GENETIC SUSCEPTIBILITY FOR LYMPHEDEMA SECONDARY TO BREAST CANCER TREATMENT: AN INVESTIGATION OF THE CONNEXIN GENES

by

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Secondary lymphedema is the accumulation of protein-rich fluid in the interstitial spaces of the extremities. It typically occurs as a result of a trauma or infection in the lymphatic system. This is a significant public health issue because lymphedema has emerged as one of the most debilitating consequences of breast cancer treatment and currently no model exists to predict who will be affected. The aim of this study was to examine genes that may increase the susceptibility to developing secondary lymphedema following breast cancer surgery and/or radiation. Perometry and bioelectrical impedance spectrometry (BIS) were also used to examine clinical and subclinical swelling in individuals.

This is a case-control study that sequenced connexin genes of 70 women with secondary lymphedema and over 100 control participants without lymphedema. Connexins form gap junction channels that facilitate communication between cells. The connexins that were sequenced include connexin 47 (*GJA12*), connexin 37 (*GJA4*), connexin 40 (*GJA5*), and exon 2 of connexin 43 (*GJA1*). Four missense mutations and one synonymous substitution were identified in connexin 47. The mutations were not found to be polymorphic in control individuals. The identification of connexin 47 mutations is compelling and warrants further research to determine if and how this gene increases the risk of secondary lymphedema after breast cancer treatment. These findings could have implications for prevention, management, and early diagnosis of breast cancer-associated secondary lymphedema.

TABLE OF CONTENTS

PREFACE ..... **IX**

1.0 INTRODUCTION **1**

1.1 PATHOPHYSIOLOGY OF LYMPHEDEMA **3**

1.2 DIAGNOSIS **5**

1.3 TREATMENT AND MANAGEMENT **7**

1.4 GENETIC ETIOLOGY OF LYMPHEDEMA **8**

2.0 SPECIFIC AIMS **11**

3.0 MATERIALS AND METHODS **15**

3.1 STUDY DESIGN **15**

3.2 PARTICIPANTS **16**

3.3 LABORATORY PROCEDURES **17**

3.4 MEASUREMENT PROCEDURES **18**

3.4.1 Bioelectrical Impedance **18**

3.4.2 Perometry **21**

3.5 STATISTICAL ANALYSIS **21**

4.0 DATA ANALYSIS **22**

4.1 DESCRIPTIVE STATISTICS **22**

4.2 RESULTS **26**

4.2.1 Gene Sequencing Analysis 26

4.2.2 Measurement Analysis 31

5.0 DISCUSSION 33

5.1 GENE SEQUENCING 34

5.2 FLUID AND VOLUME MEASUREMENTS 36

5.3 LIMITATIONS 37

5.4 CONCLUSIONS 38

BIBLIOGRAPHY 39

LIST OF TABLES

Table 1. Group A demographic variable means. 22

Table 2. Group B demographic variable means and standard deviations. 23

Table 3. Group B independent t-tests for equality of means of demographic variables. 23

Table 4. Participant recall of risk factor exposure. 25

Table 5. Analysis of the number of risk exposures reported. 26

Table 6. Connexin 47 mutations identified in individuals with or at-risk for secondary lymphedema. 27

Table 7. Description of patients with primary lymphedema and the H198H synonymous substitution. 30

Table 8. BIS and perometer measurement means and standard deviations. 32

Table 9. Comparison of impedance ratios. 32

LIST OF FIGURES

Figure 1. A Cole plot illustrating the relationship between impedance (Z), resistance (R), and reactance (X_c)..... 13

Figure 2. The perometer frame and computer generated image from perometer data. 14

Figure 3. Placement of bioelectrical impedance spectrometry (BIS) electrodes. 20

Figure 4. Group B time after breast cancer surgery. 24

Figure 5. Amino acid structures. 27

PREFACE

I would like to thank my thesis advisor, David Finegold, MD, my thesis committee chair, Robert Ferrell, PhD, and my committee members Adam Brufsky, MD, PhD and Elizabeth Gettig, MS, CGC for their encouragement and guidance throughout the completion of this research project. I would also like to extend my thanks and appreciation to Mark Kimak for his unlimited patience in the lab and his willingness to answer any and all questions throughout the process. A special thanks goes to Diana Campbell and Jennifer Bradley, our very talented undergraduate research assistants. Their positive attitudes and enthusiasm for the project helped make this a very enjoyable experience. In addition, I would like to acknowledge the nurses in the Clinical Research Center at Magee-Womens Hospital for their assistance in making the study run smoothly, and the medical oncology nurses who helped in the recruiting process. Lastly, thanks to my entire family and to my husband, Jared, for their support, love, and friendship.

1.0 INTRODUCTION

Every year in the United States approximately 200,000 women are diagnosed with breast cancer. It is estimated that 80% of these women will survive this diagnosis, with the survival rate continuing to improve as treatments improve.[1] Because women are now living longer after having breast cancer there has been increasing attention on the adverse effects of therapy. Current treatment options include surgery, radiation, chemotherapy, and/or hormone therapy. Advancements in these therapies have led to successful outcomes for many patients with breast cancer. Unfortunately, lymphedema has emerged as a debilitating and devastating consequence for a substantial number (~20%) of these individuals.

Breast cancer-associated lymphedema, which is characterized by an accumulation of lymph fluid in the interstitial space of one or both arms, is often attributed to surgery or radiation as an iatrogenic side effect. Axillary lymph node dissection is the most common cause of lymphedema in the United States.[2] As many as one in four women who receive this surgery will develop edema of the arm and subsequently be diagnosed with lymphedema.[3] Of women who are affected, 80% develop it within the first three years after surgery and the remainder develop it at a rate of 1% per year.[4] It is well recognized that treatment to the axilla, either with surgery and/or radiation, is the initiating cause of swelling; however, the mechanistic factors that lead to lymphedema for some women but not others are poorly understood.[5] The inability to identify

predisposing factors has created growing fear and frustration in patients who are at a high risk of developing lymphedema by virtue of being treated for breast cancer.[6]

Lymphedema is associated with a considerable degree of functional impairment, psychological morbidity, and diminished quality of life.[7] Many women express frustration with the lack of information they received before and after surgery concerning their risks of developing lymphedema. This is likely a combined function of the limited scientific evidence that is available, and the reduced opportunity for healthcare workers to provide education due to minimal postoperative hospital stays.[8] Much of the literature regarding the risk of lymphedema is centered on severity of disease, extent of surgery, such as axillary clearance versus sentinel node biopsy, and the use of chemotherapy or radiation. In the past, it was thought that the trauma of treatment was responsible for the pathophysiology of lymphedema;[9] however, a study of 200 patients failed to find a relationship between lymphedema and a number of factors including extent of surgery or radiation to the breast, dose of radiation, surgical or radiation complications, time since presentation, age, menopausal status, handedness, or drug therapy.[9] In addition, a review of 36 studies on secondary lymphedema after breast cancer therapy was also unable to identify compelling predisposing factors.[10]

It is apparent that there is a lack of understanding of why women develop lymphedema after breast cancer treatment. Preventative measures such as the avoidance of infection, injury, and arm constriction are often recommended based on anecdotal evidence, but scientific verification is still lacking and these measures do not guarantee that a woman will remain symptom-free.[11] Many studies have attempted to identify factors that increase or decrease a woman's risk with little success. Obesity and infection are the only two factors that are consistently associated with secondary lymphedema, but they still do not explain the majority of

cases.[12; 13] At the present time, there is no cure for lymphedema, but it is important to identify women in the early stages of the condition in order to better manage the swelling and prevent it from progressing to the later stages. Examining mechanisms that have not received much attention, such as inherent lymphatic variation and genetic etiology, may lead to the development of screening tools that could help to identify women at the greatest risk for this debilitating condition.

