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# Formulating a composite scaffold with tissue regeneration and anti-cancer activity

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# FORMULATING A COMPOSITE SCAFFOLD WITH TISSUE REGENERATION AND ANTI-CANCER ACTIVITY

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# ABSTRACT

Breast cancer affects 1 in 8 women in the United States and is the second most commonly diagnosed cancer among women. Many treatment options, such as chemotherapy or surgery, are invasive, ineffective, or detrimental to the body.

It has been previously demonstrated that tannic acid has anti-cancer properties and is effective in killing cancer cells while not adversely affecting healthy cells. Tannic acid crosslinks collagen type I. We have proposed using tannic acid crosslinked collagen type I beads to fill in the void left after surgery while simultaneously treating any remaining cancer cells after a breast cancer patient has a lumpectomy. This will aid in killing any remaining cancer cells while helping to regrow the void left in the tissue by first growing the patient's adipocytes on the beads before delivery.

Matrigel®, an extracellular membrane-based (ECM) hydrogel is thermoresponsive, which is ideal for the matrix that will be used. Expanding on previous work, adipocyte-seeded beads are mixed with Matrigel® and placed in Transwell inserts should allow us to determine if TA is capable of diffusing out the matrix and treating the $HER2^+$ breast cancer cells in the bottom of the wells. Imaging and cell counting determined that tannic acid had an effect on the $HER^+$ breast cancer cell. Western blots were conducted to determine changes proteins in protein expression after three days of treatment with varying concentrations of tannic acid. FC assays determined the amount of tannic acid in the media after the treatment period. The permeability of tannic acid and ability to diffuse through the gel is important for efficient treatment of breast cancer cells.

The findings from this proof of concept experiment will allow for further research to be performed on novel cancer treatments that are less invasive for patients. Future research includes finding an alternative matrix in which to deliver these cells that has properties like Matrigel®. Additionally, ideal bead size and specific concentration of tannic acid will be identified to ensure beads are deliverable and effective.

# DEDICATION

I would like to dedicate this research to my parents, Kim and Kelly Williams. Words cannot describe how thankful I am to have you both as my support system, but I'm going to try anyway. We share more than just the same initials and I am so thankful to take after both of you. Whenever people ask me what inspired me to major in bioengineering, I always tell them that my mom is a biology major and my dad is an engineering major. Thank you for exposing me to so many different things when I was little, whether it was Take Your Child to Work Day, playing on the Kubota, or sending me to Alabama for space camp.

Mom, although I went to college 300 miles from home, I knew you were never more than a phone call away. Whether it was help writing an email or a random "how do I be an adult" question, you have always been there for me when I needed the most. I love all of our shopping trips, failed baking attempts, and Whose Line marathons.

Daddy, thank you for always being there for me, whether it is to help me with homework or talk me through how to set up an awesome sub system. You've taught me so much about how to solve problems and be more independent (even if I do need you to be my handyman sometimes). I can always count on you to cheer me up, whether it's with selfies with Ari or one of your analogies.

Thank you both for always believing in me, sending me kitty pictures, and being a shoulder to lean on. I love you more than you love riding Toy Story at Disney.

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I would first like to thank my parents for all the support they have provided me throughout my entire life. Thank you for always believing I could do whatever I set my mind to, and for always encouraging me to go after what I want. I would not be the person I am today without the both of you. I would also like to thank my advisor, Dr. Brian Booth, for his direction and support throughout this entire project. I have been so grateful to work in a lab that allows for students to learn so much and become better scientists. I would also like to thank my fellow Institute for Biological Interfaces of Engineering lab and staff members. Heather Gregory, Bailey-Jean Walker, and Chris Moody helped me tremendously throughout this process and I could not have done it without their assistance. I would also like to thank Scheen Thurmond for always being more than willing to help whenever I ran into problems. I would like to thank the Clemson University Bioengineering Department for all the opportunities they have provided me throughout both my undergraduate and graduate careers. I could not ask for a better place to have earned my degrees. The professors and departmental staff provided so much help and support and I could not be more thankful. In particular, I would like to thank Maria Torres for all of her help and guidance, and always remaining calm in my times of panic. Lastly, I would like to thank Dabo's All In Team ® Foundation for making this research possible with their funding.

TABLE OF CONTENTS

Page

TITLE PAGE ..... i

ABSTRACT ..... ii

DEDICATION ..... iv

ACKNOWLEDGMENTS ..... v

LIST OF FIGURES ..... viii

CHAPTER

I. INTRODUCTION AND BACKGROUND ..... 1

1.1 Project Overview ..... 1
1.2 Breast Cancer Overview ..... 1
1.3 Breast Cancer Statistics ..... 2
1.4 Breast Cancer Stages ..... 3
1.4.1.1 Stage 0 ..... 3
1.4.1.2 Stage I ..... 4
1.4.1.3 Stage II ..... 4
1.4.1.4 Stage III ..... 5
1.4.1.5 Stage IV ..... 6
1.5 Hormone Receptor and HER2 Classifications ..... 6
1.6 Clinical Treatment Options ..... 7
1.7 Polyphenols ..... 8
1.7.1 Tannins ..... 9
1.8 Previous Work ..... 9
1.8.1 Tannin Effects on $ER^+$ Breast Cancer ..... 9
1.8.2 Tannin Effects on $HER2^+$ Breast Cancer ..... 10
1.8.3 Tannin Effects on Triple Negative Breast Cancer ..... 10

II. MATERIALS AND METHODS ..... 12

2.1 Collagen Bead Preparation ..... 12
2.2 2D Cell Culture ..... 12
2.3 Cell Seeding on Collagen Beads ..... 13

Table of Contents (Continued) Page

2.4 Co-culture of BT474 Cells with TA cross-linked beads with SW872 cells ..... 13
2.5 Imaging of Cells ..... 14
2.6 Media Collection ..... 14
2.7 Protein Collection ..... 14
2.8 Western Blotting ..... 15
2.9 Folin-Caiocalteau Assay ..... 15
2.10 Cell Counts ..... 15
2.11 Statistical Analyses ..... 16

