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A Study of Breast Cancer Comorbidities and Apoptosis in T47D Breast Cancer Cells

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**A Study of Breast Cancer Comorbidities and Apoptosis in T47D
Breast Cancer Cells**

A Major Qualifying Project Report,

submitted to the faculty of

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the Degree of Bachelor of Science

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April 25, 2019

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ABOUT THIS PROJECT

This project was conducted in fulfillment of the Major Qualifying Project (MQP) in satisfaction of two major degree requirements: Biology and Biotechnology and Professional Writing. The goal was to combine both disciplines to demonstrate the technical and social research aspects gained in my four years at Worcester Polytechnic Institute. The first chapter of this project is an epidemiological study of breast cancer comorbidities to further examine the discrepancies of rural and urban healthcare. The second chapter of this project is a laboratory study of breast cancer cells treated with a common over-the-counter supplement to examine the effects the supplement has on the cells.

ACKNOWLEDGEMENTS

I would like to say thank you to the Canton-Potsdam Hospital for approving and allowing me to use their deidentified breast cancer patient data to complete an epidemiological study of breast cancer comorbidities in rural New York. I would like to say thank you to Brenton Faber for pushing me into a second-degree path and for advising the Professional Writing aspect of this project.

Additionally, thank you to Michael Buckholt and Jill Rulfs for advising and aiding in the Biology & Biotechnology proportion of this MQP. Without their help, this MQP would not be possible and the research experience gained was invaluable. A last thank you to Louis Roberts for helping with troubleshooting and providing advice regarding protein assays and concentrating.

CHAPTER ONE

Disease and the Human Experience: Evaluating Breast Cancer Comorbidities

ABSTRACT

Breast cancer is the most common form of cancer among woman. There is evidence that breast cancer patients in rural areas have different medical experiences than those in urban areas. This project aims to examine the healthcare of 202 rural breast cancer patients from the Canton-Potsdam Hospital (CPH). Comorbidities associated with breast cancer were examined before and after breast cancer diagnosis. Data showed that their comorbidities are consistent with those identified in larger national studies and include hypertension, type 2 diabetes, high cholesterol, and cardiac history. Rates of hypertension for CPH were higher than Saint Lawrence Country and New York State, indicating a need to improve treatment options for these patients. While post-treatment infection remains a concern for this population, it appears well managed.

INTRODUCTION

Epidemiology

The Centers for Disease Control and Prevention (CDC) defines epidemiology as “the study of distribution and determinants of health-related states or events in specified populations and the application of this study to the control of health problems” (*An introduction to applied epidemiology and biostatistics*). In other words, epidemiology is the study of the state of disease within and across populations in an effort to understand, monitor, and influence public health. Epidemiology has been practiced informally since Hippocrates, about 400 B.C., albeit the idea was not coined as epidemiology. Given the importance of disease, humans have long been concerned with disease occurrence and transmission; however, such interest has long been complicated by superstition, religious bias, and poor understanding of the nature of disease and illness. As the knowledge of disease and health care has improved so has the desire to understand the mechanisms and relationships of disease to daily life (*An introduction to applied epidemiology and biostatistics*).

In the 1980s, six tasks of epidemiology were instantiated as a foundation for public health. They are public health surveillance, field investigation, analytic studies, evaluation, linkages, and policy development. Public health surveillance is monitoring public health to understand the patterns of disease through reporting. Field investigation may include interviews or characterizing populations who seem to contract a certain disease. Analytic studies are hypothesizing and testing why, how, or where disease takes place. Evaluation examines the effectiveness of a current public health program. Linkage is the idea of working together; physicians, epidemiologists, governmental and private agencies, and academia work together in public health settings. These five tasks allow for the sixth task to happen: policy development. Together, field work, investigating, and understanding provide the foundation for health care policies to be developed (*An introduction to applied epidemiology and biostatistics*).

Health Disparities in Rural Medicine

Geographic distance, poverty, lack of resources, and lack of specialty care present unique challenges for rural healthcare infrastructures. Rural patients often face more complications, more untreated conditions, longer waiting times to treatment, dated or less current treatment protocols, and higher mortality rates than urban patients (National Rural Health Care). According to an article from the Association of American Medical Colleges, heart disease, cancer, unintentional injury, chronic lower respiratory disease, and stroke, which are the five leading causes of death in the United States, are higher in rural communities. Additionally, rural residents are more likely to have cancer due to higher tobacco use, human papillomavirus (HPV), and lack of cancer screening (Warshaw 2017).

Due to these challenges, specialty organizations have emerged to augment recognition and development of rural area medicine. The National Rural Health Association's (National Rural Health Care) goal is "to provide leadership on rural health issues through advocacy, communications, education and research" (National Rural Health Care). As the NRHA notes, one of the biggest challenges to rural health care is the lack of physicians compared to urban areas. Patients may have a harder time getting appointments or adequate care since there are a limited number of physicians available to account for clients. Rural patients often face economic hardships, as more rural residents live below the poverty line. Unemployment often leads to a lack of insurance and rural residents are more likely to have diabetes and heart disease. Rural patients often have a hard time treating and taking care of their health due to the lack of accessibility to health care and money-related problems (National Rural Health Care).

Rural Healthy People 2020 was a study conducted and supported by Texas A&M to evaluate health priorities for rural America. Using an electronic questionnaire, respondents were asked to list their top 10 highest priorities that are needed in terms of health care. Residents in Ohio, Texas, Missouri, West Virginia, Michigan, and Indiana had the highest number of respondents to the survey. Forty-two percent (42.2%) of respondents were from the Midwest. From the survey, the researchers found that access to quality health care centers was the single most important priority. Cancer ranked as health concern for 35% of respondents. From this brief questionnaire, the Rural Healthy People 2020 program was able to identify the highest concerns of people living in rural America, with health care center access being the highest concern (Bolin et al. 2015). Other researchers have identified that many people who live in rural areas have higher travel time and more difficult access to cancer center treatments.

This project examines the experience of breast cancer patients in rural geographies. These patients face most of challenges outlined above. A 2014 study of Iowa cancer patients found that 12.3% of patients diagnosed with invasive cancer were not treated with therapy from the first course and only 31.6% diagnosis were treated with chemotherapy first course. The researchers used data from the Iowa Cancer Registry (ICA), to look at the incidences of cancer from 2004-2010, as well as the Iowa Physician Information Systems (IPIS) to track physicians. The median travel time to a chemotherapy center was 31 minutes and there were limited options of treatment centers in the state (Ward et al. 2014).

Baldwin et al. (2008) conducted a descriptive study measuring travel patterns and distances between colorectal cancer patients in urban and rural areas. They used data from Surveillance, Epidemiology, and End Results (SEER) cancer registry from 5 states to look at patients 66 and older who were fully enrolled in a fee-for-service Medicare following their diagnosis with stages 1-3 colorectal cancer. Overall, the researchers found that about 40% of patients living in small isolated rural areas had radiation services within 30 miles and about 73% had services within 50 miles. Yet, 25% of patients did not have an oncology service area

within 30 miles of their residence. They also found that patients who did not have a service within their rural area often traveled to more urban areas to receive radiation or chemotherapy but were least likely to travel for surgery. Patients traveled between 47.8 and 67.0 miles for treatment from isolated areas, whereas patients who lived in large rural areas traveled up to 70.0 miles for care (Baldwin et al. 2008).

Breast Development and Cancer

Breast cancer is a form of cancer that more typically affects women more than men and it occurs when breast epithelial cells mutate and have uncontrolled growth. Today, breast cancer is the second most commonly diagnosed cancer in woman and the leading cause of death in woman. The American Cancer Society (ACS) estimates that at least one in eight women will be diagnosed with breast cancer in her lifetime. While the mortality rates of breast cancer have reduced over the years, ACS suggests that at least 39,000 cases of breast cancer a year will result in death (National Library of Medicine-Breast Cancer; Chalasani 2018).

Anatomy and Development

A woman's breast goes through a series of changes throughout her lifetime in response to hormonal changes from birth, puberty, menstrual cycles, pregnancy, breastfeeding, and menopause. Breast anatomy is quite complex, which can be seen in Figure 1.