1.1 PATHOPHYSIOLOGY OF LYMPHEDEMA

The lymphatic system functions to remove waste material and excess fluid from tissues throughout the body. It consists of small lymphatic capillaries where interstitial fluid is initially drained from the tissues, larger precollector vessels, collecting lymphatic vessels in which the precollectors drain, and lymph nodes. The lymphatic capillaries are distributed throughout the tissue spaces and are formed as interconnected branches. The lymphatic capillaries do not have an obvious opening for lymph fluid to enter, but instead are made up of single cells that overlap in a way that causes them to separate slightly when pressure from interstitial fluid is present. These openings allow fluid into the capillaries, but do not allow it to leak back out. One-way valves are present to keep the flow of fluid unidirectional away from the tissues and into the collecting lymphatic vessels. The afferent lymphatic vessels utilize one-way valves and smooth muscle contractions to propel lymph fluid to the lymph nodes where it is filtered then returned to the circulatory system via efferent lymphatic vessels.

Lymphedema develops when there is a failure within the lymphatic system. This can occur due to rare congenital malformations in the lymph pathway, referred to as primary

lymphedema, or as a result of outside trauma such as surgery or an infection, which is referred to as secondary lymphedema. The pathophysiology of primary lymphedema is not completely understood, but is thought to result from congenital or acquired malformations such as lymph vessel hypoplasia or aplasia, atrophy of the smooth muscle layer within the vessels, or hyperplastic lymph vessels.

The pathophysiology of secondary lymphedema depends on the cause of the lymphedema and is still not fully known for most cases. In less developed parts of the world secondary lymphedema is often caused by filariasis, a parasitic worm that obstructs the lymphatic system and can reduce lymphatic contractility. Other cases of secondary lymphedema are precipitated by lymphangitis, a bacterial infection of the lymph vessels, and cellulitis, a bacterial skin infection. If these conditions are recurrent they can lead to progressive destruction of the lymphatic vessels.[14]

Cancer treatment, specifically surgery and/or radiation, is the most common cause of secondary lymphedema in developed countries.[14] It is thought that the trauma of surgery or radiation to the axilla interrupts the lymphatic drainage pathways from the arm. This creates a rise in lymphatic pressure with subsequent lymphatic dilation. If the lymph system is overwhelmed by the influx of fluid or if the valves are incompetent, fluid is less likely to move in the appropriate unidirectional manner and backflow can develop towards the skin. The inability to clear protein from the interstitial fluid combined with a reduction in the colloid osmotic pressure gradient results in continued leakage of fluid from the capillaries until the interstitium reaches a new equilibrium.[15]

Postoperative arm swelling is not uncommon following surgery and is likely a result of trauma as described, but it tends to resolve spontaneously after a few weeks.[14] Lymphedema,

however, develops months to years after treatment, with sudden or gradual onset.[15] Because of this delay in lymphedema development and the fact that nearly 75% of women do not experience it, the explanation based solely on trauma of treatment is insufficient and leaves many questions still unanswered. Several studies have attempted to create an animal model of surgery-induced lymphedema without much success. Transient edema in a canine model has been produced after transection and resection, but was found to resolve within 2-4 weeks.[16] Surgical ablation with irradiation produced lymphedema more reliably in canines, although it was not present in every case and it required far greater surgical trauma than what is involved in breast cancer treatment.[17] These results further support the idea that other variables are likely playing a role in the development of lymphedema beyond the interruption of the lymphatics because of surgery.

More recently, clinical studies of patients with lymphedema have indicated that inherent variation in lymphatic function may predispose an individual to secondary lymphedema. Investigations of lymphatic clearance rates using lymphoscintigraphy reveal abnormalities in the "normal" contralateral arms of breast cancer survivors who have developed lymphedema, further suggesting that genetic susceptibility may play a role in the development of secondary lymphedema.[18; 19]

1.2 DIAGNOSIS

The true incidence of secondary lymphedema following breast cancer treatment is difficult to determine because of the variety of diagnostic methods and definitions currently being used by clinicians and researchers. Universal standards for the diagnosis of secondary lymphedema have yet to be established. Currently, the gold standard for lymphedema diagnosis is

lymphoscintigraphy. It is also helpful in differentiating between lymphedema and other causes of edema such as heart, renal or hepatic failure, venous thrombosis, phlebitis, chronic venous insufficiency, and lipedema.[20] Lymphoscintigraphy is performed by injecting a water-soluble colloidal radiotracer subcutaneously into a patient's hand or foot. A gamma camera is then used to capture serial images of the path of the radiotracer through the lymphatic vessels in order to monitor the flow of lymph fluid. This method is the most effective technique for imaging the structure and function of the lymph system, but it is not ideal for routine clinical practice.[21]

There are a number of other existing procedures used to aid in the diagnosis of lymphedema which include: (1) limb girth via tape measurements, (2) water displacement, (3) perometry, and (4) bioelectrical impedance. Taking physical measurements at specified points along the limbs can be performed in many different settings and is useful for continuous monitoring; however, the assessment of secondary lymphedema may require more sensitive modes of measurement to identify it earlier. Water displacement techniques correlate well with physical measurement[22], but are difficult to perform and may not be practical in every clinical or research setting.

Perometry is an automated method of determining limb volume that utilizes infrared light transmitters and photo sensors inside a moving frame. The frame takes measurements every half-centimeter along the length of the limb. Perometer measurements are less likely than physical measurements to be affected by inter-operator variability and have been found to be consistently reproducible.[23; 24] Bioelectrical impedance spectrometry (BIS) is another automated technique that has shown promise for quantitatively measuring lymphedema in patients with breast cancer.[25] BIS is based on the principle that as the fluid volume in the extremities increases, the impedance to electrical current decreases. Further research is necessary, but BIS could prove to

be the most effective and convenient method for early detection of breast cancer-related lymphedema. In addition, BIS assesses extracellular water content, which is thought to be the earliest sign of lymphedema.[26] Although advances have been made in technology available to measure the limbs of women at-risk for developing secondary lymphedema, the question still remains as to whether these measurements have the ability to detect phenotypic differences between women who will develop lymphedema and those who will not.

1.3 TREATMENT AND MANAGEMENT

For many women who experience edema after breast cancer surgery the swelling resolves without the need for treatment. Women who do eventually develop irreversible lymphedema require immediate treatment and consistent management for life. Compression bandaging is often the first step in managing fluid accumulation. Compression bandages provide a low amount of pressure at rest and enhance the clearance of lymphatic fluid during muscle movement. As swelling progresses it is often necessary for a woman to be fitted for a compression sleeve. Patients with mild lymphedema use compression sleeves in order to maintain volume reduction in the affected limb. The sleeves are custom fitted for the patient's arm and wrist and function to apply approximately 20-60 mmHg of pressure.[27]

In addition to near constant compression, specially trained massage therapists or physical therapists can perform gentle massage known as manual lymphatic drainage (MLD). The goal of MLD is to stimulate the flow of lymph fluid and direct it from areas of stasis to the functioning lymphatics for clearance. The most common treatment plan for a woman after a diagnosis of

lymphedema involves a combination of the previously mentioned therapies along with patient education, meticulous skin hygiene, and exercises.[27]

If left untreated, lymphedema will progress through three well-defined stages. Grade 1 lymphedema is typically mild and is managed successfully with physical therapy. At this point in development the skin will appear to pit when pressure is applied. Grade 2 lymphedema is characterized by changes in the affected tissue that include hardening of the skin and an increase in fibrous tissue. Patients with grade 2 lymphedema are at an increased risk for infections and experience a decrease in the efficacy of therapeutic interventions. The final stage, grade 3 lymphedema, is the most profound and is commonly referred to as lymphostatic elephantitis. It occurs when the affected limb is left untreated. Limb function is significantly compromised and excess skin may appear to hang in folds. Less than 1% of patients will develop lymphangiosarcoma, a dangerous malignancy, or another secondary tumor.[15]

1.4 GENETIC ETIOLOGY OF LYMPHEDEMA

Investigation into the potential genetic etiology of secondary lymphedema after breast cancer treatment began in response to the identification of genes responsible for the onset of primary lymphedema. Many genes are involved in lymphatic development, but only three are known to play a role in primary lymphedema. Mutations in *FLT4 (VEGFR3)*, *FOXC2*, and *SOX18* are associated with Milroy disease[28; 29], lymphedema-distichiasis syndrome[30], and hypotrichosis-lymphedema-telangiectasia[31] respectively. The majority of affected individuals and families, however, do not have a mutation in these genes and researchers are continuing to search for others that may be involved.