III. RESULTS AND DISCUSSION ..... 17

3.1 Imaging ..... 17
3.2 Cell Counts ..... 18
3.3 Folin-Ciocalteau Assay ..... 18
3.4 Western Blotting ..... 20

IV. CONCLUSIONS ..... 22

4.1 Conclusions ..... 22
4.2 Future Research ..... 23

REFERENCES ..... 25

LIST OF FIGURES

Figure Page

1.1 TA Crosslinked Collagen Type I Seeded with Adipocytes ..... 1

2.1 Experimental Setup ..... 13

3.1 Images of BT474 breast cancer cells after treatment ..... 17

3.2 Cell Counts after 3 days of treatment ..... 18

3.3 TA levels in media below SW872 seeded TA crosslinked collagen beads ..... 19

3.4 Western Blots for Bcl-2, β-actin, and P-SAPK ..... 20

3.5 Apoptosis Pathway ..... 21

CHAPTER ONE

# INTRODUCTION AND BACKGROUND

## 1.1 Project Overview

A current treatment for breast cancer is surgery to remove the tumor from the breast, which results in a void. This project aims to create collagen type I beads crosslinked with tannic acid (TA) to prevent the recurrence of breast cancer and to help fill the void left after surgery. Figure 1.1 shows the hypothetical method in which beads are created and seeded with a patient's adipocytes, loaded into a syringe, and injected into the surgery site. The collagen is remodeled as the adipocytes grow and attach to the beads. This allows for the release of the TA, which then interacts with any remaining cancer cells. The TA then induces apoptosis in the cancer cells.

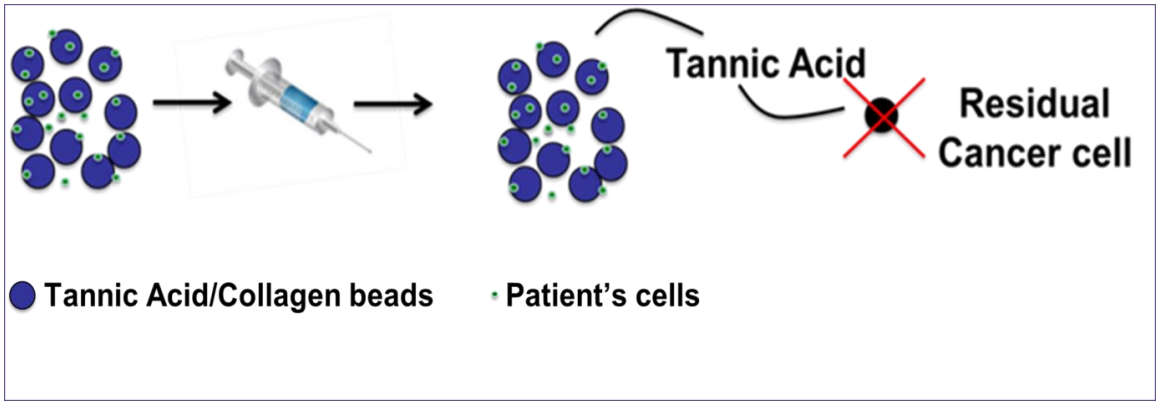


Figure 1.1: Tannic acid collagen type I-crosslinked beads with patient's adipocytes seeded form the basis for a tissue regeneration matrix with anti-cancer properties.

## 1.2 Breast Cancer Overview

Breast cancer is a type of cancer that develops from the cells within the breast (American Cancer Society, 2017). Although it can present itself differently in patients,

typical signs and symptoms of breast cancer are skin changes (such as redness or swelling), an increase in size or change in shape of the breasts, general pain in/on any part of the breast, irritated or itchy breasts, changes in breast color, and many others (Cancer Treatment Center of America, 2017). Briefly, breast cancer begins with a mutation in cells within the breast that allows these mutated cells to grow out of control, which results in a tumor. Most forms of breast cancer begin in the ducts that carry milk to the nipple, while others start in the alveolar cells that make milk (Cancer Treatment Center of America, 2017). Breast cancer spreads once the cancer cells reach the blood or lymph system and are carried to other parts of the body (American Cancer Society, 2017). Tumors can be benign, meaning they are not a threat, or malignant, meaning they are cancer and can spread to different parts of the body (Cancer Treatment Center of America, 2017). Though breast cancer is predominantly found in women, it can also affect men (National Breast Cancer Foundation, 2017).

Breast cancer is diagnosed several different ways, including imaging tests such as mammograms or ultrasound, or a biopsy, which is completed after an abnormal mammogram (American Cancer Society, 2017). Some biopsies use a needle, while others use an incision. A tissue sample is taken to a lab where a pathologist analyzes it and determines if the tissue is cancer. A biopsy is the only sure way to diagnose a patient with breast cancer (American Cancer Society, 2017).

### 1.3 Breast Cancer Statistics

Breast cancer is the most commonly diagnosed cancer among women in the USA

and has the second highest death rate for cancers among women (American Cancer Society, 2017). Data from 2009-2013 indicate that there were 123.3 new cases and 21.1 deaths per 100,000 women, making this a relevant problem in modern society (American Cancer Society, 2017). Recent improvements in mammography screening, improved treatments, and early detection have helped to decrease the mortality rate in the past several years. However, there is still a need to develop better treatment methods as it is predicted that there will be 252,710 new cases of invasive breast cancer in 2017 (American Cancer Society, 2017).

## 1.4 Classifications of Breast Cancer

### *1.4.1 Breast Cancer Stages*

The stages of breast cancer are determined based on the size of the tumor within the breast, the number of lymph nodes affected, and signs indicating whether the breast cancer has invaded other organs (American Cancer Society, 2017).

#### *1.4.1.1 Stage 0*

Stage 0 breast cancer is characterized as a non-invasive cancer where abnormal cells have been found in the breast. In this stage, there is no evidence of cancer cells or non-cancerous abnormal cells invading other parts of the breast (American Cancer Society, 2017).

*1.4.1.2 Stage I*

Stage 1 breast cancer is an invasive breast cancer in which cancer cells are invading the normal surrounding breast tissue. It can be divided into two subcategories: Stage IA and Stage IB (American Cancer Society, 2017).