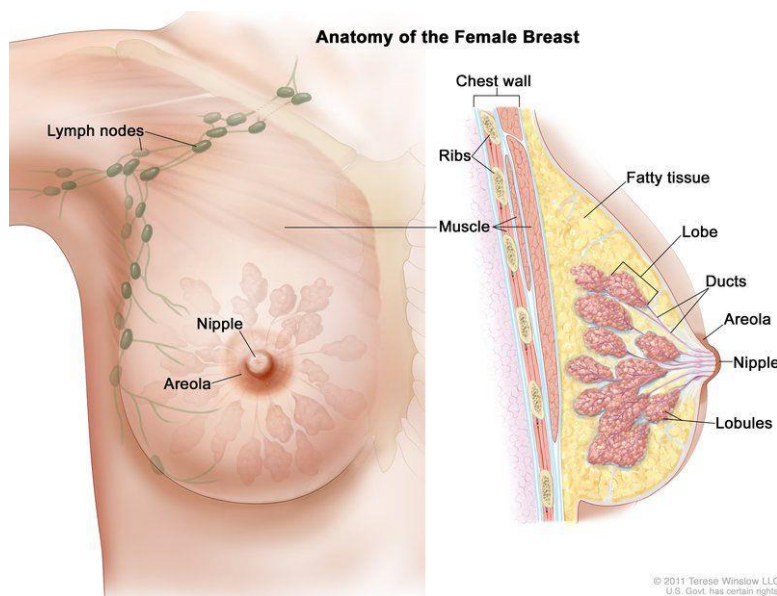


Figure 1. Anatomy of the female breast (National Breast Cancer Foundation)

Each breast contains about 20 glands that are responsible for producing milk at the time that a woman is breastfeeding. The lobes are connected to the nipple by ducts that carry milk (National Breast Cancer Foundation). Lymph nodes are also present near breast tissue that

carry lymph fluid and white blood cells; these components are an important part of the body's immune system (Cleveland Clinic). The rest of the breast tissue is fatty tissue (National Breast Cancer Foundation).

Breast tissue develops in response to estrogen and progesterone changes in the body, both of which are steroid hormones produced by the ovaries. During puberty, estrogen is responsible for breast enlargement through the excessive fatty tissue and duct system development. More breast changes occur during ovulation and menstrual cycle due to a rise in estrogen and progesterone levels to prepare the body for the possibility of pregnancy. Estrogen stimulates the growth of ducts while progesterone stimulates milk gland formation. These hormonal changes cause swelling, soreness, and pain. If a woman does not become pregnant, these symptoms reduce, and the cycle begins again. Once a woman is pregnant, the breasts swell and enlarge further as estrogen and progesterone stimulate the final formation of milk ducts. As women age, their bodies change once more as they go through menopause. During menopause, hormone levels drastically decrease, causing breast tissue to lose and change shape (John Hopkins Medicine). The body responds to these decreasing levels of hormones by causing a multitude of symptoms, including hot flashes, sleep problems and night sweat, and vaginal and urinary changes (The American College of Obstetricians and Gynecologists).

Breasts are constantly growing and dividing in order to satisfy the needs of the body. Sometimes, abnormal breast tissue growth can develop as a response to a genetic mutation that goes undetected in normal breast cell cycle. This growth causes a tumor. Not all abnormal growth may be cancerous unless the tumor invades surrounding tissues. Typically, these breast cancers can be found in the milk ducts (ductal cancer) or structures within the lobes (lobular cancer) (John Hopkins Medicine).

Triple-Negative Breast Cancer

BRCA1 and *BRCA2* genes are normally encoded as tumor suppressors in the human genome (Breast Cancer). In its normal pathway, *BRCA1* prevents cells from dividing too rapidly or uncontrolled. Additionally, Brac1 protein plays a role many DNA repair pathways, by assisting in fixing mutated DNA. When *BRCA1* is mutated, the protein transcribed is abnormally short and is unable to perform to properly repair DNA or cease cell growth. This causes the cells to grow uncontrollably with mutated copies of DNA (*BCRA1* gene). Those with mutations in *BCRA1* have a 46% to 87% risk of breast cancer in their lifetime. *BRCA1* is considered triple-negative breast cancer, meaning the cells responsible for the tumor do not have hormone receptors (estrogen receptors (ERs), progesterone receptors (PRs) or human epidermal growth factor receptor 2 (HER2)) (Pertucelli et al. 2016).

Triple-negative breast cancer is often referred to basal-like breast cancer as it typically is found on the basal layer of breast tissue. The ACS suggests that premenopausal (under the

age of 50) are more likely to be diagnosed with triple-negative breast cancer and that African-American women are two times as likely to be diagnosed (Alteri et al. 2018). Additionally, mutations in *BRCA1* are more likely to be passed down to a family member (Pertucelli et al. 2016).

ER+, PR+, and HER+ Breast Cancer

Some studies have shown that breast cancer cells may proliferate in response to hormones. Breasts grow and develop in response to hormonal changes produced by the ovaries (estrogen and progesterone) (National Breast Cancer Foundation). Luminal A breast cancer is associated with having ER or PR positive receptors. About 2/3 of people diagnosed with breast cancer have Luminal A breast cancer (Arteri et al. 2018). Hormone positive breast cancers are more commonly seen in older women who are post-menopausal and oftentimes can be less aggressive at an earlier diagnosis due to targeted anti-hormonal therapies (Chalasani 2018). This trend of older women being diagnosed with breast cancer may likely be due to the hormone therapy many older women take to alleviate symptoms of menopause (The American College of Obstetricians and Gynecologists). Some studies have shown trends that hormone replacement therapy is associated with increased risk of breast cancer, specifically hormone receptor breast cancer, despite it is still commonly used for menopausal symptoms (Shook 2014).

HER2+ breast cancer is responsible for 1/5 of all breast cancer diagnosis. *HER2* is a growth hormone that regulates cell growth and differentiation. Mutations of *HER2* may breast cancer due to overexpression of the protein. Thus, *HER2* is unable to regulate and instead promotes cellular growth by stimulating breast cancer cells to make more HER2 receptors. Oftentimes, HER2 positive breast cancer tends to grow faster and spread, unlike ER+ or PR+ breast cancer (breastcancer.org). When HER2 positive breast cancer is associated with hormone receptor positive breast cancer, it is called Luminal B breast cancer. Luminal B breast cancer tends to be more aggressive than Luminal A.

Clinical Presentation and Diagnosis

The most common symptom of breast cancer is detection of lumps or mass in the breasts. Other physical signs can be change in breast shape, skin tethering, nipple inversion, ulceration, hardness, asymmetry between breasts, swelling, and redness. Breast cancer signs associated with breathing difficulties, bone pain, jaundice, altered cognitive function, headaches, and other neurological symptoms may indicate metastatic spread of the cancer (Chalasani 2018). The disease is often associated with lymph nodes in the armpits and may be swollen upon diagnosis.

Breast cancer can be classified in five stages, ranging from non-cancerous to severe (Stage 0 to IV). Not all breast cancer begins in the breasts, but instead occurs in the neighboring lymph

nodes. Lymph node swelling around the chest or armpits is a strong indicator of the disease, even if there are no symptoms in the breasts (National Breast Cancer Foundation).

The ACS recommends that woman over the age of 40 years old should be screened annually for breast cancer. Average risk factors that include general screening are women without a familial history of breast cancer, confirmed *BRCA1* mutations, or history of radiotherapy to the chest at a young age. Annual screening includes mammograms and breast exams (Mayo Clinic 2018; Oeffinger et al. 2015). Mammograms are an X-ray designed to examine the breast tissue for any abnormalities. Yearly screening allows earlier diagnosis of breast cancer (National Breast Cancer Foundation-Mammogram). A study by the ACS shows that by screening yearly for breast cancer there is a reduction in mortality from breast cancer. While yearly screening is important, false-positives may be common, which require additional testing, including ultrasounds, diagnostic mammograms, MRIs, or biopsies (Oeffinger et al. 2015; National Library of Medicine-Breast Cancer).