Milroy disease (congenital lymphedema) is well characterized as a type of primary lymphedema that presents with lower limb lymphedema at birth or early in childhood. The swelling is often bilateral and symmetric, but the severity varies between individuals and within families. Milroy disease is inherited in an autosomal dominant pattern with approximately 85-90% of individuals with a *FLT4* mutation developing the condition before the age of three.[32] The *FLT4* gene is located on chromosome 5q35.5. It encodes the vascular endothelial growth factor receptor-3 (VEGFR3) protein, a tyrosine kinase receptor for vascular endothelial growth factors C and D that is thought to be involved in lymphangiogenesis and maintenance of the lymphatic endothelium.

Lymphedema-distachiasis syndrome is another autosomal dominant primary lymphedema condition that presents with lower limb lymphedema and distichiasis. Other associated features include varicose veins, congenital heart disease, ptosis, and ocular findings such as corneal irritation, recurrent conjunctivitis, and photophobia.[33] Approximately 25% of individuals are asymptomatic. Distichiasis is often present at birth and lymphedema develops between late childhood and puberty. Sequence analysis of the *FOXC2* gene detects 95% of mutations in individuals who have been clinically diagnosed with lymphedema-distachiasis. *FOXC2* is located on chromosome 16q24.3 and it encodes the forkhead box protein C2. This protein functions as a transcriptional regulator during embryonic development and is expressed in adult adipose tissue and lymphatics.[34] *FOXC2* mutations have also been implicated in phenotypes attributed to other lymphedema syndromes.[35]

Other primary lymphedema conditions include hypotrichosis-lymphedema-telangiectasia syndrome, Meige disease (lymphedema praecox), and lymphedema with yellow nails. Hypotrichosis-lymphedema-telangiectasia syndrome is characterized by lower limb

lymphedema, hair loss, and telangiectasias. It is very rare and has been found to be caused by mutations in the *SOX18* gene in both an autosomal dominant and recessive inheritance pattern.[31] Meige disease primarily affects women during puberty, and lymphedema with yellow nails often develops in individuals over the age of 50. Genes have not been identified in association with these two conditions.

Because several genes function together during lymphangiogenesis and lymphatic endothelial maintenance it is likely that other genetic pathways are involved in primary lymphedema. These same pathways may also contribute to a predisposition to developing secondary lymphedema after surgery for breast cancer. The recent identification of mutations in the hepatocyte growth factor receptor MET (HGF/MET) pathway has provided evidence for a parallel mechanism contributing to both primary and secondary lymphedema. Finegold *et al*. (2008) discovered a *MET* mutation that is shared in patients with breast cancer-associated secondary lymphedema and patients with primary peripheral and intestinal lymphangectasia.[36] This same research group has found that among 239 individuals with both a diagnosis of primary lymphedema and a family history of lymphedema, 5% have mutations in the *VEGFC/VEGFR3* pathway and 7% have mutations in *FOXC2*. *HGF/MET* mutations likely contribute to a similarly small proportion of individuals with lymphedema.[36] It is possible that alterations in any of these known lymphedema pathways and those that have yet to be described could account for an increased risk for secondary lymphedema in certain patients undergoing treatment for breast cancer, consistent with the findings in *HGF/MET*.

2.0 SPECIFIC AIMS

Specific Aim 1: To examine additional genes that may predispose a woman to developing secondary lymphedema following breast cancer surgery.

Hypothesis: Women with breast cancer-associated secondary lymphedema will have previously unrecognized variations in connexin genes that will not be found in women who do not develop lymphedema.

We chose to sequence a selection of the gap junction genes including connexin 47 (*GJA12*), connexin 37 (*GJA4*), connexin 40 (*GJA5*), and exon 2 of connexin 43 (*GJA1*). Connexins are a very complex family of genes. Their functional end products, gap junctions, serve as intercellular conduits or channels that provide electronic and metabolic communication between cells. There are approximately 20 different types of connexins that cluster together to form gap junctions. A single gap junction channel can be comprised of multiple connexin subunits and the types of connexins determine the permeability, conductance, and mode of regulation of the channel.[37] Gap junctions are found in a wide variety of tissues and have been implicated in numerous developmental processes. The connexin genes are regulated at the transcriptional, translational, and posttranslational levels although the mechanisms by which they maintain tight regulation across cell types are not currently understood.[38] This study is the first to examine the role that gap junctions may play in the onset of secondary lymphedema.

Specific Aim 2: To examine the arm volumes of participants with and without secondary lymphedema using bioelectrical impedance spectrometry (BIS) and perometry.

Hypothesis: Participants with lymphedema will have increased fluid and volume measurements in the affected arm compared to the contralateral unaffected arm. Participants who have not developed lymphedema are not expected to exhibit measurement differences between arms, although BIS may detect subclinical swelling in individuals who later develop lymphedema.

BIS and perometry are sensitive techniques to investigate swelling in the limbs. Our aim was to examine these measurements in affected and at-risk arms compared to contralateral arms. We were also interested in examining results of participants who were unaffected at the time of measurement but later developed lymphedema.

BIS is a relatively new tool to measure fluid volume, and it may be able to identify subclinical swelling earlier than other techniques. BIS utilizes electric current passed through specific regions of the body to measure the impedance to flow. The amount of impedance is inversely proportional to the volume of fluid in the tissue. Data for bioelectrical impedance measurements is recorded over a frequency range of 4-1,000 kHz with a bioimpedance spectrometer (SFB7, Impedimed Ltd., San Diego, CA). Low frequencies (<30 kHz) travel through extracellular fluid and high frequency currents travel through intracellular fluid. Impedimed software is used to process the data and provide values for the resistance of extracellular water (R_o), resistance of total tissue fluid (R_∞), and resistance of intracellular water (R_i). For the assessment of lymphedema we are primarily interested in extracellular fluid, which is optimally measured at zero frequency (R0). At R0 current is unable to penetrate cell membranes and is forced to move around the cells. Practical limitations exist, however, that prevent a direct measure of resistance at R0. BIS uses data collected over the range of

frequencies to extrapolate impedance at R0. Across each frequency two components of impedance are measured, resistance (opposition to the current from fluid in the body) and reactance (opposition to the current due to cell membranes and tissue).[39] To estimate R0 reactance is plotted against resistance on a Cole plot (Figure 1). Unilateral arm swelling or lymphedema is assessed with a relative index of extrapolated R0 values comparing the affected to the unaffected limb, or in the case of this study comparing the at-risk limb to the limb that is not at risk for lymphedema.[39]

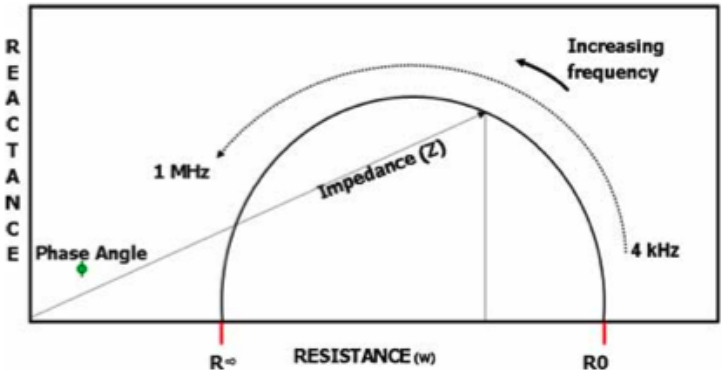

Figure 1. A Cole plot illustrating the relationship between impedance (Z), resistance (R), and reactance (X_c).
Bioelectrical impedance spectrometry (BIS) measures impedance to flow of electric current through regions of the body to estimate fluid volume. Extracellular fluid is optimally measured at zero frequency (R0), which must be estimated based on reactance and resistance plotted on a Cole plot.