Stage IA is characterized by:

- tumors that are up to 2 cm
- the cancer has not spread outside the breast or into any lymph nodes

Stage IB indicates:

- there is not tumor in the breast, but rather small groups of cancer cells in the lymph nodes
- or there is a tumor in the breast no more than 2 cm
- or there are small groups of cancer cells in the lymph nodes

*1.4.1.3 Stage II*

Stage 2 breast cancer is divided into two subcategories: IA and IB (American Cancer Society, 2017).

Stage IIA implies:

- no tumor can be found in the breast, but cancer is found in 1-3 axillary lymph nodes
- or the tumor measures greater than 2 cm and has spread to the axillary lymph nodes
- or the tumor is between 2-5 cm but has not spread to the axillary lymph nodes

Stage IIB is characterized by

- a tumor between 2-5 cm and small groups of breast cancer cells in the lymph nodes
- or a tumor between 2-5 cm along with cancer in 1-3 axillary lymph nodes or to lymph nodes near the breastbone
- or a tumor larger than 5 cm that has not spread to the axillary lymph nodes

*1.4.1.4 Stage III*

Stage III breast cancer can be divided into three subcategories: IIIA, IIIB, and IIIC (American Cancer Society, 2017).

Stage IIIA indicates:

- no tumor is found in the breast or the tumor may be any size
- cancer is found in 4-9 axillary lymph nodes or lymph nodes near the breastbone
- or the tumor is larger than 5 cm and there are small groups of breast cancer cells found in the lymph nodes
- or the tumor is larger than 5 cm and the cancer has spread to 1-3 axillary lymph nodes or to the lymph nodes near the breastbone

Stage IIIB denotes:

- the tumor may be any size and has spread to the chest wall and/or skin of the breast and has caused swelling or an ulcer
- or the cancer may have spread to up to 9 axillary lymph nodes
- or the cancer may have spread to lymph nodes near the breast bone

Stage IIIC is an invasive cancer in which:

- there may be no sign of cancer in the breast
- or if there is a tumor, it may be any size and have spread to the chest well and/or the skin of the breast
- cancer may have also spread to 10 or more axillary lymph nodes
- or the cancer has spread to lymph nodes above the collarbone
- or the cancer has spread to axillary lymph nodes or to lymph nodes near the breastbone

*1.4.1.5 Stage IV*

Stage IV breast cancer indicates that the cancer is invasive and has spread beyond the breast and nearby lymph nodes to other organs of the body. This stage can be described as "advanced" or "metastatic" (American Cancer Society, 2017).

## 1.5 Hormone receptor & HER2 classifications

Breast cancer can be classified by the presence or absence of hormone receptors (such as estrogen receptor and progesterone receptor) and the status of the HER2 protein (human epidermal growth factor receptor-2) (American Cancer Society, 2017). After a pathological examination, the cancer is classified as hormone receptor-positive or receptor-negative. For example, a cancer could be assigned the classification of estrogen receptor-positive ($ER^+$) and progesterone receptor-positive ($PR^+$), HER2-positive, triple positive (presence of all three receptors), or triple negative (absence of all three receptors). Breast

cancers that are classified as $ER^+$ have the best prognosis, while those that are classified as triple negative have the worst prognosis (American Cancer Society, 2017).

### 1.6 Clinical Treatment Options

Current breast cancer treatment options include surgical intervention, radiation therapy, chemotherapy, hormone therapy, and targeted therapy (American Cancer Society, 2017). Many factors influence the choice of treatment option, such as patient's age, general health, patient preferences, the stage of the tumor, the tumor's subtype, and genomic markers. Surgery as a treatment for breast cancer involves the removal of the tumor and some surrounding healthy tissue (American Cancer Society, 2017c). Radiation therapy uses high-energy x-rays or other particles to kill cancer cells. Chemotherapy is the use of drugs to destroy cancer cells by stopping the cancer's ability to grow and divide. Chemotherapies are administered intravenously or orally. Hormonal therapy is commonly used for tumors that test positive for either estrogen or progesterone receptors and involves the blocking of the hormone receptors (American Cancer Society, 2017). Lastly, targeted therapy involves the treatment of the cancer's specific genes, proteins, or tissue environment. This is a very focused treatment that works differently than chemotherapy to block the growth and spread of cancer cells while minimizing damage to healthy cells (American Cancer Society, 2017).

While these procedures and treatments have shown to be effective, each carries its own set of disadvantages. Surgery is prone to infection or excessive bleeding, along with long recovery times. There is also the threat of damage to nearby tissues. Radiation therapy

can result in the hardening of the arteries, soreness, skin irritation, and swelling (American Cancer Society, 2017). Chemotherapy has side effects such as heart or nerve damage, damage to normal cells, fatigue, hair loss, and nausea and vomiting (American Cancer Society, 2017). Hormonal therapy side effects include bone and joint pain, bone thinning, nausea, headaches, and weight gain. The side effects of targeted therapy include pain, seizures, changes in mood or thinking, fatigue, and swelling (American Cancer Society, 2017). This extensive, incomplete list of side effects from existing therapies leads to the necessity for a novel treatment of breast cancer to lessen the recurrence of cancer and to rebuild functional tissue within the breast.

### 1.7 Polyphenols

Polyphenols are naturally occurring compounds that are characterized by their multiple hydroxyl groups attached to an aromatic ring (Vermerris & Nicholson, 2008). They are a secondary metabolite of plants and have been of increasing interest for their defense against UV radiation or aggression by pathogens. There have been more than 8,000 polyphenolic compounds identified in various plant species, and all arise from a common intermediate, phenylalanine, or shikimic, which is a close precursor (Chung et al., 1998). Classification of polyphenols is determined by the numbers of phenol rings they contain and on the basis of structural elements that bind these rings together. The four main classes of polyphenols are phenolic acids, flavonoids, stilbenes, and ligands (Pandey & Rizvi, 2009).

### *1.7.1 Tannins*

Tannins are a subset of polyphenols that are water-soluble and can be classified as hydrolysable or condensed (Petridis, 2006). The hydrolysable groups exhibit a polyhydric alcohol core with hydroxyl groups that have been either partially or wholly esterified by gallic acid or hexahydroxydiphenic acid. The condensed group of tannins are usually products of polymerized lavan-3-ols and flavan-3,4-diols (Chung et al., 1998). Tannins are present in many plant-based foods, such as wine, green tea, and coffee (Pandey & Rizvi, 2009). Research indicates tannins are anti-carcinogenic and are a possible source of treatment for many different types of cancer (Chung et al., 1998). As tannins are widely available, they are a promising candidate for a novel, therapeutic anticancer agent (Stich, H. & Rosin, 1984).