Treatment

Appropriate breast cancer treatment should be determined by the originating cause of disease. Commonly, many patients undergo surgery to remove the mass or breast tissue in which the breast cancer has originated. Breast-conserving surgery (BCS) or partial mastectomy, removes the cancerous tissue and some healthy tissue surrounding the tumor. BCS is often accompanied by radiation therapy, which is the use of high-energy rays to kill the cancer cells as rapidly dividing cells are more susceptible to cell death (Diagnosis). Mastectomy removes the entire cancerous breast. In some cases, lymph nodes or even both breasts may be removed in mastectomy procedures.

Hormone therapy is a common treatment option for those who have hormone receptor positive breast cancers. Hormone therapy works by blocking the body's natural ability to produce hormones so that the cancer cells will not grow in response to the hormone. Some drugs, such as aromatase inhibitors, are used to block estrogen production and are often used in postmenopausal woman. Some Food and Drug Administration (FDA) approved aromatase inhibitors are anastrozole, letrozole, and exemestane. Other drugs are designed to specifically block estrogen's ability to interact with cancer cells. Selective Estrogen Receptor Modulators (SERMs) are antagonists of estrogen receptors; they block the ERs so that estrogen cannot bind. Tamoxifen, for example, was the first FDA approved chemopreventative and SERM. It is metabolized by the body and broken down to smaller molecules capable of binding to ERs to prevent proliferation (Oseni et al. 2008). These hormone therapies can used together or separately, often for 5 years following surgery (National Cancer Institute).

Comorbidities Surrounding Breast Cancer

Breast cancer is often associated with a coexistent chronic disease, due to immunocompromising effects of cancer treatments or old age. Fu et al. (2015) looked at woman who were diagnosed with breast cancer (stages I-III) at the New York University Langone Medical Center. They examined the comorbidity rate, in this case an existing diagnosis prior to treatment, in patients before and all the way up to 12 months after treatment surgery. The researchers used the Charlson Comorbidity Index, which is a means of scoring comorbidities based on disease weight, in order to determine the mortality rate of the various comorbidities. Fu et al. (2015) found that there was no significant difference in cancer treatment with comorbidity, including biopsy, dissection, mastectomy, lumpectomy, radiation, and chemotherapy. Twenty-eight comorbidities were identified: 32.8% was hypertension, 32.8% arthritis, 22.4% was thyroid issues, 12.7% hypercholesterolemia, and 12% was diabetes. Overall, it was found that comorbidities had a negative correlation with quality of life for breast cancer patients (Fu et al. 2015).

Another study by Wu et al. (2014) used a questionnaire to look at the various comorbidities associated with breast cancer with data from the California Breast Cancer Survivorship Consortium (CBCSC). The CBCSC had collected data from various agencies to specifically look at the comorbidities associated with non-white females who were diagnosed with breast cancer. From the questionnaire, the researchers obtained information on comorbidities of conditions: diabetes, hypertension, and myocardial infarctions (MI). Patients who had these various conditions prior to breast cancer diagnosis were considered. 27.7% of patients were diagnosed with hypertension, followed by previous cancers, diabetes, and MIs prior to breast cancer. However, they found that diabetes (for 15 years prior) and MIs were associated with breast cancer mortality. Additionally, there was an increased risk of mortality for those not treated with radiation therapy or chemotherapy (Wu et al. 2014).

Breast Cancer in Rural Medicine

As part of the CDC's National Breast and Cervical Cancer Early Detection Program, in 1992, Missouri established the Show Me Healthy Woman program to increase cancer screening in high-risk female populations, such as those who live below the federal poverty level, are less than 65 years of age, have disabilities, or have little health insurance. Despite this program, there are still discrepancies in access to breast cancer screening in the rural populations versus urban ones. Williams et al. (2015) conducted a study in 2015 that focused on the differences between urban and rural breast cancer screening in Missouri. The researchers used reported cases from the Missouri Cancer Registry and Research Center as their sample population from 2003-2008. Rural county codes were used to determine if the patients lived in rural areas. Williams et al. (2015) found that the breast cancer incident rate between 2003 and 2008 was 289.6 per 100,000 women and more white woman were diagnosed than African-

American. They also found that woman from non-metro areas (22 of the 115 counties) had about 45-75 minutes of travel time to reach medical care. Of these 22, 13 areas are considered rural. They also found that although there are 180 cancer screening facilities, the facilities are clustered in one area, and some women do not have access to a center (Williams et al. 2015).

Another study by Jacobs et al. (2008) looked at whether a patient living in a rural setting was more likely to have a mastectomy than an urban patient. The researchers used data from the 2006 Surveillance, Epidemiology, and End Results (SEER). The criteria for the study was females with either Stage I, II, or III breast cancer without any prior cancers. Additionally, patients were excluded if they had any diseases that may require a mastectomy or stage IV metastatic cancer. Codes from the Economic Research Service (ERS) of the US Department of Agriculture were used to define urban versus rural populations. The researchers found that 44.92% of urban woman received a mastectomy whereas 59.9% of rural woman did. They also concluded that access and distance to radiation therapies influenced the rate of mastectomies. The average of radiation of technologists and radiation oncologists per county were 2.25 and 2.57 respectively. Urban populations had significantly higher averages for specialty oncologists. Married woman typically preferred having breast-conserving surgery than mastectomies than unmarried or widowed woman. Furthermore, the researchers verified that the lack of academics, treatments, oncology consultations, and high costs of healthcare results in a higher mastectomy rate in rural populations (Jacobs et al. 2008).

Examining Breast Cancer in Rural Medicine

The study was approved by the Worcester Polytechnic Institute (WPI) Institutional Review Board (IRB#19-0217) and Canton-Potsdam Hospital (CPH) Institutional Review Board (IRB# IORG0007792) to examine the rate of breast cancer in rural New York. From previously mentioned studies, there is a lack of financial resources, treatment options within acceptable driving distances and available physicians in many rural healthcare settings. This causes a desire for improvement of health care in rural settings from those living in remote areas, especially those suffering from cancer. Breast cancer effects 1 in 8 women from all over the United States (Chalasani 2018). Using de-identified patient data from Canton-Potsdam Hospital, breast cancer comorbidities were examined if to determine if there are any difference between this rural hospital to the rest of New York State and other studies.

METHODS

Since this study required the use of patient data, Institutional Review Board (IRB) approval from both Worcester Polytechnic Institute (WPI) and Canton-Potsdam Hospital (CPH) was obtained before proceeding with the project. After approval was granted, the following data from the hospital database was obtained: patient ID, visit ID, admit date, discharge date, age, diagnosis 1 IDC9 or IDC 10 code, name of diagnosis 1, procedure, chemotherapy date, diagnosis 2 IDC9 or IDC 10 code, name of diagnosis 2, diagnosis 3 IDC9 or IDC 10 code, and name of diagnosis 3. The data was reaggregated and patient ID were renamed and randomized before the data was moved to WPI. Data was transferred to Microsoft excel.

After receiving the data, the first task was to analyze the general population of the study data. To determine the number of patients and the age of each of them, patient IDs were removed using the *duplicate deletion* function so that only one patient ID remained. The mean age was determined. Using a *countif* function, the sum of ages within five-year bins was counted. Since the youngest patient was 27, bins were broken up into five-year categories from age 25-30, 31-35, 36-40, 41-45, etc.

Each patient had multiple entries for one unique emergency room visit. Therefore, more duplicates had to be deleted in order to see each comorbidity one time to determine the rates. Similar to finding the ages of the patient population, duplicates were deleted using the *duplicate deletion* function of each patient and diagnosis ID individually. Using this function deleted most of the duplicates, but some remained and had to be deleted by hand. Diagnosis considered as comorbidities were grouped together in categories. Hypertension was considered as any diagnosis with hypertension in the diagnosis. All hypercholesteremia or hyperlipidemia like diagnoses were categorized as hypercholesteremia. Neoplasms that were not breast cancer were categorized as “other neoplasm” or “lymph neoplasm”. The reason for categorizing neoplasms in the lymph nodes as separate was because many breast cancers are associated with cancer in the surrounding lymph nodes. All pain, regardless of location or type, were categorized as “pain”. Type 2 Diabetes had a multitude of diagnoses and were all categorized under one name. Infections were any diagnoses with the word “infection”, sinusitis or bronchitis. Any circulatory disorder was “cardiac disease”, excluding arrhythmias or atrial fibrillation. Hypothyroidism was a separate diagnosis than thyroid disorders because they are different diseases. Kidney stones were categorized as any diagnosis that referred to calculus of any part of the urinary system. Hypothyroidism was diagnosed differently than other thyroid disorders and were counted separately. COPD was only considered as patients with COPD and not emphysema so the rate of COPD patients could have been higher than reported. Lastly, depression and anxiety were often referred to under different diagnoses and were categorized as one. All comorbidities were calculated to be number over patient population count and shown as a percentage of 100 on a bar graph.

