THE ROLE OF CONNEXIN 40 IN SUSCEPTIBILITY TO SECONDARY LYMPHEDEMA FOLLOWING BREAST CANCER TREATMENT

by

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BA Biology, BA Psychology; Boston College, 2010

Submitted to the Graduate Faculty of

Department of Human Genetics

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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April 10, 2012

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Sarah Elizabeth Noon, M.S.

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Approximately 1 in 8 women will be diagnosed with breast cancer during their lifetime; however, continuing advances in the field of medicine have increased the expected survival rate. With such an increase in survival among breast cancer patients, there has been an emphasis on quality of life post-surgery. One of the most feared complications following breast cancer surgery is the development of lymphedema. Approximately 20-30% of women treated for breast cancer are affected by the onset of secondary lymphedema. Unfortunately, there is no cure and only a portion of women witness improvements in arm symptoms from the available treatments.

Currently, there is no accepted model to successfully predict which women are at higher risk for development of lymphedema post treatment. An individual's genotype as a risk factor is not typically considered when studying patient specific risk information. Since treatment for lymphedema is most successful when initiated early, identification of high risk women through detection of genetic risk factors can aid in early diagnosis, improved treatment outcome, and even prevention which is significant to the field of public health.

Mutations in the HGF and MET genes had been previously identified in patients who developed lymphedema secondary to breast cancer treatment suggesting the possibility of a genetic predisposition to development of lymphedema. More recently, findings of mutations in the connexin 47 (GJA12/GJC2) gene in breast cancer patients diagnosed with secondary

lymphedema confirmed a genetic predisposition. T here are several connexins expressed in lymphatics and further investigation of their involvement with lymphedema is warranted.

The purpose of this study is to continue the investigation of the connexin genes and their involvement in secondary lymphedema. This is a case-control study designed to sequence the connexin 40 gene in women treated for breast cancer with and without a diagnosis of secondary lymphedema. In this study, 91 cases and 168 controls were sequenced for the connexin 40 gene. No previously unidentified connexin 40 mutations were found in this cohort of women analyzed. Despite no mutations being identified in connexin 40, further studies of the connexin genes are warranted given their expression and involvement in the lymphatic system.

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PREFACE

I would like to thank the members of my committee David Finegold, MD, Robert Ferrell, PhD, Adam Brufsky, MD, PhD, and Robin Grubs, PhD, CGC for their support and guidance during these past two years. I am greatly appreciative of having the opportunity to work on this project it has been a wonderful and enjoyable experience. Thank you to Dr. Ferrell for allowing me to use his laboratory for the completion of this project. A special thanks is given to Elizabeth Lawrence and Nancy Petro for sharing their laboratory expertise and for their guidance and assistance in lab.

I would like to acknowledge the medical oncology nurses and Dr. Inna Belfer who referred patients for involvement in this study. I would also like to extend my appreciation to the nurses in the Clinical Translational Research Center at Magee Women's Hospital for assisting during patient visits. Last but not least, I would like to thank my classmates, family, and friends for all of their constant love and support.

1.0 INTRODUCTION

1.1 ANATOMY AND PATHOPHYSIOLOGY OF LYMPHEDEMA

The lymphatic vascular system has been described as a mass transport system[1] responsible for the drainage and return of lymph fluid to the intravascular circulatory system. Fluid of the lymphatic systems contains water, proteins, lipids, foreign matter, cellular debris and a variety of cells[2]. Approximately 90% of interstitial fluid enters the venous capillaries and returns to circulation[3]. The other 10% of interstitial fluid, lymph fluid, contains macromolecules which are too large to pass through the venous capillaries. Therefore, this fluid enters the lymphatic capillaries and is filtered by the lymph nodes before returning to venous circulation. There are three main physiological functions of the lymphatic system which include maintenance and homeostasis of interstitial fluid, uptake of dietary lipids, and progression of the immune cell response[4].