## 1.8 Previous Work

### *1.8.1 Tannin Effects on $ER^+$ Breast Cancer*

It has been previously demonstrated that $ER^+$ breast cancer is susceptible to the anti-carcinogenic effects of TA in numerous studies (Bawadi, et al., 2005; Booth et al., 2013; Fujita, et al., 2001; Ngobili et al., 2015). Some tannins, such as ellagic and gallic acid, do not exhibit estrogenic activity (Zand, et al., 2000), therefore, more recent studies have focused on other mechanisms of interference.

We (Booth et al., 2013) have shown that TA causes changes to the morphology of MCF7 $ER^+$ breast cancer cells from spindle shaped to round cells. We also found that TA induced significant apoptosis in these cells after 24 hours of treatment. This indicates that

these cells are sensitive to the apoptotic effects of TA. In addition, cell viability was decreased and apoptotic activity was increased due to TA exposure. It was determined that this mechanism is through the activation of caspases 3/7 and 9.

### *1.8.1 Tannin Effects on HER2$^+$ Breast Cancer*

It has been shown that tannins are cytotoxic to HER2$^+$ and trastuzumab-resistant HER2$^+$ breast cancer cells in a dose-dependent manner (Huang, et al., 2011; Moongkarndi et al., 2004; Signoretti et al., 2002). The mechanism of tannin action has been widely studied in HER2$^+$ breast cancer cells (Booth et al., 2013; Jordan and Booth, 2016;submitted; Ngobili et al., 2015).

We (Jordan and Booth, 2016; submitted) showed that TA induces apoptosis in HER2$^+$ breast cancer cells through a mechanism responsible involving activation of caspases 3/7 and 9. There was a significant reduction in cell proliferation and growth in the HER2$^+$ breast cancer cells after exposure to TA.

### *1.9.3 Tannin Effects on Triple Negative Breast Cancer*

Triple negative breast cancer cells do not respond to normal hormonal therapies (Triple Negative Breast Cancer Foundation, 2017). The effects of tannins and other chemopreventative agents have been of particular interest and have been the subject of many different studies using triple negative breast cancer cell lines.

We previously published results (Booth et al., 2013) of a study that used collagen type-1 beads crosslinked with TA. This was done to further understand the effects of

tannic acid on different types of breast cancer cell lines. Though the effects of TA were less significant on the triple negative breast cancer cells when compared to other types of breast cancer cells, a change in morphology was still observed. In addition, the proliferation of the treated cells was lower than that of the untreated cells. This indicates that apoptosis was induced in the triple negative cells that were treated. It was postulated that this was done via caspase activation. Although not as dramatic as other types of breast cancers, it was shown that there were elevated levels of caspases 3/7 and 9 when the cells were treated with TA. The effects of TA on these cells were shown to be both dose- and time-dependent.

# CHAPTER TWO

# MATERIALS AND METHODS

## 2.1 Collagen Bead Preparation

TA crosslinked collagen beads were prepared as previously described (Booth et al., 2013; Ngobili et al., 2015). Briefly, a 1mg/ml collagen type I solution was prepared using a stock solution of 3.1 mg/ml (Advanced Biomatrix, Poway, CA) (Vernon, et al., 2005),. The TA cross-linked beads were prepared using a Nisco Encapsulation Unit VAR V1 electrostatic syringe pump loaded with a 40:60 of 1.4% alginate:1mg/ml collagen solution in MilliQ water (Sigma Aldrich, St. Louis, MO). The pump was programmed with the following settings: Volume: 20 mL, Rate: 10 mL/hr, Dia: 15. The solution was pumped into a 1.5% $CaCl_2$ (mass/volume) solution (Fisher Scientific; Fair Lawn, NJ). One hour after formation, the beads were filtered out using a mesh strainer and placed in a TA crosslinking solution comprised of either 0.1%, 1.0%, or 10.0% (wt/vol) TA/MilliQ water. Twenty-four hours later, the beads were strained once again in a mesh strainer and then placed in a 50 mM sodium citrate solution (Fisher) for 3 hours, then strained and stored in phosphate buffered saline (PBS) at 20$^{o}$C.

## 2.2 2D Cell Culture

The pre-adipocyte SW872 cell line and the human $HER2^{+}$ breast cancer BT474 cell line were obtained from ATCC (Manassas, VA). The SW872 cells were cultured using Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (ATCC), and the BT474 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (ATCC).

To enhance each media type, 10.0% fetal bovine serum (FBS) (Corning Life Sciences), 1.0% antibiotic-atimycotic (AA) (Gibco; Great Island, NY), and 0.2% fungizone (Gibco) were added. The cells were incubated at 37 $^{o}$C with 5.0% $CO_2$.

### 2.3 Cell Seeding on Collagen Beads

Confluent 2D cultures of SW872 cells were trypsinized and counted. For each experiment, 1.0 x $10^6$ cells were added to 50 ml roller tubes with 10 ml medium containing TA-crosslinked collagen type I beads. The roller tubes were rotated at 3 rpm and incubated at 37 $^{o}$C with 5.0% $CO_2$.

### 2.4 Co-culture of BT474 cells with TA cross-linked beads with SW872 cells

Confluent 2D cultures of BT474 cells were trypsinized and counted and 1.0 x $10^5$ cells were seeded per well in a Costar 24-well Transwell® plate (Corning Life Sciences; Corning, NY). The plate was incubated at 37$^{o}$C with 5.0% $CO_2$ for 24 hours. Each of the three concentrations of tannic acid crosslinked beads were put in the Transwells in triplicate. Growth factor-reduced Matrigel® (Corning Life Sciences) was placed in the well on top

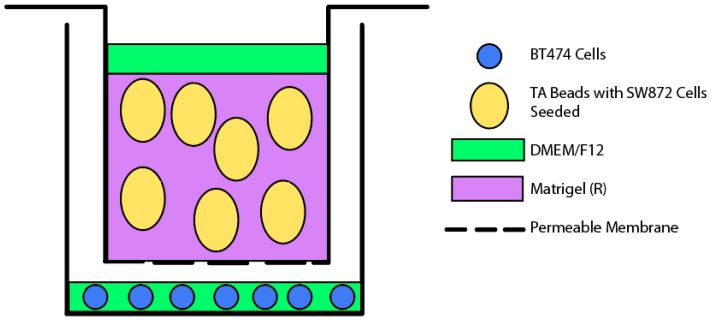


*Figure 2.1: Co-culture of cells and beads*

of the beads and incubated at 37 $^{o}$C with 5.0% $CO_2$ until the Matrigel solidified. DMEM/F12 was added to each well and the plate incubated at 37 $^{o}$C with 5.0% $CO_2$.