Once comorbidities were categorized and calculated, the first date of breast cancer diagnosis was found. For some patients, there was no mention of when they were diagnosed because the data was from emergency room. Therefore, the advisor of this project went back into the database and looked up the first date of diagnosis for each of the patients without any mention of breast cancer. The rest of the population's date of diagnosis was considered to be the first mention of breast cancer in the emergency room records. From there comorbidities from the categorizes listed above were counted before and after this date of diagnosis. Comorbidities were counted and divided over the total population and shown a percentage of 100 on a bar graph.

Since the infection rate was low in this population, a more detailed investigation of the infections was conducted. Infections were categorized into the most commonly mentioned infections: viral, post-operative, urinary tract infections (UTI), respiratory, gastrointestinal, sinusitis, and bronchitis. Bronchitis was chosen to be separate from the respiratory infections because it was distinctly mentioned as a separate infection in the diagnoses. Infection data was displayed on a pie chart in percentages of the population of patients who had infections. Some patients had multiple infections. Each infection was considered as one to understand the different types of infections that were occurring in the population.

RESULTS

After receiving patient data, the first goal was to determine the number of patients in the data set and the average age for each of them. This was done by using the first date of emergency room records in the dataset, not necessarily their first date of diagnosis. Distribution of ages can be seen in Figure 2.

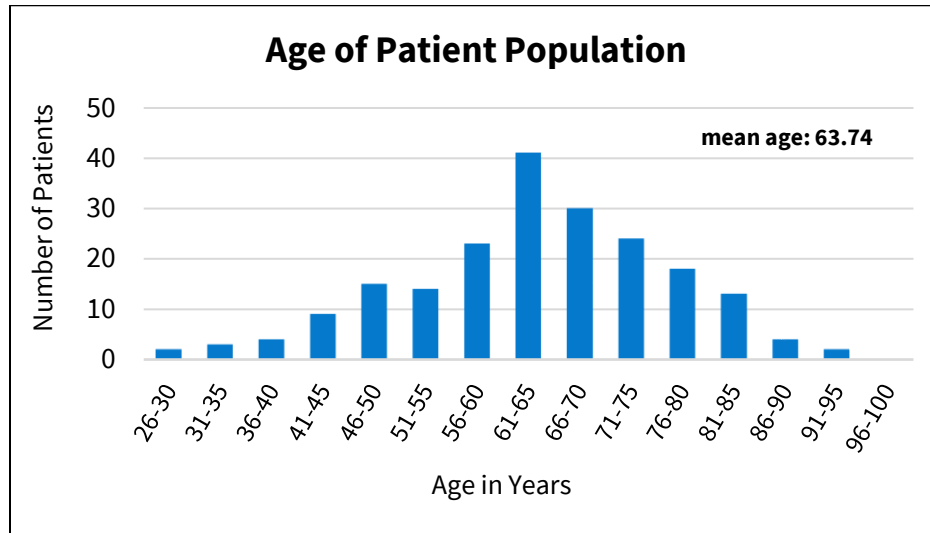


Figure 2. Histogram of the ages of the patient population. Ages are in bins of five years.

According to Figure 2, the patient data for breast cancer has a normal distribution with a mean age of 63.74 from a total population of 202 patients. The majority of the patients are between the ages of 56 and 80, reiterating that breast cancer in this population is typically a disease of older women. Rarer cases of breast cancer can be seen between 26 and 45 years old.

Once the population was examined, the diagnostic data was grouped into broader categories of comorbidities. The rate of occurrence in this study population can be seen in Figure 3.

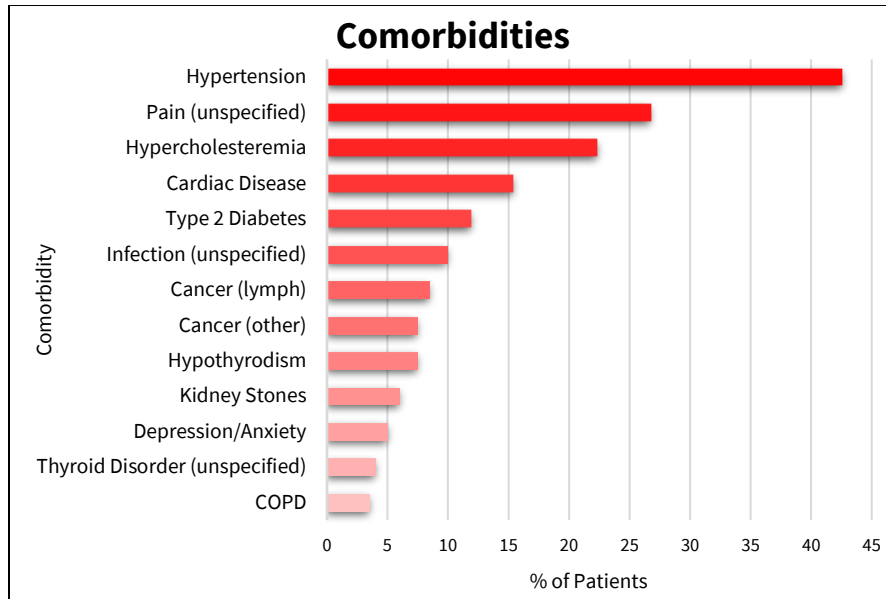


Figure 3. Occurrences of comorbidities in study cohort.

Figure 3 shows that hypertension has the highest percentage of comorbidities at 42.5% in the CPH breast cancer patients. Following hypertension, is pain (unspecified) (26.7%), hypercholesteremia (22.2%), cardiac disease (15.3%), and type 2 diabetes (11.8%). Infections remained lower than expected at 9.9%. To gather a better understanding of the association between breast cancer and its comorbidities, the comorbidities were counted from before and after breast cancer diagnosis. Breast cancer diagnosis was when breast cancer was first mentioned in the patient data from the emergency department unless otherwise obtained. Figure 4 shows a bar graph of the comorbidities before and after diagnosis.

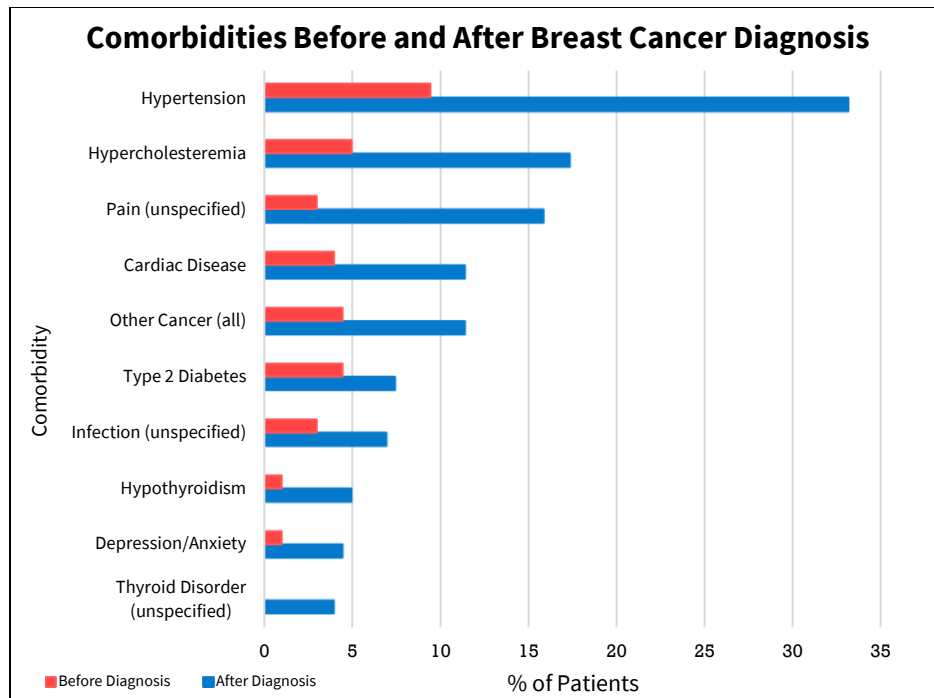


Figure 4. Occurrence of comorbidities before and after breast cancer diagnosis.