The lymphatic vascular system consists of a network of branched capillaries and ducts[4] in which two main types of lymphatic vessels exist: the smaller initial lymphatic vessel and the collecting lymphatic vessel. The smaller initial lymphatic vessel includes the smallest lymphatic capillary and the larger precollector vessel. The one-way, open-ended small lymphatic capillaries are where interstitial fluid is initially drained from tissue. The small lymphatic

capillaries funnel into precollector vessels which drain into the collecting lymphatic vessels[5]. The collecting lymphatic vessels are composed of lymphangion units separated by intraluminal one-way valves. These valves open in response to positive pressure or upstream flow of fluid while reverse flow closes the valves. Collecting lymphatic vessels are covered by a continuous basement membrane and smooth muscle cells. Endothelial cells present in these vessels have an overlapping elongated shape thereby forming continuous junctions preventing any leakages during lymph transport[4]. The structure and mechanism of collecting lymphatic vessels allow for coordinated opening and closing of the valves resulting in efficient unidirectional flow of lymph transport.

Collecting lymphatic vessels are the major limb lymphatic vessels which provide flow to the lymph nodes[5]. Movement of interstitial fluid is dependent upon inherent forces of pressure as the basic motor function for the lymphatic system. Changes in capillary hydrostatic pressure and tissue oncotic pressure drive filtration while interstitial hydrostatic pressure and plasma oncotic pressure promote absorption[1]. Movement of tissues in response to pressure and contraction of smooth muscle cells in the collecting vessels result in compression and expansion of the lymphatics. Compression and expansion of the lymphatics regulate lymph flow through the lymphatic system[1, 5].

Lymphedema occurs in response to failure of the lymphatic system. It is caused by a decrease in lymph circulation as the result of an imbalance between the rates of interstitial fluid production and removal. Lymphedema is thus characterized by chronic accumulation of protein-rich fluid within the interstitial space[1, 6, 7]. Lymphedema is typically a consequence of either increased lymphatic flow or an intrinsic reduction in transport capacity. Lymphatic flow may be

increased by the presence of deep vein thrombosis, heart failure, malnutrition hypoproteinemia, cirrhosis, nephritic syndrome, or local inflammatory responses. Such conditions lead to a net increase in capillary pressure serving as a driving force for lymph fluid production[1, 6]. On the other hand, intrinsic reduction in transport capacity may be caused by a vascular disruption or malformation. There are multiple causes that may be responsible for impaired transport including trauma, infections, tumor invasion, external sources of compression, and heritable disorders affecting lymphatic pathogenesis[6]. The exact etiology of lymphedema is unknown in the majority of cases. Despite the various potential causes the implication is the same: entry of fluid exceeds the capacity of the lymphatics to remove the fluid resulting in disease.

1.2 CLASSIFICATION OF LYMPHEDEMA

Lymphedema is estimated to affect approximately 2-3 million individuals in the United States[8]. It is a chronic and progressive disease which is often debilitating for many affected individuals. Lymphedema can be classified into two main categories: primary and secondary.

1.2.1 Primary Lymphedema

Primary lymphedema is characterized by an inborn error of lymphatic development or function[6]. Primary lymphedema can be further classified and is most commonly grouped based upon age of onset into three subcategories: congenital lymphedema, lymphedema praecox,

and lymphedema tarda[5]. Congenital lymphedema presents at birth or within the first two years of life. Congenital lymphedema more commonly presents as a bilateral manifestation with both right and left lower extremities being affected. Lymphedema praecox commonly appears around puberty; however, it can occur any time up to the third decade of life. The majority of lymphedema cases are lymphedema praecox. Lymphedema praecox, unlike congenital lymphedema, typically exists as a unilateral presentation affecting an individual's foot and calf[5, 7]. Lymphedema tarda is recognized as onset after the age of 35. It is the rarest of the primary lymphedema forms accounting for less than 10% of reported cases[7]. Additionally, there are particular forms of inherited primary lymphedema which occur less commonly. There are several recognized inherited lymphedema syndromes with identified gene mutations. The genetic basis in many of these syndromes continues to be investigated.

1.2.2 Secondary Lymphedema

In addition to primary lymphedema, lymphedema that is acquired is referred to as secondary lymphedema. It is the most common form of lymphedema with an incidence of greater than 100 million worldwide[2]. Secondary lymphedema is the result of disruption to the lymphatic system caused by an external event. Surgery, tumor formation, trauma, radiation therapy, and filarial infection are just a few examples of events which can result in lymphatic obstruction and subsequential fluid accumulation[6, 8]. Filarial infection is the most common cause of secondary lymphedema worldwide.