Perometry operates on an optoelectronic system that uses infrared light beams to take measurements and calculate volume. The perometer is composed of a four-sided frame that moves along a rail in the long axis of the limb being measured. Light transmitters are located on two sides of the frame and project light towards photosensors on the opposing sides (Figure 2). The light transmitters are activated when a limb is placed inside the frame and blocks the transmission of light. The perometer creates a series of electronic images every half-centimeter along the limb in order to calculate volume (Fig. 2).

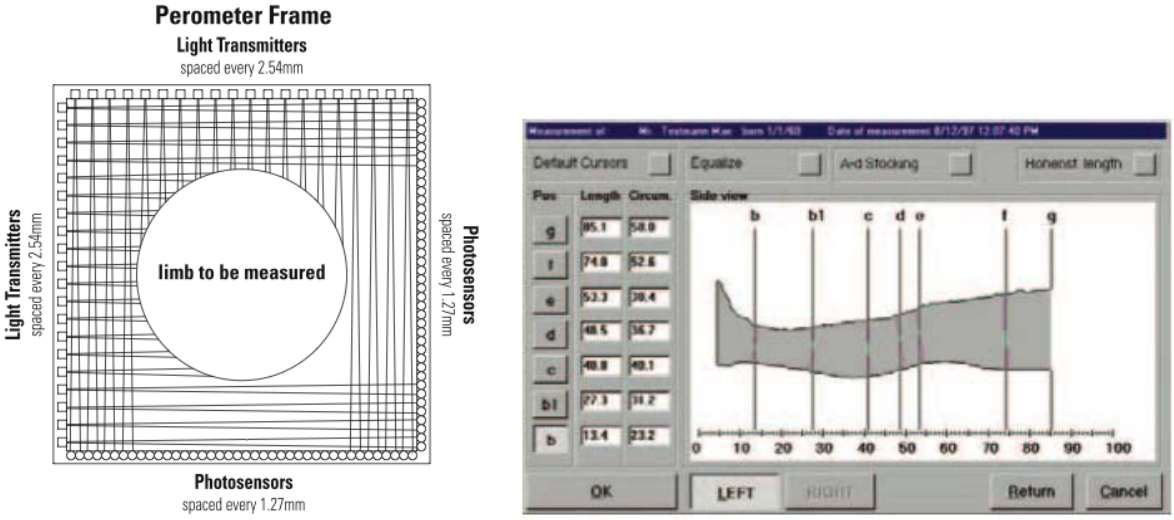

Figure 2. The perometer frame and computer generated image from perometer data.
The perometer uses infrared light beams to take measurements every half-centimeter along the limb and calculate volume. Information collected from the perometer is displayed as an image on the computer.

3.0 MATERIALS AND METHODS

3.1 STUDY DESIGN

This study is designed as a case-control study of women who have been treated for breast cancer. It was approved by the Institutional Review Board of the University of Pittsburgh. Cases are defined as women who have been diagnosed with lymphedema, and controls are defined as women who are at-risk for developing secondary lymphedema but have not developed it at the time of analyses. The control participants are considered to be at-risk because they have had breast cancer surgery. Participants were recruited from Magee-Womens Hospital.

DNA from two cohorts was sequenced in an attempt to identify mutations in the connexin genes. The first group (Group A) consists of individuals who received breast cancer treatment between the years of 1988 and 1999. This provided a retrospective look at the development of lymphedema. The second group (Group B) consists of individuals who received breast cancer treatment in the past four years, which represents a prospective view of lymphedema development.

3.2 PARTICIPANTS

Potential participants in the first retrospective group (Group A) were sent letters from Magee-Womens Hospital and were contacted by phone if they responded with an interest in participating (as described in a master's thesis presented by Roxanne Miller, 2003[40]). Participants were eligible to participate if they had a breast cancer diagnosis between 1988 and 1999. Letters were sent to 160 potential cases and 500 controls from the Physical Therapy and Medical Records departments at Magee-Womens Hospital. A total of 69 cases and 65 controls were recruited. Medical and family histories were obtained and participants were asked to provide a 30cc blood sample and/or a buccal swab. They were also asked to complete a questionnaire and a medical release form to verify the type of breast cancer treatment and the diagnosis of lymphedema when applicable. Sequencing of the connexin genes was analyzed for 59 cases.

Participants in the second group (Group B) were recruited from flyers posted in Magee-Womens Hospital, referred by oncology physicians, and were approached in the Medical and Surgical Oncology departments by researchers to determine interest in participating. Women in the medical oncology suite were approached if they had signed the research registry consent form indicating that they were receptive to learning about research studies. Researchers spoke with women in the surgical oncology office after a nurse or physician obtained permission. Women between the ages of 18 and 100 were eligible if they had undergone surgery for breast cancer within the past four years. Family members between the ages of 18 and 100 were also eligible to participate, although we only included those who have had a personal history of breast cancer (n=4) in this analysis. Interested individuals were given information about the study and were scheduled to have limb measurements in the Clinical Research Center (CRC) of Magee-

Womens Hospital. A total of 77 women participated in the study (n=52 controls, n=15 cases, n=10 unsure). Participants were classified as "unsure" if they reported having symptoms of lymphedema, but had not been clinically diagnosed at the time of data analysis.

During their appointment at the CRC participants were asked to sign the consent form, complete a medical history questionnaire and medical release form, and provide information about their family history. They also provided either a mouthwash sample or a 30cc blood sample. Participants had their blood drawn by a CRC phlebotomist or by a nurse in the chemotherapy suite in the Department of Medical Oncology. Limb measurements were performed with assistance from the nurses in the CRC and included physical measurements with a tape measure, perometry, and bioelectrical impedance. Sequencing of the connexin genes was analyzed for 35 women from this group (n=11 cases, n=24 controls).