**2.5 Imaging of Cells**

After 24 h, images were acquired of the four different groups of BT474 cells (those treated with 10% TA, 1.0% TA, 0.1% TA, and those that were untreated). Each well was imaged 3 times using an Axiovert 40 CFL microscope and Axiocam MRc5 (Zeiss; Overkochen, Germany). This procedure was repeated after 48 and 72.

**2.6 Media Collection**

Conditioned media was removed from the cultures and stored in the freezer at -20$^{o}$C until analysis.

**2.7 Protein Collection**

BT474 cells were cultured and treated as described above. The protein lysates were collected using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific; Rockford, IL) and Halt Phosphatase Inhibitor Cocktail (Thermo Scientific) and Halt Protease Inhibitor Cocktail (Thermo Scientific) were added. Lysates were cleared by centrifugation at 10,000 rpm for 10 min at 4 $^{o}$C. Cleared lysates were transferred to new tubes and stored in the freezer at -20$^{o}$C until analysis.

### 2.8 Western Blotting

Western analysis was also performed as previously described (Booth et al., 2013). Using a BCA Protein Assay Kit (Thermo Scientific), protein concentration was determined. Lysates were combined with Laemmli's SES Sample Buffer (Boston Bio-Products, Boston, MA) and heated for 10 min. Proteins were separated using a 4-15% Criterion TGX gel (Bio-Rad), and then transferred to nitrocellulose. After blocking for one hour with 5% non-fat milk in PBS, the membranes were probed with anti-BCL-2, anti-P-SAPK, and anti-β-actin overnight at 4°C with shaking. The membranes were washed three times with 0.1% Tween-20 in PBS and incubated with secondary HRP-conjugated antibodies for 2 hrs at room temperature. The membranes were washed twice with 0.1% Tween-20 in PBS and rinsed in di$H_2O$. Bands were detected using ECL (enhanced chemiluminescence; Cell Signaling Tech) and images collected using Fluor-Chem M (ProteinSimple, San Jose, CA).

### 2.9 Folin-Ciocalteu Assay

The Folin-Ciocalteu (FC) assay was used as described previously to quantify TA concentrations in collected conditioned media samples (Ngobili et al., 2015).

### 2.10 Cell Counts

After treatment and removal of media, cells were trypsinized and counted using a hemocytometer.

### 2.11 Statistical Analysis

The Student's *t*-test was used to analyze cell counts and TA concentrations. A p-value of 0.05 was used.

CHAPTER THREE

RESULTS AND DISCUSSION

## 3.1 Imaging

Imaging of the cells after various time points showed from a qualatative standpoint that the TA is capable of diffusing out of the matrix and killing the breast cancer cells. After 24 hours, there is a visible reduction in cells in the 10% TA group (Figure 3.1). After 72 hours, there is a clear reduction in cell number between all three TA treated groups (Figure 3.2). Brown particulates are also present in some of the samples, which is inherent of cell death. The control group has had some natural cell death occur, but there has not been as clear of a reduction in number.

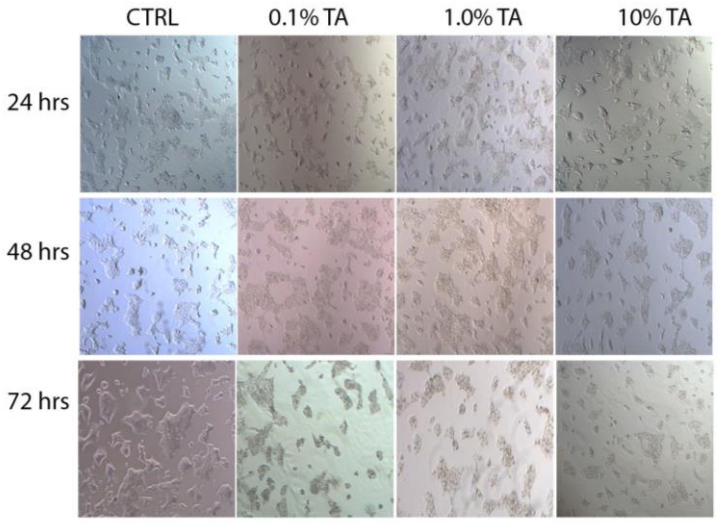


Figure 3.1: Images of BT474 breast cancer cells after treatment time points. Total magnification: 1000X

### 3.2 Cell Counts

To obtain a quantitative assessment of the effects of TA on the cells, cell counts were performed after 72 hours of a treatment. Figure 3.2 shows that there is a correlation between the numbers of cells vs. the TA concentration. The 10% TA treatment group exhibited the fewest number of cells, while the control group exhibited the greatest numbers of cells. The differences in each group when compared to the control group are statistically significant, with each having a value of $p < 0.05$ using the Student's t-test.