1.2.2.1 Secondary Lymphedema Related to Breast Cancer

Breast cancer is the most common malignancy occurring in women in the United States[2]. Approximately 1 in every 8 women are diagnosed with breast cancer during their lifetime[9]. In 2011, it was estimated that 288,130 w omen received a diagnosis of breast cancer of which 39,520 women will die from the disease[10]. Advances in breast cancer treatment over the years have improved the long term survival of many women by use of surgery, radiation, chemotherapy, and hormone therapy. However, development of secondary lymphedema remains a common complication following cancer treatment.

In treating breast cancer, lymph node status serves as a significant marker in determining the best methods of treatment[2]. Axillary lymph node dissection (ALND) involves removal of the axillary lymph nodes and such surgical excision can obstruct the lymphatic system. Studies have shown a correlation between the extent of lymph nodes removed and the severity of lymphedema[8]. Incidence of lymphedema following ALND can be as a high as 45%-56%[2, 11]. Newer techniques such as sentinel node (SLND) biopsy involve excision of the first lymph node(s) to receive drainage from the breast. T he risk of lymphedema after SLND is lower compared to that of ALND with estimates of lymphedema incidence being approximately 7%-15%[12, 13]. Differences in surgical procedures also affect risk estimates as secondary lymphedema is more prevalent in women treated with mastectomy compared to breast-conserving surgery techniques. Approximately 20% of women treated with mastectomy will develop lymphedema compared to 8% of women who received breast-conserving surgery[14]. Risk of lymphedema may also be increased by adjuvant radiation therapy typically received by patients following lumpectomy or mastectomy procedures. Theories propose secondary

lymphedema induced via radiation exposure is related to fibrosis of the lymph nodes resulting in constraint of lymphatic channels[15]. However, the exact etiology of radiation induced lymphedema is unknown. Even with improvements in surgical techniques and treatment options, lymphedema remains a common debilitating complication post treatment[16, 17].

Secondary lymphedema effects on average approximately 20-30% of patients treated for breast cancer[18-20] with onset occurring anywhere from weeks to years after treatment[21]. The estimates of lymphedema vary due to the lack of a universal diagnostic definition and the application of a variety of measurement techniques. One study examining a cohort of 287 women found nearly one in two patients report at least moderate to extreme upper body symptoms at 6 years following a breast cancer diagnosis[19]. Development of secondary lymphedema can cause a significant impact on the quality of life for such individuals[3]. Not only does lymphedema cause physical impairment and dysfunction but it also may result in altered body image, anxiety, and depression. The psychological distress caused by this disease can ultimately affect social relationships as well as lower self-esteem. Findings in a study by Hayes et. al. (2011) suggest that lymphedema may also have an influence on survival following breast cancer treatment.

1.3 EVALUATION AND DIAGNOSIS

The majority of lymphedema diagnoses are made by clinical observation through physical examination[1]. Lymphedema can be classified as mild (Grade I), moderate (Grade II), or severe

(Grade III)[21]. Grade I pitting occurs in response to pressure and edema improves with elevation of the limb. With grade II classification the affected limb does not pit with application of pressure and the edema continues to worsen. Grade III is characterized by continuous swelling and associated skin changes such as a thick appearance with the presence of folds.

When the diagnosis of lymphedema is not clearly distinguished by physical exam, there are a variety of measurement evaluations which can establish a diagnosis. The most sensitive marker involves finding at least a 200 ml difference in arm volume by water displacement volumetry[22]. However, breast cancer related lymphedema does not typically present with uniform edema throughout the entire length of the arm and there is evidence that segmental differences in drainage may result in segmental presentation of swelling[23, 24]. In this case, diagnosis based on a total volume difference may not be as sensitive. More commonly used is circumferential measurement criteria including at least a 2 cm difference in arm circumference between any two points when the measurements are made every 4 cm along the axis[2, 15, 22]. Women treated for breast cancer who self-report symptoms of heaviness are reported to correlate with this 2 cm or greater difference in limb circumference[25]. However, limitations exist with circumferential measurement use such as difficulty with control of intrarater and interrater reliability[21, 26]. Approximately 12% of individuals are given a false diagnosis based on circumferential measurement alone[27].