Figure 4 above shows before and after breast cancer diagnosis comorbidities. Again, hypertension has the highest comorbidity rate after diagnosis compared to before diagnosis. Hypercholesteremia (17.3%) is higher than pain (15.8%) after diagnosis, followed by cardiac disease (11.3%) and other cancer (11.3%). Hypertension rates followed by hypercholesteremia rates after diagnosis is not surprising because oftentimes both comorbidities can be associated with each other. Additionally, other cancer after breast cancer diagnosis is relatively low, but also high compared to the other comorbidities after diagnosis.

Since the number of occurrences of infections seemed to be relatively low compared to the other comorbidities, a closer look of infections was inquired. Infections were broken up into categories that appeared the most the patient data. While bronchitis could be considered a respiratory disease, many of the study population had diagnoses indicated as respiratory diseases different than bronchitis. Figure 5 shows the infection occurrence for each individual infection.

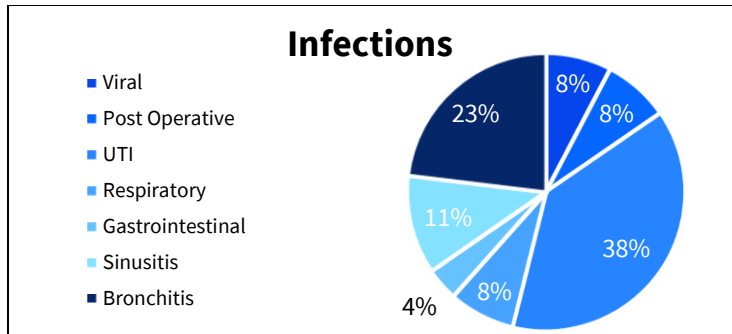


Figure 5. Pie chart of infection occurrences.

As shown in Figure 5, urinary tract infections (UTIs) have the highest rate of occurrence (38%). Following UTIs, bronchitis has the highest occurrence rate, 23%. This may likely due to the volume of patients with COPD (Figure 3). Additionally, 16.3% of the population has a tobacco or nicotine use history, likely resulting in various respiratory functions. Post-operative and viral infections are both 8%, which is relatively low for this population.

DISCUSSION

The overall goal of this study was to explore comorbidities related with breast cancer in rural areas. Use of data from Canton-Potsdam Hospital made it possible to do this. Often it is seen that cancer patients in rural areas experience different medical encounters than those in urban settings. This accounts for treatment, diagnosis, screening, and health care availability (National Rural Health Association). From this study, it was seen that breast cancer patients in rural New York have similar comorbidities to national averages and other studies. The age distribution in this study provided a wide range of patient ages that showed a very normal model. This provided a better understanding of how the disease affects people from all ages.

From prior research, it was evident that in general breast cancer patients tend to have higher rates of hypertension and thyroid disorders. In this study cohort, 43% of patients were shown to have hypertension (Figure 3), with about 34% of patients experiencing symptoms of hypertension post breast cancer diagnosis (Figure 4). Hypertension rates are high in this small population of 202 patients. Compared to Saint Lawrence County (county in New York in which CPH is located) and New York State, hypertension rates in the study population are 13% higher than in the general population (Expanded Behavioral Risk Factor Surveillance). The increased rate in the study was higher than expected but may be likely due to the course of the disease. It has been shown that cancer patients undergoing chemotherapy are more likely to have higher rates of hypertension than before treatment (Fraeman et al. 2013).

Twenty-three percent (23%) of the study cohort had been diagnosed with hypercholesterolemia (Figure 3). Saint Lawrence County reported that 38% of people living in the county were diagnosed with hypercholesterolemia. This rate is almost identical to the rate of entire New York State (Expanded Behavioral Risk Factor Surveillance). The lower rate in the patient population indicates that many of the patients are sufficiently being treated for their hypercholesterolemia. Although the number of comorbidities per patient in this study are not listed, it was observed that many of the patients had both hypercholesterolemia and hypertension as comorbidities with breast cancer.

Hypothyroidism and other thyroid disorder made up a smaller portion of comorbidities. 7.5% of patients experienced hypothyroidism (Figure 3), with 5% of those being diagnosed with hypothyroidism after diagnosis (Figure 4). Similarly, 4% of patients had an unspecified thyroid disorder (Figure 3). Comparatively, other studies have found that breast cancer patients tend to have higher rates of thyroid disorders. Fu et al. (2015) found that of their 134 participants, 22.4% of the population had reported having a thyroid disorder (Fu et al. 2015).

The small percentage of people in this study (10%) (Figure 3) who were diagnosed with some indication of infection was surprising. A closer investigation of people who were diagnosed with infections was conducted (Figure 5). Bronchitis and other respiratory infections were

likely high due to the percentage of people who had COPD (Figure 3) or the rate of patients who had a history of nicotine or tobacco use (not shown). Urinary Tract Infections (UTIs) infected 38% of those with infections (Figure 5). The prevalence of UTIs may be due to hormonal therapies the patient may be taking. Sometimes, the decrease in estrogen via anti-estrogens (to cease proliferation of ER positive breast cancers) can cause vaginal atrophy subsequently causing an impact on the bladder (Chesapeake Urology Associates). The low presence of UTIs in this population indicates that the condition is well managed. This can be said for the overall small percentage of infections in the study cohort. It was thought that breast cancer patients would have an increased presence of infections due to the impact chemotherapy, radiation treatments, and hormone treatments can have immune cells (American Cancer Society 2019).

Other cancers, including lymphatic cancer, affected 16% of the total population (Figure 3). Many times, breast cancer patients have cancer in the associated lymph nodes surrounding the breast tissue (National Breast Cancer Foundation). Additionally, in some cases, breast cancer can spread, becoming metastatic. That was seen in 7.5% of patients diagnosed with various cancers affected different parts of the body (Figure 3). Of those diagnosed with other cancers, 11% of patients were diagnosed with another cancer after breast cancer diagnosis (Figure 4). This number was not surprising as cancer is a brutal disease that can spread rapidly.

While this study only analyzed the comorbidities occurring with breast cancer, there were several limitations that should be considered when understanding the data. From this study, there were only 202 patients, which is a relatively small number of patients compared to other retrospective studies on the subject. In order to have a more expansive understanding of comorbidities in the CPH population, a larger sample size would be needed. Additionally, the data set available for this study was from emergency room visits. All dates of breast cancer diagnosis were considered to be the primary diagnosis from the emergency room from that date. This may have not been the first date that these patients had breast cancer so their comorbidities before and after diagnosis may have been different. Additionally, the emergency room database only had the first three diagnoses of the patient's medical history so there likely could be more comorbidities that was not listed that could impact the rates found in this study.

Other cancer rates were considered in this study, while remission of breast cancer was not. When going back to collect dates of first breast cancer diagnosis, some of the patients likely had remission of breast cancer (not listed in this study). Comorbidities associated with breast cancer remission or other cancers would make an interesting study in this population.

Overall, the goal of this project was to examine if there is any difference in comorbidities associated with rural breast cancer patients compared to breast cancer patients in urban

settings. It was found that breast cancer patients at CPH have higher rates of hypertension than in the rest of New York but was consistent with other studies (Expanded Behavioral Risk Factor Surveillance; Fu et al. 2015). This population also showed lower rates of infection than expected breast cancer populations (American Cancer Society 2019). Despite some limitations in this study, this study provided insight into CPH breast cancer patients and their quality of health.