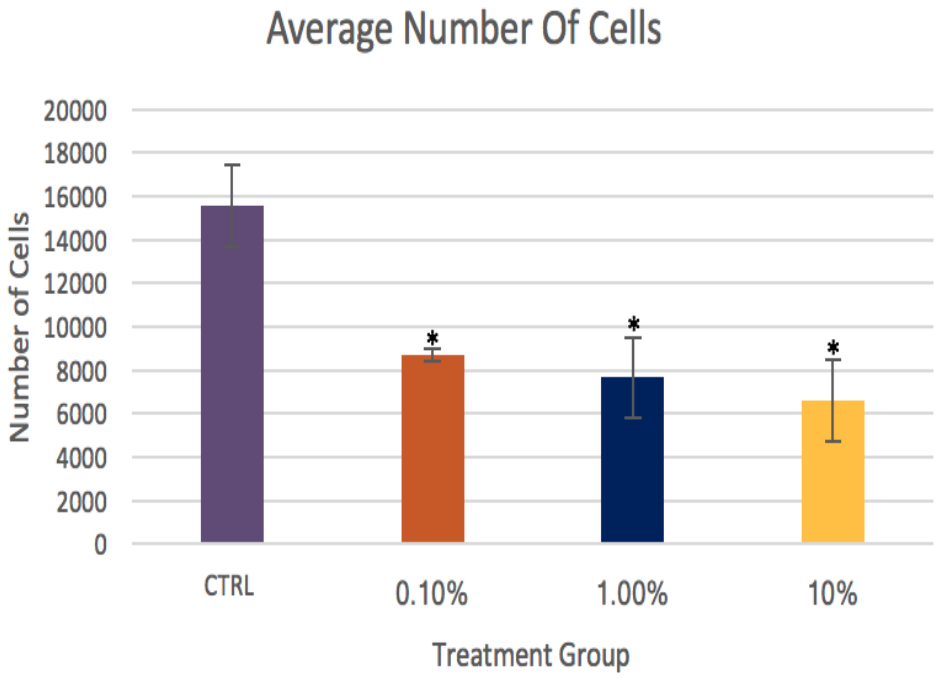


Figure 3.2: Average number of BT474 breast cancer cells in wells after 3 days of treatment. *p<0.05 using Student's T-test

### 3.3 Folin- Ciocalteau Assay

The Folin-Ciocalteau assay is a colorimetric assay conducted to quantify the concentration of TA found in the media below the Transwell® inserts containing the TA

crosslinked collagen type I beads. While it demonstrated above that the TA was diffusing out of the Matrigel® and killing the BT474 cancer cells (Figure 3.1), it is important to further understand the release profile and determine the minimum concentration that is effective in inducing apoptosis. The results of this assay are shown in Figure 3.3.

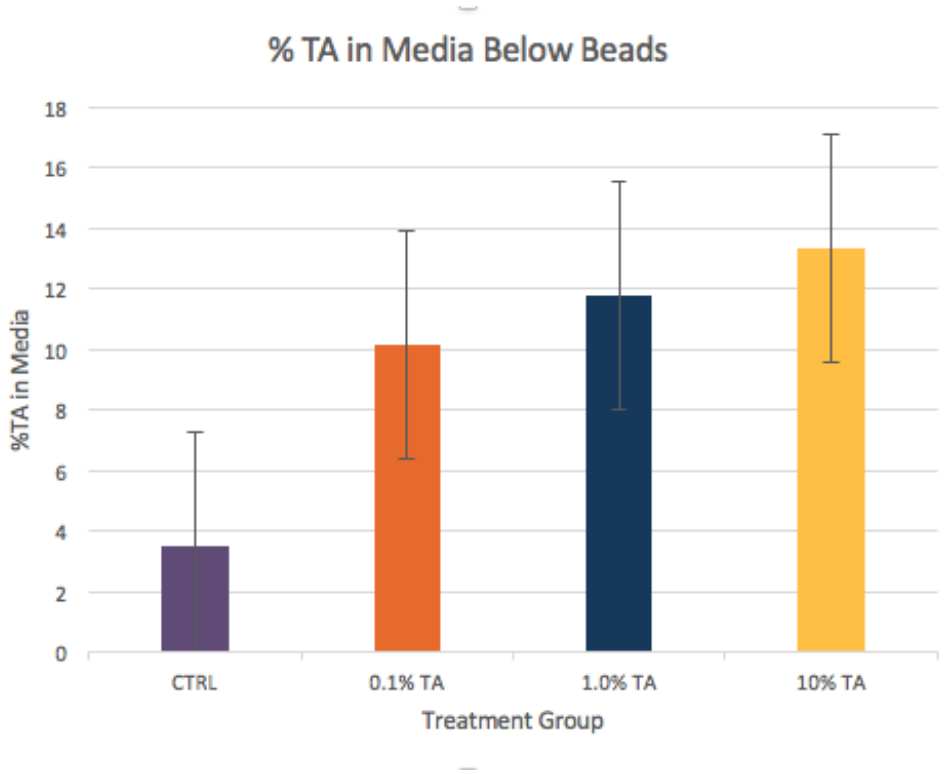


Figure 3.3: %TA found in media under wells after 3 days of treatment. No statistical difference was found between groups.

Figure 3.3 indicates there is an increasing trend observed among the increasing %TA treatment groups. However, it is important to note that the reagent used in this assay reacts with the phenol red component of the media. This creates a false positive for

the presence in the control group that contained no TA. For instance, the chart indicates that there is almost 4% TA in the group that contained no TA. The presence of phenol red in the media could be the reason for these seemingly high levels of TA in all of the groups. It can also be hypothesized that more TA had been metabolized by cells in the 10%TA treated group, which can produce misleading results. If more TA has been metabolized by the cells, less will be present in the media. This could explain why the varying concentrations of TA found in the experimental groups do not increase by the same magnitude as the values they were treated with. While not entirely accurate, this assay does show that there is an increasing amount of TA that correlates with an increase in the concentration of TA that the groups were treated with and serves as a good visual representation of this.

### 3.4 Western Blotting

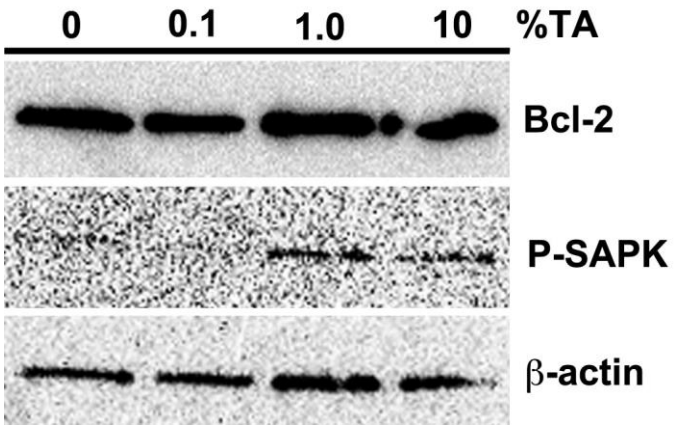


Figure 3.4: Western Blot results for Bcl-2, P-SAPK, and β-actin. Lane 1: Ladder. Lane 2: Control. Lane 3: 0.1%TA treated cells. Lane 4: 1.0% TA treated cells. Lane 5:10% TA Treated cells.