3.3 LABORATORY PROCEDURES

DNA was isolated from EDTA anti-coagulated peripheral blood by the salting out procedure as described by Miller *et al*. (1988).[41] Amplification and sequencing primers were generated based on reported human connexin cDNA sequences. Sequences were downloaded from GenBank (accession numbers NM_010288, NM_002060, NM_005266, and NM_020435). Primers flanking each exon were designed to amplify the target sequences. The following primers were used to screen the coding exons of the connexin genes:

Connexin 43 (*GJA1*), exon 2 only: 2F, 5'-TGGGACAGGAAGAGTTTGCA-3' with 2R, 5'-GCCAGGGACACCAAGGACAC-3' and 2F2, 5'-TGCTGCGAACCTACATCATCA-3' with 2R2, 5'-CACCGGATCAAAATTAACACCTG-3' (53°C T_a; 1.75 mM Mg^{++})

Connexin 37 (*GJA4*): 2F, 5'-CGAACGAGAAGGATGCCA-3' with 2R, 5'-GATGAAGATGGTCTTCTCC-3' (54°C T_a; 1.5 mM Mg^{++}) and 2F2, 5'-GTGTTTGTGTGCCAGCGA-3' with 2R2, 5'-CCAGGCAGCCAGACTTCT-3' (54°C T_a; 1.5 mM Mg^{++})

Connexin 40 (*GJA5*): 2F, 5'-CCATTGGATGGATGGATC-3' with 2R, 5'-CCGTAGATGAAGTACTGG-3' (54°C T_a; 1.5 mM Mg^{++}) and 2F2, 5'-GGAAGGGAATGGAAGGAT-3' with 2R3, 5'-CAGTTCAGAAGGGACACG-3' (54°C T_a; 1.5 mM Mg^{++})

Connexin 47 (*GJA12*): 2F, 5'-AGGCGGTAAGCTCCACGTCATT-3' with 2R7, 5'-CAGTGGTGACCAAGCCAG-3' (59.6°C T_a)

The amplimers were treated with shrimp alkaline phosphatase and Exonuclease I then sequenced in both directions using ABI Big Dye 3.1 chemistry (Applied Biosystems, Foster City, California 94404). An ABI 3730 DNA analyzer was used to sequence the fragments, and the sequences were aligned with Sequencher V5.0 software (Gene Codes Corp., Ann Arbor, Michigan 48108).

3.4 MEASUREMENT PROCEDURES

3.4.1 Bioelectrical Impedance

In order to control for variation in fluid levels due to external factors, participants were instructed to refrain from caffeine and alcohol at least 12 hours before their appointment and not to engage

in strenuous activity or exercise at least two hours beforehand. BIS was performed according to methods previously described.[39; 42]

To summarize, participants were asked to remove all jewelry from their wrists and ankles and to lie in a supine position on a non-conductive bed. Arms were placed pronated and slightly abducted with no skin-to-skin contact. Skin on the wrists, hands, ankles, and feet were cleansed with an alcohol swab before the surface electrodes were attached. We used bony landmarks to standardize the placement of the electrodes. Arm impedance was measured from the wrist to the axilla. As illustrated in Figure 3, the current drive electrodes were attached to the dorsal surface of the third metacarpal of the hand and the dorsal surface of the third metatarsal of the foot. Voltage sensing electrodes were fixed on the dorsum of the wrists midway between the styloid processes. The measurement was performed on the right and the left arm.

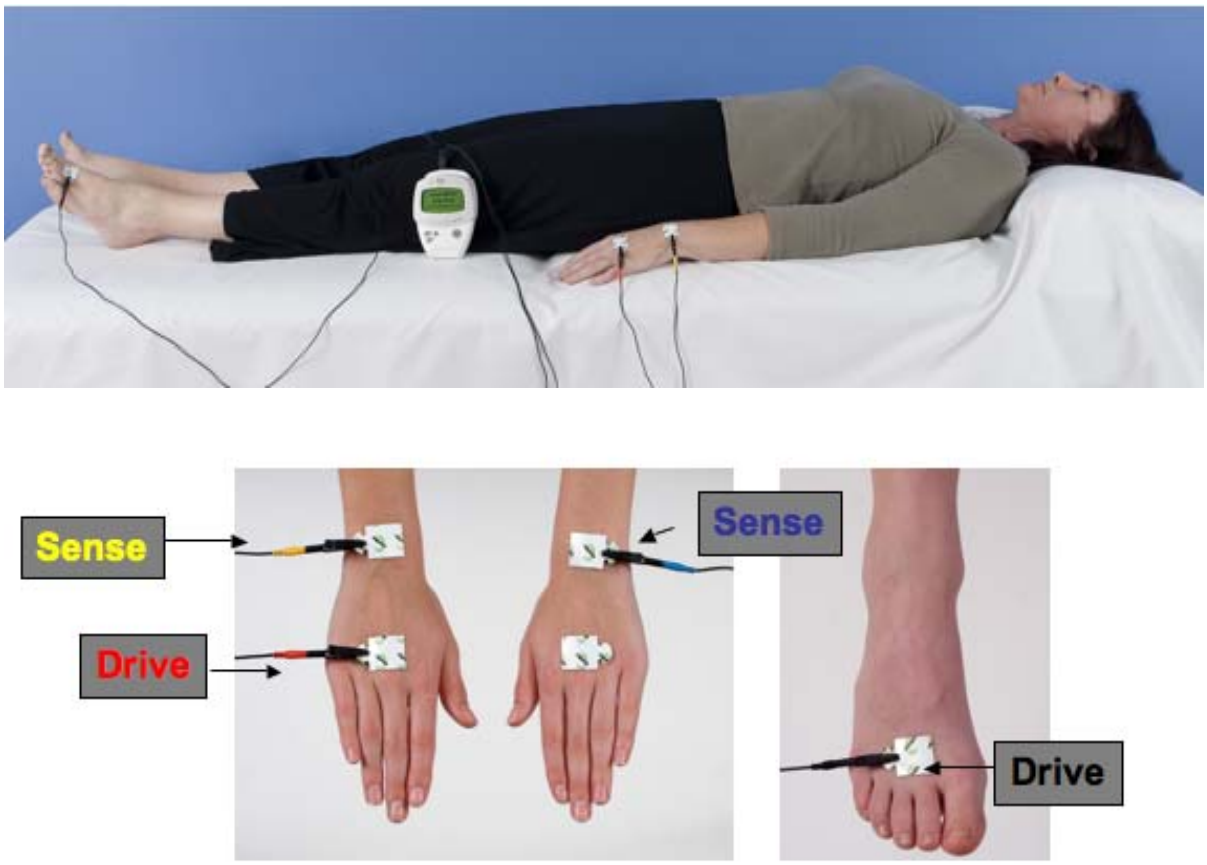

Figure 3. Placement of bioelectrical impedance spectrometry (BIS) electrodes.
The placement of electrodes determines the limb that is being measured with BIS. Arm impedance is measured by placing the current drive electrodes on the dorsal surface of third metacarpals and the dorsal surface of the third metatarsals. Voltage sensing electrodes are fixed to the dorsum of the wrists midway between the styloid processes.

3.4.2 Perometry

Perometry (Perometer 1000M, Juzo) was performed with the participants standing and reaching towards the middle of the frame. They were instructed to reach down until their middle finger touched the hand rest and to keep their arm as vertical and straight as possible. The frame was lifted in a slow consistent motion up the arm then lowered back down. Measurements were obtained for both arms.

3.5 STATISTICAL ANALYSIS

Data analysis was performed using SPSS version 17.0 for Mac OS. Descriptive statistics and frequency distributions were obtained for each variable of interest. All descriptive statistics for Group A were provided by the master's thesis of Miller (2003).[40] Independent t-tests for Group B were conducted to examine demographic differences between the cases and controls. Fisher's exact tests were performed to examine differences in potential risk exposures associated with secondary lymphedema between cases and controls. To investigate the total number of exposures, an analysis of variance (ANOVA) was performed using the reported number of exposures as the dependent variable and the lymphedema classification (case, control, or unsure) as the independent variable. ANOVAs were also conducted to compare measurement data. When applicable, post-hoc analyses were conducted with Tukey's post-hoc test. Pedigree analysis was performed using Progeny V6.0 software.