CHAPTER TWO
EVALUATING APOPTOTIC AFFECTS FROM PROMENSIL IN BREAST
CANCER CELLS

In collaboration with Daniel Crosby (BBT) and James Velez (BBT) for project work and writing the methods chapter

ABSTRACT

Phytoestrogens are compounds from plants that are structurally similar to estrogen. Often, they are found as over-the-counter supplements marketed for post-menopausal symptoms and have been associated with increased risk of breast cancer. Other MQPs have shown that Promensil, a phytoestrogen supplement, has been shown to have antiproliferative effects on the T47D breast cancer cell line. However, the mechanism of action of the antiproliferative effects is unknown. It has been reported that when isolated, genistein, a component of Promensil, has induced apoptosis in breast cancer cell lines mediated through caspase-3. This project aims to examine if the antiproliferative effects of Promensil on the T47D breast cancer cell line may be due to activation of caspase-3, causing apoptosis of the cells.

INTRODUCTION

Breast Cancer

Breast cancer occurs when breast cells rapidly grow out of control, beginning in the ducts or lobules of the breasts and can eventually spread to the rest of the body and typically affects more woman than men (Center for Disease Control and Prevention). As woman age, their risk of breast cancer increases, likely due to the change of hormones during menopause. The average age of diagnosis for woman is 61 and is the most commonly diagnosed cancer among women in the United States, second to skin cancer. There are various forms of breast cancer that can be more severe than others (Center for Disease Control and Prevention; Chalasani).

Breast cancer cells can develop in response to a variety of genetic changes that must be identified to prescribe the proper treatment. Some breast cancers may be due to mutations in HER2, human epidermal growth factor receptor 2, or BRCA1, a tumor suppressor (HER2 Status; National Library of Medicine). However, about two-thirds of all breast cancers can be identified by a positive hormone receptor, either estrogen receptor (ER) or progesterone receptor (PR) positive. ER positive breast cells grow in response to estrogen, whereas PR positive cells grow in response to progesterone. These hormones can bind to the cell receptors as a signal for cell proliferation. Hormone receptor positive breast cancer is the most common form of breast cancer, especially in older woman (American Cancer Society).

Estrogen and its receptors

Estrogens are a group of steroids that are responsible for many growth and developmental changes that occur a woman's life time. It is responsible for changes to a woman's body during puberty, menstrual cycles, pregnancy, breast development, bone growth, and other metabolic processes. The most abundant estrogen is 17 β -estradiol (E2), which is produced in the ovaries and placenta (Women's Health Updates).

Studies have shown that ERs regulate cell proliferation and development in both men and women. There are two main ERs, ER α and ER β , which are encoded by different genes and vary in quantity, depending on the type of cell in which they are found (Lee et al. 2012). ER α is more commonly present in breast, ovary, bone, liver, male reproductive organs, and prostate cells, whereas ER β is more commonly found in bladder, colon, and immune system cells, as well as some overlap between the two. ER α acts as a growth receptor for E2 more often than ER β , which typically has more anti-proliferate affects. (Paterni et al. 2014). ER positive breast cancer cells can have both estrogen receptors, ER α and ER β . Yet, ER α is more likely responsible for increased proliferation in breast cancer cells, than ER β (Lee et al. 2012).

Menopause

As woman age, their bodies change, and their ovaries begin to stop producing estrogen. Decrease in estrogen eventually causes a woman's menstrual cycle to end. During menopause, women may experience a vast number of symptoms and other health risks associated with a loss of estrogen. Some symptoms of menopause may include hot flashes, sleeping problems and night sweat, and vaginal and urinary changes. Menopause also increases the risk of long-term health problems, like osteoporosis and cardiovascular disease. To alleviate the symptoms of menopause, many women resort to taking estrogen supplements- either hormone therapy with progestin or plant-based estrogen substances (The Menopause Years).

Hormone Replacement Therapy

The Food and Drug Administration (FDA) approved the use of estrogen as a supplement to alleviate symptoms of menopause in 1942. Since then, estrogen has become widely used as a hormone replace therapy (HRT) to relieve the side effects of menopause. In 2002, the Women's Health Initiative (WHI) conducted a study to look at the risks of HRT. They found that women who had taken HRT were more likely diagnosed with breast cancer at an advanced stage (Chlebowski et al. 2003). Yet, despite this association of HRT with breast cancer, it is still unknown if breast cancer is a direct result of HRT (Shook 2011). The American College of Obstetricians and Gynecologists (ACOG) explains some of the risks of hormone replacement therapy. They say that estrogen therapy may cause uterine cancer, deep vein thrombosis, or stroke. In addition to systemic estrogen therapy, some woman may choose to take over-the-counter estrogen supplements that may be plant based to supplement the loss of estrogen (The American College of Obstetricians and Gynecologists).

Phytoestrogens

Phytoestrogens are compounds, structurally similarly to estrogen, that are derived from plants. These compounds can be found in commonly consumed foods, such as soy products, peas, beans, flaxseeds, red wine, and tea. A review by Bilal et al. (2014) describes the role phytoestrogen plays on ERs. Phytoestrogens typically have a lower binding affinity to ERs than estrogen, but one study showed phytoestrogens genistein and daidzein have a higher binding affinity specifically to ER β (Kuiper et al. 1998). Other studies have shown that some phytoestrogens can interact with cell growth factors and pathways (Choi and Kim et al. 2008). Particularly, one study showed that at high concentrations genistein was able to inhibit growth factor cyclin D1 in breast cancer cells (Lavigne et al. 2008). Others have shown that

phytoestrogens may inhibit estrogen synthesis all together in ER positive breast cancer cells (Bilal et al. 2014).

Some phytoestrogens are being studied as Selective Estrogen Receptor Modulators (SERMs), which are compounds that can bind to the ER and act as antagonists. Tamoxifen, for example, was the first chemopreventative and SERM approved for breast cancer by the FDA. It is metabolized by the body and broken down into smaller molecules capable of binding to ERs to prevent breast cancer cell proliferation. Like tamoxifen, some phytoestrogens have been shown to have antiproliferative properties or act as antagonists that are able to bind to the estrogen receptor. It is also suggested that some phytoestrogens may be able to bind to ER β preventing stimulation of ER α and further cell proliferation (Oseni et al. 2008).

Isoflavones

Isoflavones are a class of phytoestrogens that are widely present in many food sources. The four isoflavones are genistein, daidzein, biochanin A, and formononetin. Genistein and daidzein are derived from biochanin A and formononetin. Isoflavones are converted in the gastrointestinal tract to structures similar to estrogens (Barnes 2004).

Genistein and daidzein are the most common isoflavones and share a similar phenolic ring structure to estrogen. Both can be found in red clover and soy beans. Genistein has been highly studied and shown to have a higher binding affinity to both ER α and ER β . Additionally, genistein treatment may inhibit cancer cell line growth, including breast cancer. The effect of genistein is determined by the concentration metabolized by the body. Concentrations less than 1 μ M act as an agonist for estrogen, inducing cell growth, whereas concentrations greater than 5 μ M act as an antagonist, shown in MCF-7 breast cancer cells (Sarkar and Li 2002).

Promensil

Promensil is an over-the-counter phytoestrogen supplement containing isoflavones from red clover. It was created to relieve symptoms of menopause and to provide dietary support. The Promensil website says that their double strength 80mg tablets contain isoflavones genistein, daidzein, formononetin and biochanin (About Promensil).

There is limited information on the Promensil website about the effects Promensil, causing other researchers to investigate this phytoestrogen supplement. One study by van de Weijer and Barentsen (2002) showed that Promensil treatments decreased hot flashes in menopausal women. In a randomized placebo-control study, the researchers gave participants either two Promensil tablets at 40 mg or two placebo tablets. The researchers

found that all groups of women had hot flash symptoms at baseline, but the Promensil treatment group had hot flashes reduced by 44% by the end of the study (van de Weijer and Barentsen 2002).

Another study by Setchell et al. (2001) investigated dietary supplements containing isoflavones, including Promensil. Their goal was to determine the content of the supplement using liquid chromatography and mass spectrometry to compare to claims of the manufacturer. They found that Promensil contains 41.7 mg isoflavones per capsule, which is consistent with the manufacturer's claim that each single strength tablet contains 40 mg (Setchell et al. 2001).