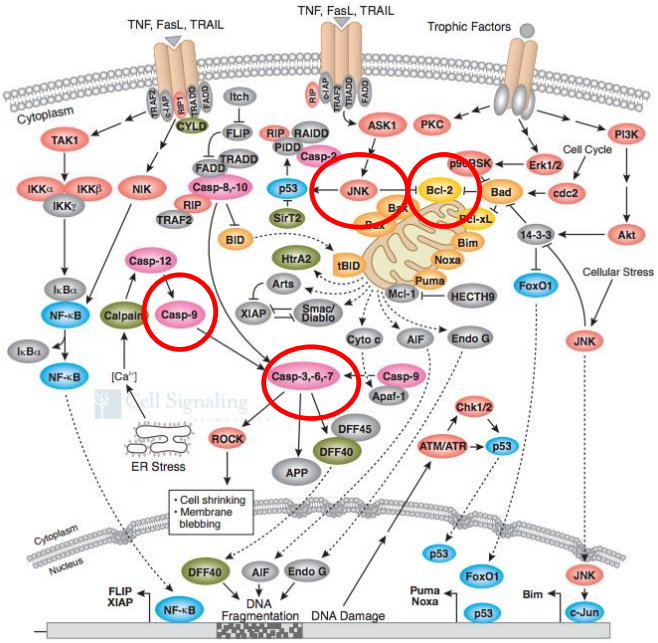


Figure 3.5: Regulation of Apoptosis Pathway

The results exhibited that not only is TA capable of diffusion out the matrix, but also that it kills the cancer cells via a different path in the apoptosis pathway than originally thought. The western blot for the Bcl-2 protein indicates that there is no difference between the controls and the TA-treated groups (Figure 3.4). It has been previously shown that TA induces apoptosis through caspase 3/7 and 9. Rather than affecting the mitochondria, as was originally thought, it would appear that the pathway is initiated due to endoplasmic reticulum (ER) stress (Figure 3.5). The western blot for P-SAPK (denoted JNK in the apoptosis pathway) indicated a difference between the 0.1% and control vs. the 1.0% and 10.0% groups. Further investigation is necessary to determine the mechanism of TA-induced apoptosis. Figure 3.4 shows the apoptosis pathway and the different proteins mentioned have been circled.

# CHAPTER FOUR

# CONCLUSIONS

## 4.1 Conclusions

Seeding TA crosslinked collagen type I beads with human pre-adipocytes forms the basis for an injectable soft tissue regeneration matrix. This matrix has chemotherapeutic properties against breast cancer. When the TA crosslinked beads of varying TA concentrations had been seeded with human pre-adipocytes were placed in a hydrogel and then used to treat $HER2^+$ breast cancer cells, it was shown that the TA was capable of diffusing out of the matrix and inducing apoptosis in the breast cancer cells. As the adipocytes attach and grow on the TA crosslinked beads, they remodel the collagen and allow for the TA to leach out. The free TA then reacts with breast cancer cells in the surrounding environment and induces apoptosis.

A Folin-Ciocalteau assay quantified TA in collected conditioned media demonstrating an increase (Figure 3.3). Qualitative imaging and quantitative cell counts demonstrated the anti-cancer effects of TA towards the $HER2^+$ breast cancer cells (Figures 3.1 and 3.2). Western blotting previously demonstrated that TA-induced apoptosis involved the caspase 3, 7, 9 pathway and we hypothesized that this involved mitochondrial degradation. Surprisingly, we found that this may not be the case. Western analysis showed that Bcl-2, a mediator of mitochondrial-controlled apoptosis, is not involved in TA-induced apoptosis of $HER2^+$ breast cancer cells (Figure 3.4). Instead, we found that SAPK/JNK is also involved in TA-induced apoptosis of $HER2^+$ breast cancer cells (Figure 3.4) suggesting endoplasmic reticulum stress as a mediator of TA-induced

apoptosis. Further analysis will need to be conducted in order to further investigate this, as the mitochondrial pathway is not ruled out.

The findings of this research conclude that TA crosslinked collagen type I beads loaded with adipocytes and placed in a matrix are a possible novel tissue regeneration platform with anti-cancer properties.

### 4.2 Future Research

While much progress has been made on this project, there is still much more to be done to improve the procedures and progress it towards clinical application.

One of the main questions that still needs to be answered is the correct size of bead to use. The beads must be small enough to be delivered through a needle without being damaged by rupturing or other structural damage, but must also be big enough for cell loading and survival *in vivo*. There is batch-to-batch variation between the beads, so it is necessary to determine a way to consistently create the same size bead.

Additionally, the most effective concentration of TA will need to be determined. The three concentrations used for my purposes encompassed a broad range and the concentration will need to be much more specific in future experiments. It is critical that the lowest concentration that is still effective in killing the cancer cells is determined. This therapeutic dose will help maximize effectiveness of the TA while minimizing local toxicity.

There is also a need to find a hydrogel that can be delivered into humans. Matrigel® is not ideal because it is derived from cancer cells. It serves well as a proof of concept, but

is not ideal for actual use. The ideal hydrogel will be thermoresponsive so it is a liquid at room temperature and solidifies at body temperature, will be porous to TA, able to hold the beads in place, and allow for cell proliferation.

Another important question that needs to be further investigated is the half-life of TA. This is important because it can help determine how long TA will stay in the body once it is injected. Once again, it is important to minimize local area toxicity. This will also aid in determining the highest adipocyte cell seeding density on the beads without taking away from the chemotherapeutic effects of TA against the breast cancer cells.

It has been previously shown that TA has a chemotherapeutic effect against different types of breast cancer by inducing apoptosis of the cells via the caspase pathways. However, there is a need to further understand the mechanism in which TA works against the breast cancer cell types that have been tested. The pathway does involve the mitochondria, but could possibly be due to endoplasmic reticulum stress, as was shown in the western blots.

