		*PMS2*
	Medulloblastoma		
	Glioblastoma		
Familial gastrointestinal stromal tumor (606764)	Gastrointestinal stromal tumors	Dominant	*KIT*
Genodermatoses with cancer predisposition			
Melanoma syndromes (155600, 155601, 609048, 608035)	Malignant melanoma	Dominant	*CDKN2 (p16)*
			CDK4
			CMM
Basal cell cancers, Gorlin syndrome (109400)	Basal cell cancers	Dominant	*PTCH*

	Brain tumors		
Cowden Syndrome	See above	Dominant	*PTEN*
Neurofibromatosis 1 (162200)	Neurofibrosarcomas	Dominant	*NF1*
	Pheochromocytomas		
	Optic gliomas		
	Meningiomas		
Neurofibromatosis 2 (101000)	Vestibular schwannomas	Dominant	*NF2*
Tuberous sclerosis (191100)	Myocardial rhabdomyoma	Dominant	*TSC1*
	Multiple bilateral renal angiomyolipoma		*TSC2*
	Ependymoma		
	Renal cancer		
	Giant cell astrocytoma		
Carney Complex (160980, 605244)	Myxoid subcutaneous tumors	Dominant	*PRKAR1A*
	Primary adrenocortical nodular hyperplasia		
	Testicular Sertoli cell tumor		
	Atrial myxoma		
	Pituitary adenoma		
	Mammary fibroadenoma		
	Thyroid carcinoma		
	Schwannoma		
Muir Torre syndrome (158320)	Sebaceous carcinoma	Dominant	*MLH1*
	Sebaceous epitheliomas		*MSH2*
	Sebaceous adenomas		
	Keratoacanthomas		
	Colon cancer		
	Laryngeal carcinoma		
	Malignant gastrointestinal tract tumors		
	Malignant genitourinary tract tumors		
Xeroderma pigmentosum (278730, 278700, 278720,	Skin cancer	Recessive	*XPA,B,C,D,E,F,G*
	Melanoma		*POLH*
	Leukemia		

278760, 74740, 278780, 278750, 133510)			
Rothmund Thomson syndrome (268400)	Basal cell carcinoma	Recessive	*RECQL4*
	Squamous cell carcinoma		
	Osteogenic sarcoma		
Leukemia/lymphoma predisposition syndromes			
Bloom syndrome (210900)	Leukemia	Recessive	*BLM*
	Carcinoma of the tongue		
	Squamous cancers		
	Wilms' tumor		
	Colon cancer		
Fanconi anemia (227650)	Leukemia	Recessive	*FANCA,B,C*
	Squamous cancers		*FANCA,D_2*
	Skin carcinoma		*FANCE,F,G*
	Hepatoma		*FANCL*
Shwachman-Diamond syndrome (260400)	Myelodysplasia	Recessive	*SBDS*
	Acute myelogenous leukemia		
Nijmegen breakage syndrome (251260)	Lymphoma	Recessive	*NBS1*
	Glioma		
	Medulloblastoma		
	Rhabdomyosarcoma		
Canale-Smith syndrome (601859)	Lymphoma	Dominant	*FAS*
			FASL
Immunodeficiency syndromes			
Wiskott-Aldrich (301000)	Hematopoietic malignancies	X-linked recessive	*WAS*
Common variable immune deficiency (240500)	Lymphomas	Recessive	Unknown
		Dominant	Unknown
Severe combined immune deficiency (102700, 300400,	B-cell lymphoma	X-linked recessive	*IL2RG*
		Recessive	*ADA*

312863, 601457, 600802, 602450)			*JAK3*
			RAG1
			RAG2
			IL7R
			CD45
			Artemis
X-linked lymphoproliferative syndrome (308240)	Lymphoma	X-linked recessive	*SH2D1A*
Genitourinary cancer predisposition syndromes			
Hereditary prostate cancer (176807, 601518)	Prostate cancer	Dominant	*HPC1*
			HPCX
			HPC2/ELAC2
			PCAP
			PCBC
			PRCA
Simpson-Golabi-Behmel syndrome (312870)	Embryonal tumors	X-linked recessive	*GPC3*
	Wilms' tumor		
von Hippel-Lindau syndrome (193300)	Hemangioblastomas of retina and central nervous system	Dominant	*VHL*
	Renal cell cancer		
	Pheochromocytomas		
Beckwith-Wiedemann syndrome (130650)	Wilms' tumor	Dominant	*CDKN1C*
	Hepatoblastoma		*NSD1*
	Adrenal carcinoma		
	Gonadoblastoma		
Wilms' tumor syndrome (194070)	Wilms' tumor	Dominant	*WT1*
WAGR: Wilms' tumor, aniridia, genitourinary abnormalities, mental retardation (194072)	Wilms' tumor	Dominant	*WT1*
	Gonadoblastoma		
Birt-Hogg-Dubé syndrome (135150)	Renal tumors	Dominant	*FLCL*