Apoptosis

Apoptosis is the process of programmed cell death. This process is normal for homeostasis and occurs as a defense mechanism in many immune cells or as a way to control cell populations. Apoptosis occurs in response to a variety of factors and stimuli, but only affects single cells or small clusters of cells. Some cells die in response to DNA damage from cancer treatments or other drugs. Such stimuli initiate apoptosis through a cascade of cysteine proteases, called caspases. Cell death causes cells to lose their natural shape, shrink and for chromatin to condense. Cell membranes remain intact with cytoplasm staying inside the membrane. Once a cell dies, macrophages, which are immune cells capable of ingesting cells, clean up the dead cells from floating around in the body (Elmore 2007).

Not all apoptosis occurs in the same way or is activated by the same signal. There are three main pathways of apoptosis: extrinsic, intrinsic, and perforin. Extrinsic pathway is activated by cell death receptors; intrinsic pathway is activated by other stimuli, including hormones, cytokines, toxins, hypoxia, or hypothermia; and perforin pathway is initiated by T-cells responsible for mediating cytotoxicity (Elmore 2007).

Cell death is essential for normal health and growth. Overpopulation of cells can cause cell crowding, which prevents cells from forming connections with one another. Apoptosis is also a means of removing pathogenic cells from the body. In some cases, apoptosis is inhibited by oncogenes, as seen in some lymphoma studies (Elmore 2007).

Caspase-3 and apoptosis

Caspases are proteases responsible for cleaving proteins to initiate cell death. They remain inactive until they are activated by an apoptosis signal or another caspase. Some caspases act as initiators for a cascaded response; others act as effectors, which are responsible for continuing an apoptotic response. Particularly, caspase 3 is an effector caspase that has been

widely studied to demonstrate their role in apoptotic events (Mooney et al. 2002). It plays an essential role in cell shrinkage, blebbing, chromatin condensation, and DNA fragmentation during cell death. Caspase-3 has also been studied in caspase-3 knockout mice. These mice died a few weeks after birth with phenotypic defects, suggesting it is not only critical for its role in apoptosis, but also general survival (Porter et al. 1999).

In general, studies have been conducted to look at apoptosis in breast cancer cell lines. T47D breast cancer cell line, for example, are caspase-3 positive and shown to undergo apoptosis in response to a protein kinase inhibitor in a manner mechanistically different than caspase-3 negative breast cancer MCF-7 cells (Mooney et al. 2002). Other studies have looked at the effect of phytoestrogens on cancer cells, particularly treating cells with genistein. One study has shown that treating breast cancer MCF-7 cells with genistein has positive proapoptotic effects with caspase-3 activation (Lecomte et al. 2017). Sarkar and Li (2002) found that genistein could also induce apoptosis in MCF-7 breast cancer cells, prostate cancer cell lines, and lung cancer cells (Sarkar and Li 2002).

T47D Breast Cancer Cell Line

T47D cells are a breast cancer cell line derived from 54-year-old female with infiltrating ductal carcinoma breast cancer isolated from mammary glands. They are an adherent epithelial cell line with both estrogen and progesterone hormone receptors that also express the WNT7B oncogene (American Type Culture Collection). T47D cells are also positive for caspase-3, therefore, they are an ideal model for examining apoptotic effects as a result of any treatment (Mooney et al. 2002).

Evaluating the Effects of Promensil on T47D cells

This project is a continuation of the phytoestrogen project at Worcester Polytechnic Institute (WPI). One project has shown that proliferation of T47D cells is inhibited at higher concentrations of phytoestrogens (Gergel et al. 2010). Another project, using T47D cells in which expression of ER β is turned off in the presence of tetracycline (Storm et al. 2004), showed no difference in the number of T47D cells affected by Promensil in the presence or absence of tetracycline (Wambach 2018).

T47D cells are used as a model breast cancer cell line to examine if there are apoptotic effects after treatment with Promensil. Studies, as mentioned previously, have shown that genistein, a component of Promensil, induces apoptosis in breast cancer cell lines. It is likely that the anti-proliferation effect that Promensil has on T47D cell line may be due to activation of apoptosis by Promensil.

METHODS

Promensil Extraction

Two double strength (80mg) Promensil tablets were crushed into a fine powder in a mortar and pestle and added to 80% methanol (100mL) in a 250 mL round bottom flask. The flask was put into a reflux condenser for one hour in a 70°C water bath. The solution was vacuumed filtered through a filter paper funnel and stored at -20°C. Extract was filter-sterilized using a bottom-top vacuum filter in the biosafety hood before use and stored at -20°C.

Cell Maintenance

T47D cells were obtained from ATCC. Cells were grown in DMEM supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin (Pen/Strep 100X). Insulin (0.01mg/mL) was added directly to the flask. Cells were plated in T25 or T75 flasks, depending on usage, and incubated at 37°C and 5% carbon dioxide. Cells were maintained and passaged as needed.

Cell Plating and Synchronization

T47D cells were plated in 12 well plates at 150,000 cells per well in regular growth media with insulin. After 24 hours, media was changed to serum-free, insulin free DMEM media and 1% Pen/Strep in order to synchronize cells in the cell cycle. 24 hours later, media was changed again to phenol-red free DMEM (PHRED) supplemented with 10% charcoal-stripped FBS and 1% Pen/Strep. PHRED media was used to limit the estrogenic effects on the cells caused by the phenol red structure that is like estrogen. Charcoal-stripped FBS is used to be certain there are no endogenous estrogens in the serum. At this time, 100nM β -estradiol dissolved in methanol or 10 μ L Promensil was added. Untreated controls also included 10 μ L of methanol. Cells were treated for 48 hours before being harvested.

Cell Harvesting

After treatment, media was aspirated, and cells were washed with 1mL cold PBS on ice. Cells were lysed by freezing at -80°C for 10 minutes. In the first well of each treatment, cells were scraped into 200 μ L cold 1X Phosphate Buffered Saline (PBS) by a rubber spatula and transferred to the next duplicate well. This was done until all replicates were pooled, and samples were transferred to 1.5mL microfuge tubes. Lysates were then incubated for 10 minutes on ice, vortexed, and incubated for 10 additional minutes on ice. Lysates were then

spun down at 13,000xg for 5 minutes. Supernatants were transferred to new 1.5mL microfuge tubes and stored at -20°C until use.

Immunoblotting

Immunoblotting was used to determine the presence of proteins of interest. Concentration of protein from lysed cells was determined using NanoDrop Lite Spectrophotometer (Thermo Scientific). 10µg or 15µg of each sample was combined with 6X Sample Buffer (Appendix A). Samples were vortexed and proteins were denatured in a 95°C temp block for 5 minutes. Samples were put on ice for 2 minutes. Samples were loaded on 12% acrylamide 10 well Mini Protean Precast Gels (Bio-Rad) based on the amount of total protein for all three samples. 1X SDS Running Buffer (Appendix A) was added to gel apparatus (Bio-Rad). Gel ran at a constant 200V until dye was about to run off the gel. Following gel electrophoresis, filter paper and polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore) were cut to fit the approximate size of the gel. Filter paper was soaked in semi-dry transfer buffer (Appendix A), drained, and layered on Semi-Dry Electroblotter (Owl Separation Systems). Precast gel was cut with a razor to remove well lanes and dye runoff. Gel was put in transfer buffer and placed on top of three filter papers of Semi-Dry Electroblotter, protein side up. PVDF membrane was wetted with methanol, then soaked in transfer buffer. PVDF was placed on top of gel, followed by three more filter papers. Gel was transferred at amplitudes equivalent to 0.8 cm² of gel area on apparatus for 1 hour. Following transfer, PVDF membranes were cut in half using a razor and put in blocking solution of 1% low fat instant dried milk in 1X Tris-Buffered Saline with 0.1% Tween-20 (TBS-T) for 30 minutes at room temperature on rocking platform. Primary antibody dilutions of 1:1000 of caspase-3 p17 (sc-271028-Santa-Cruz Biotechnology) were made in 1X TBS-T and kept cold. Blocking buffer was discarded and primary antibody added and incubated at 4°C on rocking platform overnight. Following incubation, immunoblots were washed 3 times for 5 minutes each in 1Xd TBS-T at room temperature on rocking platform. Secondary antibody, IgGk BP-HRP (sc-516102-Santa-Cruz Biotechnology), was diluted 1:5000 in 1X TBS-T. Immunoblots were incubated in secondary antibody for 1 hour at room temperature on rocking platform. Blots were washed again 3 times for 5 minutes in 1X TBS-T at room temperature on rocking platform. Blots were stained with colorimetric 1-Step Ultra TMB Blotting-Solution (ThermoFisher Scientific) for 5-30 minutes or until bands had developed. Reaction was stopped by added MilliQ water two times the amount of the blotting solution. Blots were imaged using ChemiDoc XRS Gel Photo Documentation System (BioRad).