Further investigation could verify that this therapy is useful as a treatment for other soft tissue cancer types such as skin cancer. A better understanding of the therapeutic mechanism in which TA kills cancer cells could lead to the discovery of its uses for many more clinical areas.

After identifying the optimal components of the injectable matrix, the next step to be taken is performing *in vivo* studies to validate the clinical potential of TA and this matrix as an alternative therapeutic treatment option for patients with breast cancer.

**REFERENCES**

American Cancer Society. (2017a). Breast Biopsy. Retrieved April 23, 2017, from https://www.cancer.org/cancer/breast-cancer/screening-tests-and-early-detection/breast-biopsy.html

American Cancer Society. (2017b). Breast Cancer: Overview. Retrieved April 12, 2017, from http://www.cancer.net/cancer-types/breast-cancer/overview

American Cancer Society. (2017c). Breast Cancer: Treatment Options. Retrieved April 12, 2017, from http://www.cancer.net/cancer-types/breast-cancer/treatment-options

American Cancer Society. (2017d). Types of Cancer Treatment. Retrieved April 12, 2017, from https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types.html

American Cancer Society. (2017e). What Is Breast Cancer? Retrieved April 23, 2017, from https://www.cancer.org/cancer/breast-cancer/about/what-is-breast-cancer.html

Bawadi, H. A., Bansode, R. R., Trappey, A., Truax, R. E., & Losso, J. N. (2005). Inhibition of Caco-2 colon, MCF-7 and Hs578T breast, and DU 145 prostatic cancer cell proliferation by water-soluble black bean condensed tannins. *Cancer Letters*, *218*(2), 153–162. https://doi.org/10.1016/j.canlet.2004.06.021

Booth, B. W., Inskeep, B. D., Shah, H., Park, J. P., Hay, E. J., & Burg, K. J. L. (2013). Tannic Acid preferentially targets estrogen receptor-positive breast cancer. *International Journal of Breast Cancer*, *2013*(1), 1–9. https://doi.org/10.1155/2013/369609

Cancer Treatment Center of America. (2017). Breast Cancer Symptoms & Signs.

Retrieved April 23, 2017, from http://www.cancercenter.com/breast-cancer/symptoms/

Chung, K.-T., Wong, T. Y., Wei, C.-I., Huang, Y.-W., & Lin, Y. (1998). Tannins and Human Health: A Review. *Critical Reviews in Food Science and Nutrition*, *38*(6), 421–464. https://doi.org/10.1080/10408699891274273

Fujita, E., Egashira, J., Urase, K., Kuida, K., & Momoi, T. (2001). Caspase-9 processing by caspase-3 via a feedback amplification loop in vivo. *Cell Death and Differentiation*, *8*(4), 335–344. https://doi.org/10.1038/sj.cdd.4400824

Huang, H.-C., Lin, C.-L., & Lin, J.-K. (2011). 1,2,3,4,6-Penta-O -galloyl-β- d -glucose, Quercetin, Curcumin and Lycopene Induce Cell-Cycle Arrest in MDA-MB-231 and BT474 Cells through Downregulation of Skp2 Protein. *Journal of Agricultural and Food Chemistry*, *59*(12), 6765–6775. https://doi.org/10.1021/jf201096v

Jordan, L. G. (n.d.). Tannic Acid Crosslinked Collagen Type I for Prevention of Local HER2-Positive Breast Cancer Recurrence. Retrieved from http://tigerprints.clemson.edu/all_theses

Moongkarndi, P., Kosem, N., Kaslungka, S., Luanratana, O., Pongpan, N., & Neungton, N. (2004). Antiproliferation, antioxidation and induction of apoptosis by Garcinia mangostana (mangosteen) on SKBR3 human breast cancer cell line. *Journal of Ethnopharmacology*, *90*(1), 161–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14698525

National Breast Cancer Foundation. (n.d.). Breast Cancer Facts. Retrieved April 19, 2017, from http://www.nationalbreastcancer.org/breast-cancer-

facts?gclid=CMmv5s_CsdMCFdQ2gQodH2IL1Q

Ngobili, T. A., Shah, H., Park, J. P., Kwist, K. W., Inskeep, B., Burg, K. J. L., & Booth, B. W. (2015). Remodeling of tannic acid crosslinked collagen type i induces apoptosis in ER+ breast cancer cells. *Anticancer Research*, *35*(3), 1285–1290.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*, *2*(5), 270–8. https://doi.org/10.4161/oxim.2.5.9498

Petridis, G. (2006). *Tannins : biochemistry, food sources and nutritional properties*. Nova Science Publishers. Retrieved from https://www.novapublishers.com/catalog/product_info.php?products_id=56550

Signoretti, S., Di Marcotullio, L., Richardson, A., Ramaswamy, S., Isaac, B., Rue, M., … Pagano, M. (2002). Oncogenic role of the ubiquitin ligase subunit Skp2 in human breast cancer. *Journal of Clinical Investigation*, *110*(5), 633–641. https://doi.org/10.1172/JCI15795

Society, A. C. (2016). What is breast cancer? *American Cancer Society*. https://doi.org/03/15/2017

Stich, H. & Rosin, M. (1984). Naturally occurring phenolics as antimutagenic and anticarcinogenic agents. *Nutritional and Toxicological Aspects of Food Safety*, *177*, 1–29.

Triple Negative Breast Cancer Foundation. (2017). Understanding Triple Negative Breast Cancer. Retrieved May 9, 2017, from https://tnbcfoundation.org/understanding-triple-negative-breast-cancer/

Vermerris, W., & Nicholson, R. L. (2008). *Phenolic compound biochemistry*. Springer.

Vernon, R. B., Gooden, M. D., Lara, S. L., & Wight, T. N. (2005). Microgrooved fibrillar collagen membranes as scaffolds for cell support and alignment. *Biomaterials*, *26*(16), 3131–3140. https://doi.org/10.1016/j.biomaterials.2004.08.011

Zand, R. S., Jenkins, D. J., & Diamandis, E. P. (2000). Steroid hormone activity of flavonoids and related compounds. *Breast Cancer Research and Treatment*, *62*(1), 35–49. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10989984