Papillary renal cancer syndrome (605074)	Papillary renal cancer	Dominant	*MET, PRCC*
Constitutional t(3;8) translocation (603046)	Renal cell cancer	Dominant	*TRC8*
Hereditary bladder cancer (109800)	Bladder cancer	Sporadic	Unknown
		Unknown	
Hereditary testicular cancer (273300)	Testicular cancer	Possibly x-linked	Unknown
		Possibly recessive	Unknown
Rhabdoid predisposition syndrome (601607)	Rhabdoid tumors (see below)	Dominant	*SNF5/INI1*
Central nervous system/vascular cancer predisposition syndromes			
Hereditary paraganglioma (185470, 115310, 16800)	Paraganglioma	Dominant	*SDHD*
	Pheochromocytoma		*SDHC*
			SDHB
Retinoblastoma (180200)	Retinoblastoma	Dominant	R*B1*
	Osteosarcoma		
Rhabdoid predisposition syndrome (601607)	Rhabdoid tumors	Dominant	*SNF5/INI1*
	Medulloblastoma		
	Choroid plexus tumors		
	Primitive neuroectodermal tumors		
Sarcoma/bone cancer predisposition syndromes			
Multiple exostoses (133700, 133701)	Chondrosarcoma	Dominant	*EXT1*
			EXT2
Leiomyoma/renal cancer syndrome (605839)	Papillary renal cell carcinoma	Dominant	*FH*
	Uterine leiomyosarcomas		
Carney complex	See above	Dominant	*PRKAR1A*
Werner syndrome (277700)	Sarcoma/osteosarcoma	Recessive	*WRN*
	Meningioma		

Endocrine cancer predisposition syndromes			
MEN1 (131100)	Pancreatic islet cell tumors	Dominant	*MEN1*
	Pituitary adenomas		
	Parathyroid adenomas		
MEN2 (171400)	Medullary thyroid cancers	Dominant	*RET*
	Pheochromocytoma		
	Parathyroid hyperplasia		
Familial papillary thyroid cancer (188500)	Papillary thyroid cancer	Dominant	Multiple loci

Syndromes of Inherited Cancer Predisposition in Clinical Oncology Syndrome.

Abbreviations: OMIM, On-Line Mendelian Inheritance in Man; HNPCC, hereditary nonpolyposis colorectal cancer; MEN, multiple endocrine neoplasia.

Appendix B: The FA-BRCA DNA Damage Response

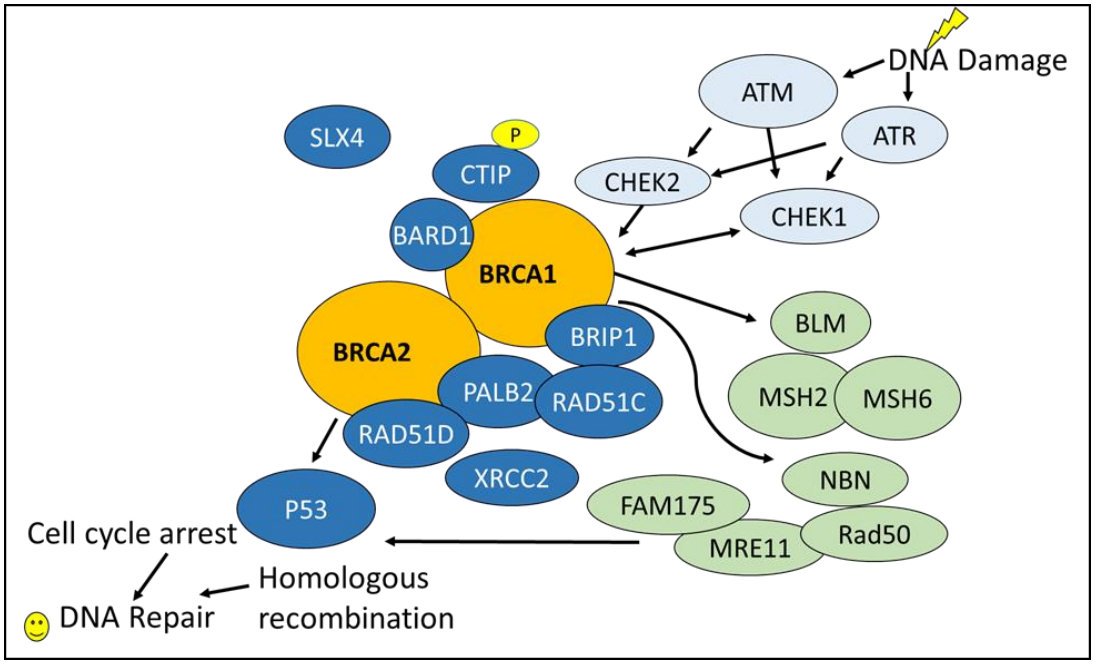

Figure B. Schematic of some of the FA genes and other interacting proteins in the DNA damage response. (Adapted from Harrell et al. (2013)). Germline loss of function mutations in DNA repair genes in 1418 patients with ovarian, peritoneal or fallopian tube cancers not selected for age at diagnosis or family history. In M. Southey (Chair), Hereditary Cancer Syndromes. Platform session conducted at the Annual meeting of the American Society for Human Genetics, Boston, MA.)

Appendix C: *CHEK2* damaging missenses resulting in loss of function as reported by the literature

Chr	Position (hg19)	NT	cDNA[a]	Protein[a]	Prediction
22	29,121,326	C>T	c.349A>G	R117G	Damaging
22	29,121,247	T>C	c.428A>G	H143R	Damaging
22	29,121,242	G>A	c.433C>T	R145W	Damaging
22	29,121,077	T>C	c.480A>G	I160M	Damaging
22	29,121,058	C>T	c.499G>A	G167R	Damaging
22	29,121,019	G>A	c.538C>T	R180C	Damaging
22	29,095,917	C>G	c.917G>C	G306A	Tolerated
22	29,092,914	G>A	c.1070C>T	S357F	Damaging
22	29,091,220	A>G	c.1270T>C	Y424H	Damaging
22	29,091,207	G>A	c.1283C>T	S428F	Damaging
22	29,090,054	G>T	c.1427C>A	T476K	Damaging
22	29,090,054	G>A	c.1427C>T	T476M	Damaging