Following transfer, gels were stained with GelCode Blue (ThermoFisher Scientific) to ensure an optimal transfer. Gels were washed 2 times for 5 minutes with MilliQ water at room

temperature on rocking platform. GelCode Blue was added to cover the gel and left to stain at room temperature on rocking platform for 15-60 minutes. Gel was de-stained in multiple washes with MilliQ water.

RESULTS

T47D cells treated with E2 or Promensil were lysed and used for immunoblotting as described in Methods. Figure 6 shows one of three immunoblots prepared as described using an antibody to caspase 3.

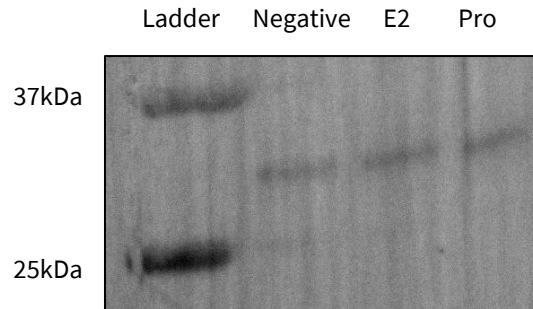


Figure 6. Immunoblot of caspase-3. Procaspase-3 has a molecular weight of 32kDa and fragment caspase-3 has a molecular weight of 17kDa. 15 ug of total protein were added to each well of a 12% polyacrylamide gel and transferred to PDVF. Negative= Methanol control, E2= β -estradiol, and Pro=Promensil.

The immunoblot in Figure 6 shows the presence of procaspase-3 in all cell treatment groups at varying absorbance intensity. Although the antibody should recognize both the procaspase and the activated fragment, the immunoblot does not show distinct bands where the fragmented activated caspase-3 would be found, suggesting that caspase was not activated in either treatment group. Immunoblotting for caspase-3 was done three times over the course of project with similar results. Absorbance intensities for the three blots were averaged, seen in Figure 7.

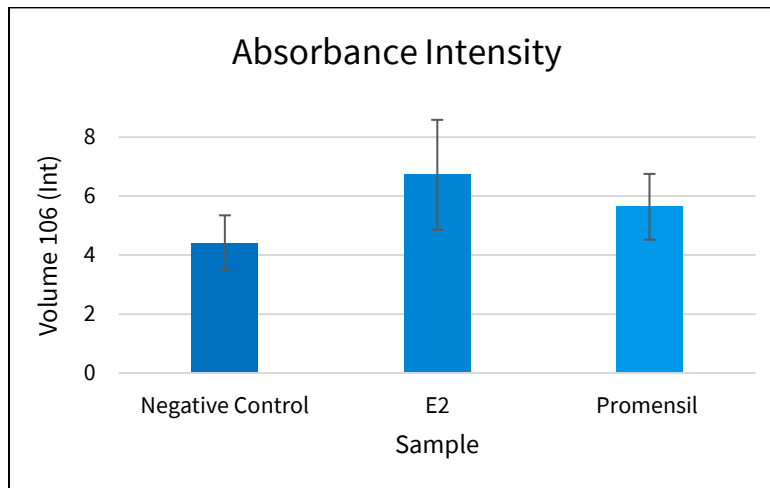


Figure 7. Absorbance intensities for immunoblots of caspase-3 shown in volume intensity. Error bars represent standard error of the mean (n=3).

Figure 7 shows the average absorbance intensity for the three caspase-3 immunoblots. Caspase 3 is evident in three treatments, consistent with expectations for T47D cells. However, cells treated with E2 show the highest average absorbance intensity. Cells with Promensil treatment show a higher average absorbance than the negative control but lower than the E2 treated cells.

DISCUSSION

The goal of this project was to test the hypothesis that Promensil decreases cell proliferation in T47D breast cancer cells by increasing the rate of apoptosis. The marker to examine apoptosis was caspase-3, an important protease responsible for initiating the final step of apoptosis in cells. It plays a role in cell shrinkage, blebbing, chromatin condensation, and DNA fragmentation, which are essential for cell death (Porter et al. 1999). Immunoblotting was the technique used to determine the level of caspase-3 in cells treated with E2 or Promensil. It was hypothesized that Promensil treated T47D cells would have increased levels of activated caspase-3, indicating that increased levels of apoptosis were the reason for the antiproliferative properties that Promensil has on T47D cells.

From the immunoblot in Figure 6, caspase-3 is present in both the negative, E2 and Promensil groups. This was seen in other immunoblots (not shown) conducted over the course of the project and is consistent with T47D cells being characterized as caspase 3 positive. When caspase-3 is activated, it is cleaved into a smaller fragment, seen at a molecular weight at 17kDa. In all immunoblots, no distinct band of fragmented caspase-3 could be seen, suggesting that the protein was not activated by either treatment group. To quantify the absorbance intensity, volume intensity from ChemiDoc XRS Gel Photo Documentation System (BioRad) was obtained. Figure 7 shows that E2 has a higher absorbance intensity of the caspase-3 band compared to the Promensil treatment or the negative control. This result was unexpected since E2 stimulates cell growth of T47D breast cancer cells. However, the variability in the data results in there being no statistically significant difference, suggesting that there is no difference in the inactivated form of the protein as a result of any treatment, including E2 and Promensil.

Final conclusions are unable to be drawn from the immunoblots done in this project. Additional experiments with increased exposure time and varying Promensil concentrations need to be conducted in order to validate the conclusion that Promensil does not increase the rate of apoptosis in T47D cells.

Previous MQPs have demonstrated that Promensil causes T47D cells to become antiproliferative (Gergel et al. 2010, Wambach 2018). However, the mechanism of action is unknown. Promensil is a substance that is unregulated by the FDA so it is difficult to understand what is in the supplement aside from the isoflavones that Promensil guarantees is in the supplement. It is possible that Promensil is cytotoxic to other human tissues, including noncancerous cell lines. Exploring the effects of Promensil on other cells lines would be necessary to determine if the antiproliferative consequences to Promensil

treatment are cell line specific in T47D breast cancer cells or if Promensil is cytotoxic to other cell lines.

Additional possibilities are that Promensil interferes with progression through the cell cycle. This possibility can be examined using similar experimental approaches, but looking at proteins such as the cyclins, CDKs or transcriptional regulators, such as p21.

The idea of using phytoestrogens as a treatment to breast cancer is promising, yet there are many complications and nuances still left to be determined. Specifically, genistein, an isoflavone found in Promensil, has been demonstrated to induce apoptosis in breast cancer cell lines, including MCF-7 cell lines (Sarkar and Li 2002). Lecomte et al. (2017) reviewed that genistein activated caspase-3 in breast cancer cell lines (Lecomte et al. 2017). While it is possible that the effects of genistein in Promensil may cause breast cancer cells to have increased rates of apoptosis, more studies will need to be conducted in order to validate any final conclusions.

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APPENDIX A

6x Sample Buffer (The Open lab Notebook):

10% B-mercaptoethanol

Glycerol

20% SDS

1M Tris-HCl

9mg Bromophenol

1x Running Buffer (Abcam):

25mM Tris-base

190mM Glycine

0.1% SDS

Semi Dry Transfer Buffer (Abcam):

48mM Tris-base

39mM Glycine

0.04% SDS

20% Methanol