4.0 DATA ANALYSIS

4.1 DESCRIPTIVE STATISTICS

Demographic information including ethnicity, age at breast cancer diagnosis, and body mass index (BMI) are provided in Table 1 and Table 2 for Groups A and B, respectively. Independent t-tests shown in Table 3 revealed that the age of breast cancer diagnosis was significantly higher for cases than for controls in Group B ($p<0.05$). BMI was not significantly different between cases and controls ($p>0.05$); however, cases tended to have a higher BMI than controls. According to the National Heart, Lung, and Blood Institute (www.nhlbi.nih.gov), BMI measurements of 19-24.9 are classified as normal, the range of 25-29.9 is considered overweight, and obesity is in the range of 30-39.9. The average BMI for cases was 31.39, which is considered obese. Obesity is a commonly reported risk factor for lymphedema.

Table 1. Group A demographic variable means.

	Cases n=69	*Controls* n=65
Ethnicity (% Caucasian)	86%	97%
Age at breast cancer diagnosis	52	51
BMI (kg/m^2)	29	27

Table 2. Group B demographic variable means and standard deviations.

	Cases n=15	*Controls* n=52	*Unsure* n=10
Ethnicity (% Caucasian)	14/15 (93%)	50/52 (96%)	9/10 (90%)
Age at breast cancer diagnosis (SD)	57.3 (8.9)	52.0 (8.4)	50.6 (10.9)
BMI (SD)	31.4 (6.1)	28.2 (5.4)	32.0 (6.4)

Table 3. Group B independent t-tests for equality of means of demographic variables.

	Group Assignment	*n*	*Mean*	*Std. Deviation*	*t*	*p*
Age at breast cancer diagnosis	Cases Controls	15 52	57.27 52.04	8.87 8.36	-2.11	0.039*
BMI	Cases Control	15 52	31.39 28.21	6.09 5.41	-1.90	0.061

***p*<0.05 (2-tailed)**

The timing of the onset of secondary lymphedema after surgery is separated into the following three categories: 0 to 3 months, 3 months to 1 year, and more than 1 year following surgery. We combined the groups to determine the distribution of lymphedema onset. Approximately 28.6% of cases developed lymphedema 0 to 3 months after surgery, 33.7% developed it 3 months to 1 year after, and 37.7% developed lymphedema more than one year after surgery. It is apparent that women are still at risk after one year, which limits the classification of participants into cases and controls in Group B because many are still within one year of their surgery (Figure 4). It is possible that in the future more participants will be reclassified as having developed lymphedema, which may alter our analyses.

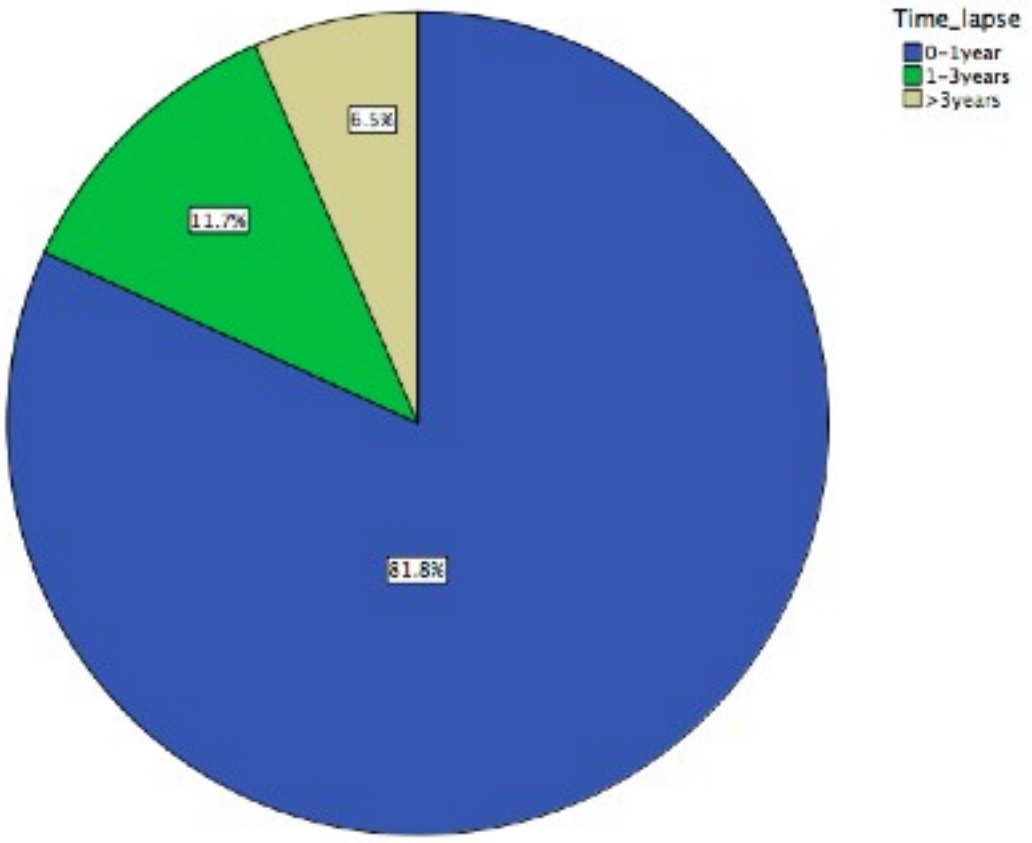

Figure 4. Group B time after breast cancer surgery.
Participants in Group B were asked how long it has been since their breast cancer surgery. The majority of women (81.8%) are still within one year of surgery. Fewer participants (11.7%) underwent surgery in the past one to three years and 6.5% reported having surgery more than three years ago.

Information was also collected from participants in Group B regarding exposures to risk factors that women are advised to avoid after breast cancer surgery. Participants were asked to recall and indicate "yes" or "no" if they had any of the listed exposures following surgery in the arm that is at-risk for lymphedema. The at-risk arm is defined as the arm on the same side as breast cancer surgery was performed. As illustrated in Table 4, there was no significant difference in exposures between cases, controls, and those who were classified as being unsure of their lymphedema status. In addition, participants were not significantly different in the number of risk factors reported (Table 5).

Table 4. Participant recall of risk factor exposure.

	Cases n=15	*Controls* n=51	*p*
Blood pressure	2	9	1.0
Blood draw	2	9	1.0
Manicure	3	14	0.74
Sunburn	1	4	1.0
Cut	5	12	0.51
Insect bite	5	8	0.15
Cat scratch	2	4	0.61

Table 5. Analysis of the number of risk exposures reported.

Group Assignment	*n*	*Mean number of exposures reported*	*Std. Deviation*	*F*	*p*
Cases	15	1.33	1.45		
Controls	51	1.18	1.42	0.075	0.93
Unsure	10	1.20	1.03		

4.2 RESULTS

4.2.1 Gene Sequencing Analysis

Sequence analysis of connexin 47 (*GJA12*), connexin 37 (*GJA4*), connexin 40 (*GJA5*), and exon 2 of connexin 43 (*GJA1*) has been completed on 59 cases in Group A and 11 cases and 24 controls in Group B. The analysis revealed five different nucleotide changes in connexin 47 (*GJA12*) that have not previously been described in patients with secondary lymphedema (Table 6). Connexin 47, located on chromosome 1q41-q42, encodes a 439-amino acid protein. The amino acids involved with the changes are illustrated in Figure 5. No nucleotide changes were identified in connexin 37, connexin 40, or exon 2 of connexin 43.

Table 6. Connexin 47 mutations identified in individuals with or at-risk for secondary lymphedema.

Mutation No.	*Participant # (group)*	*Location*	*Nucleotide change*	*Amino acid change*	*Frequency in controls*
1	1(A)*	cDNA bp 445	G → A	G 149 S	0/194
2	2(A)	cDNA bp 556	G → T	G 186 C	0/210
3	3(A)	cDNA bp 1234	C → T	H 412 Y	0/138
4	4(A)	cDNA bp 1150	C → T	P 384 S	0/137
5	5(A), 6(A), 7(A), 8(A), 9(A), 10(B), 11(B)*	cDNA bp 594	C → T	H 198 H	1/35

***Mutations also identified in individuals with primary lymphedema.**

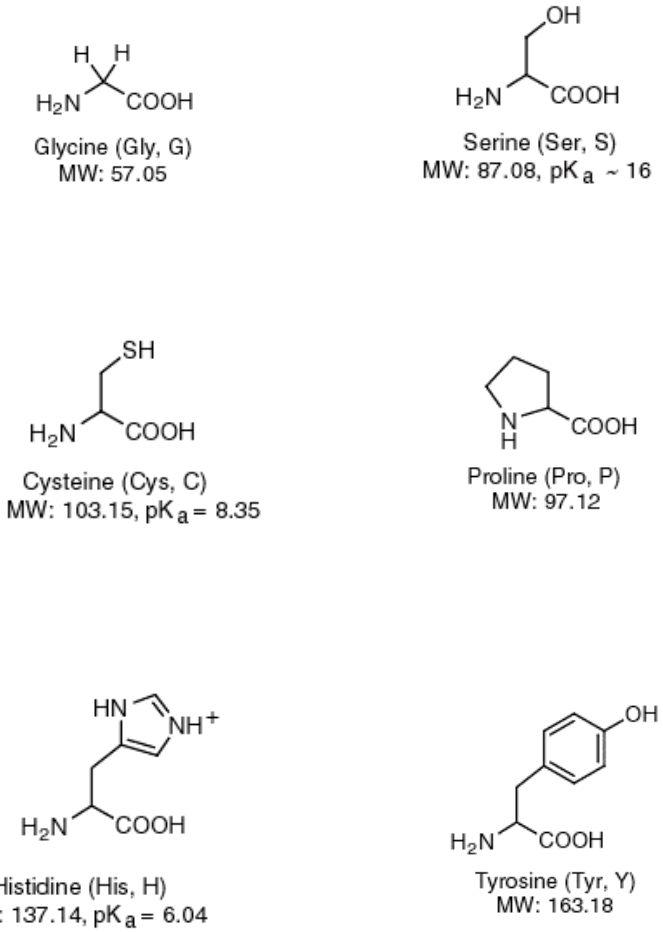

Figure 5. Amino acid structures.

Four of the nucleotide changes (No. 1-4) are missense mutations that lead to an amino acid substitution in participants from Group A. The peptides involved do not appear to be conserved across species. The mutation found in participant 1(A) has also been seen in an individual with primary lymphedema. One of the nucleotide changes (No. 5) did not alter the amino acid and is likely a synonymous substitution; however, it is not listed in the public databases as a polymorphism present in the population (HapMap, dbSNP). The synonymous substitution was found in five participants with secondary lymphedema from Group A and two participants who are at-risk for lymphedema but have not developed it from Group B. It has also been identified in 9 out of 140 individuals with primary lymphedema.

Mutation No. 1 changes a glycine to a serine at amino acid 149 (G149S). This change was found in a woman with secondary lymphedema who has no other family history of secondary lymphedema, although of her 10 siblings she is the only one to have undergone breast cancer treatment. She did report that her brother and father suffered from swelling in the feet, which may or may not be an indication of primary lymphedema. The mutation was also identified in a woman with primary lymphedema and her unaffected son. Her family history is significant for lymphedema praecox.

The second mutation (No. 2) changes a glycine to a cysteine at amino acid 186 (G186C). The mutation was found in a participant with secondary lymphedema who has no other family history of lymphedema or breast cancer. Mutation No. 3 results in a substitution of histidine for tyrosine at amino acid 412 (H412Y). This change was identified in an individual with secondary lymphedema who reports having a sister with breast cancer without lymphedema. Mutation No. 4 was identified in an individual with secondary lymphedema and distichiasis, but with no other

family history of lymphedema or breast cancer. This nucleotide change substitutes a proline for a serine at amino acid 384 (P384S).

The synonymous substitution (No.5) was identified in the following participants from Group A: three affected women with a family history of breast cancer and no family history of secondary lymphedema, one affected woman without a family history of breast cancer, and one affected woman who reports having a brother and father with secondary lymphedema and a paternal uncle with primary lymphedema. The participants from Group B who were found to have the substitution have not developed secondary lymphedema and they do not have a family history of breast cancer or lymphedema. Table 7 describes the families of the individuals with primary lymphedema found to have the H198H synonymous substitution.

Table 7. Description of patients with primary lymphedema and the H198H synonymous substitution.

Family number	*Primary lymphedema condition of proband*	*Family members affected with lymphedema*
1	Congenital lymphedema, pulmonary lymphangiectasia, hydrops	Proband and sibling died from this condition
2	Lymphedema praecox	Mother, maternal grandfather, maternal great grandmother
3	Lymphedema tarda (late-onset) with yellow nails	Mother, 2 sisters, brother, and niece with lymphedema tarda without yellow nails
4	Lymphedema praecox	Mother with lymphedema praecox and daughter with congenital lymphedema
5	Congenital lymphedema	Son and maternal aunt
6	Congenital lymphedema	Brother, nephew, cousins
7	Congenital lymphedema	Sister with lymphedema, paternal uncle and paternal grandmother unaffected but with ptosis
8	Congenital lymphedema	Brother
9	Lymphedema praecox	Sister, mother (with distichiasis), 3 maternal aunts (1 with yellow nails), cousins, maternal grandmother

4.2.2 Measurement Analysis

The BIS and perometer data are described in Table 8. BIS measurements were analyzed by creating a ratio of the unaffected (or not-at-risk) arms to the affected (or at-risk) arms. The ANOVA results comparing the impedance ratios of cases, controls, unsure participants, and cases without lymphedema at the time of measurements are shown in Table 9. A post-hoc test revealed that cases had a significantly higher impedance ratio, indicating increased fluid volume in the affected arm, compared to controls and unsure participants ($p<0.05$). The impedance ratios of cases with lymphedema at the time of measurements were not significantly different from those who had yet to develop lymphedema ($p>0.05$); however, these results should be interpreted with caution because of the small sample size and limitations in the power of the analysis.

Perometer measurements were compared by obtaining the mean differences between the unaffected (not-at-risk) and the affected (at-risk) arms for each group. The mean differences were not statistically different between groups, but the trend indicated that the cases had a greater difference between arms than the unsure participants, with the controls having the smallest difference between arms. Limitations exist, however, that complicate the interpretation of this data. It has been well established that arm dominance is a significant factor and should be considered when comparing the right and the left arms. The dominant arm is typically larger than the non-dominant arm within the range of 1.5% - 4.2%.[25] Unfortunately, we only have arm dominance information for seven control participants, and are not able to control for arm dominance in these analyses.

Table 8. BIS and perometer measurement means and standard deviations.

	Cases	*Controls*	*Unsure*
Affected or at-risk limb (Right:Left:Both)	6R:7L:2B	23R:26L:3B	4R:5L:1B
Impedance ratio (SD)	1.139 (0.15) 1.076 (0.063)[+]	1.008 (0.054)	1.021 (0.025)
Arm volume[++] (SD)	Affected Unaffected 2857 (879) 2754 (701) 2827 (1178)[+] 2863 (1274)[+]	At-risk No risk 2461 (655) 2433 (737)	At-risk No risk 2590 (346) 2531 (318)
Volume difference between arms (SD)	103 (278) -36 (211)[+]	28 (188)	59 (189)
Limb dominance[+++] (R:L)		6R:1L	
Volume difference between dominant and non-dominant arm (SD)		48 (122)	

[+] **Cases who did not have lymphedema at the time of measurements (n=3).**
[+ +]**Arm volumes measured in ml.**
[++ +]**Only 7 participants were evaluated for limb dominance.**

Table 9. Comparison of impedance ratios.

Group Assignment	*n*	*Impedance ratio*	*Std. Deviation*	*F*	*p*
Cases	10	1.139	0.15	11.994	<0.01*
Cases[+]	3	1.076	0.063		
Controls	46	1.008	0.054		
Unsure	7	1.021	0.025		

[+]**Cases who did not have lymphedema at the time of measurements**.
***p<0.05 (2-tailed)**

5.0 DISCUSSION

Secondary lymphedema is one of the most under-researched and poorly understood complications of breast cancer treatment. Approximately 20% of breast cancer survivors will develop lymphedema and be at risk for debilitating arm swelling, functional impairment, psychological stress, and diminished quality of life.[6] In the past the assumption was that breast cancer treatment, specifically the trauma of surgery as well as exposure to radiation, was solely responsible for lymphedema onset. While it is accepted that these treatments can cause swelling initially, research has failed to provide strong evidence linking treatment alone to the development of secondary lymphedema.

Currently there is no model that exists to predict who will be affected with lymphedema after treatment for breast cancer. Obesity and infection are the only two external factors that have consistently been shown to increase a woman's risk for this condition. It is important to identify and better understand additional predisposing factors, such as inherent lymphatic variation and genetic etiology, in order to help prevent the onset, diagnose it as early as possible, and minimize the effects of this debilitating condition.

5.1 GENE SEQUENCING

The investigation of the genetic etiology of secondary lymphedema is an extension of the investigations into the genetics of primary lymphedema. Researchers have discovered that primary lymphedema is caused in part by mutations in specific genes, *FLT4 (VEGFR3), FOXC2*, and *SOX18*. In addition, the recent identification of mutations in the HGF/MET pathway provides evidence for a parallel mechanism contributing to both primary and secondary lymphedema.[36] The primary aim of this study was to identify additional genes that may increase susceptibility to developing secondary lymphedema. We anticipated that previously unidentified genetic variations in connexin genes would be found in women with secondary lymphedema that would not be present in control individuals. The connexins are a family of complex genes that serve as intercellular channels and facilitate communication between cells. Connexins are present in many different tissues and have been implicated in several developmental processes, but the details of their regulation are not well understood.

Four different connexin genes were sequenced: connexin 47 (*GJA12*), connexin 37 (*GJA4*), connexin 40 (*GJA5*), and exon 2 of connexin 43 (*GJA1*). Five previously unrecognized nucleotide variations were found in connexin 47 in women who had developed lymphedema after breast cancer treatment (Table 6). Four of these changes are missense mutations and the other is a synonymous substitution. The missense mutation that results in a G149S substitution was also seen in a woman with primary lymphedema and her unaffected son. In addition, the H198H synonymous substitution has been identified in 9 out of 140 individuals with primary lymphedema, and was found in one individual out of 35 without lymphedema.

A mutation is considered causal when it meets the following four criteria: (1) it is found only in lymphedema probands and their at-risk family members, (2) it is not present in over 300

chromosomes from ethnically matched controls, (3) it causes a truncation or missense mutation in evolutionary conserved regions of the protein, and (4) it is not present in public databases of population polymorphisms. At this point, more research is necessary in order to classify the missense mutations as being causal, but the results from this study indicate that their role in lymphedema is compelling and warrants further investigation. The mutations have not been previously identified and were not found in any of the over 100 controls that were tested, which implies that they are not common polymorphisms. Future studies are necessary to determine whether the mutations are located in a functional domain of the protein and whether the peptide changes cause alterations in the protein structure or the channel kinetics. It is known that all of the altered peptides in this study are in a region of the protein channel that is located in the cytoplasm (UniProtKB/Swiss-Prot). Certain mutations involved in altering peptides located in the transmembrane region of the protein channel cause the autosomal recessive condition, Pelizaeus-Merzbacher-like disease type I (PMLD1). PMLD1 is a hypomyelinating leukodystrophy that is associated with impaired motor development, ataxia, progressive spasticity, and nystagmus.[43] Further investigations into the effects peptide substitutions have in other parts of connexin 47, including the cytoplasm region, are necessary to provide an understanding of the role this protein may play in the development of lymphedema.

The synonymous H198H substitution was identified in five women with secondary lymphedema and two who are at-risk but have not developed it. It was also identified in individuals with a variety of different forms of primary lymphedema including congenital lymphedema, lymphedema praecox, lymphedema tarda with yellow nails, and congenital lymphedema with pulmonary lymphangiectasia. The nucleotide change involved is not a splice site mutation, which makes it unlikely, although not impossible, that the variation alters the

protein. Only 35 controls have been sequenced and many more will need to be evaluated before an interpretation of the effects of this nucleotide change can be made with more certainty.

5.2 FLUID AND VOLUME MEASUREMENTS

The second aim of this study was to examine the arm volumes of participants with and without secondary lymphedema using BIS and perometry. We expected that participants with lymphedema would have increased fluid and volume measurements in the affected arm compared to the contralateral arm, and that the differences between arms would be greater than what is present in women who have not developed secondary lymphedema. Statistical analysis of the BIS data was consistent with our hypothesis. Interestingly, fluid measurements of women who did not have lymphedema at the time of BIS measurement but developed it later were not significantly different from women with lymphedema. It is possible that BIS could be sensitive enough to detect subclinical swelling prior to the onset of lymphedema; however, these results should be interpreted with caution because of the limited number of women in the sample (n=3). Further research on BIS measurements are necessary to examine the potential benefits of its use in identifying individuals at the highest risk of developing lymphedema based on early fluid levels in the upper extremities.

A comparison of arm differences with the perometer was not significant between cases and controls, although cases tended to have the highest volume difference between their arms. This was expected because arms affected with lymphedema are typically greater in volume than contralateral arms due to the accumulation of fluid. The analysis of the perometer data is

complicated by several limitations. One of the limitations is that women with lymphedema did not remove their compression garments until the time of measurement. Compression garments function to reduce fluid build-up and keep the volume of the arm much lower than it would be without the garment. It is likely that without compression the affected arms would have had increased volume and fluid measurements than what we were able to capture. These analyses are also limited by the absence of information regarding dominance. Dominant arms are often larger than non-dominant arms and should be controlled for in future studies examining perometer measurements.

5.3 LIMITATIONS

This study is limited by the number of cases available for sequencing and the uncertainty of identifying individuals as cases or controls. Many women who are considered control participants because they have not developed lymphedema are still at risk and may become affected in the future, which would alter some of these analyses. This is a particular concern in the prospective sample (Group B) because the majority of participants are still within one year of surgery. Group B also had a number of individuals who were suspected of having symptoms of lymphedema but had not been clinically diagnosed. We accounted for this in most of the analyses by creating a separate “unsure” category. We will continue to follow this group in order to adjust for changes in their lymphedema status. Difficulty in accurately diagnosing lymphedema in research participants has been a complication for many studies and hopefully will improve as technology used to diagnose lymphedema advances. It will also be important to continue sequencing known cases in order to provide more information about the connexins and

to investigate additional genes of interest. As previously discussed, the interpretation of the measurement data is limited by the absence of information on arm dominance and the use of compression garments by affected participants. Arm dominance will need to be accounted for in future studies and analyses.

5.4 CONCLUSIONS

Despite the limitations, this study does contribute to the present knowledge of lymphedema genetics and advances our understanding of the role of connexins in secondary lymphedema. If the connexin mutations discussed are in fact causal, it could have an impact on how women with breast cancer are currently treated and managed. The ability to predict who is at the greatest risk based on genetic factors will help to identify the condition earlier, improve treatment, and possibly prevent the onset. This study brings the field closer to having this ability, but more research is necessary to expand on these results in order for them to eventually lead to clinical benefits.

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