An alternative method measures the volume of fluid in each limb and the entire body through use of bioimpedance spectroscopy (BIS) technology[28]. BIS uses electrodes attached to the skin of the hands and feet which send a small electric current throughout each limb and the entire body. The resistance of flow in response to this electric current is measured at multiple

frequencies. By measuring a range of frequencies, BIS derives the resistance or impedance to flow at a frequency comparable to the impedance of extracellular fluid (0 HZ). BIS is one of the few techniques analyzing body composition and distinguishing extracellular fluid from total limb volume. In turn, BIS does not provide a measurement of volume but rather provides a measurement of electrical impedance. When the ratio of the impedance measurement of the affected limb compared to the unaffected limb is greater than 1.00, a clinical diagnosis of lymphedema is made. When comparing BIS to other diagnostic techniques, approximately 40%-60% of patients measured by circumferential means or by self-report went undetected[27].

A third common approach to diagnosis of lymphedema is use of optoelectric perometry. A perometer uses 360 light beams emitted from both sides of a movable frame positioned horizontally above a base platform[13, 21]. The space the limb occupies inside the frame disrupts the light beams creating a shadow. The frame is moved up and down along the length of each limb and the diameter is measured every 3.1 mm while indirect volume measurements are calculated by computer processing software (Peroplus^{TM})[29]. Perometry is noted as one of the most reliable methods; however, several of the disadvantages include difficulty in measuring the entire length of each limb including the hands and feet as well as the limited accessibility to use such equipment outside of a clinic setting due to its size and complexity[22, 26].

Additional radiological studies including CT, MRI, lymphangiography, and lymphoscintigraphy also aid in the analysis and diagnosis process[2]. CT and MRI scans show a distinctive pattern to the lymphatic system which can help distinguish between differential diagnoses. Lymphangiography is a technique which uses a water soluble solution to observe the filling of the lymphatics. Radiographic images of the filling process are obtained at intervals

every several minutes[30, 31]. Unlike lymphangiography, lymphoscintigraphy gathers structural and functional data of lymphatic drainage. Lymphoscintigraphy measures the rate at which radiolabelled molecules, which are injected into tissue, are cleared from the lymphatic system[32].

With each measurement technique there are reported difficulties and inconsistencies[26, 33]. As a result, the reported incidence of women affected by secondary lymphedema following breast cancer treatment varies greatly depending upon the population studied and the methods used to diagnosis disease. Reported incidence of breast cancer related lymphedema ranges in the literature from 2% to 83%[34]. Currently there is no gold standard method for clinical evaluation and diagnosis that is consistent and non-invasive[35].

1.4 MANAGEMENT AND TREATMENT

Presently, there is no cure for lymphedema; however, there are several options for treatment and medical management to help control symptoms. Current physiotherapeutic management includes complete or complex decongestive therapy (CDT) and exercise therapy[27]. Additional management options include pharmacologic treatment, pneumatic pumps, low level laser therapy, and surgery.

Complex decongestive therapy (CDT) is recognized as one of the optimal strategies for management of lymphedema[36]. CDT consists of two phases. The first phase includes CDT therapy which is implemented by a hospital or provided by a lymphedema therapist while the

second phase involves at home self-care provided by the patients themselves[37]. CDT therapy encompasses four techniques: manual lymphedema drainage (MLD), compression therapy, exercises, and patient education regarding skin care[27, 38].

MLD is a massage technique used to increase lymph flow. MLD is based on facilitating the passage of lymph from affected to unaffected areas of the body[39]. The massage therapy typically begins at the neck and trunk area with the goal of assisting in the progression of lymph from the main lymphatic pathways and ultimately drainage from the arm. MLD is typically performed for 45-60 minutes four or five times a week during a 2-4 week period.

Compression therapy includes the use of compression bandages or compression garments which contribute to a gradual reduction in volume of the affected limb[3]. Compression bandages, made from a gauze sleeve and layer of stretch fabric, are designed to protect and constrict the skin. The bandages compress the covered area averting the counter flow of lymph fluid. Compression garments are similar in function to compression bandages; however, the pressure exerted by compression garments is progressive. Compression garments have a lesser amount of compression or resistance located at the proximal end of the arm while the greatest amount is located at the distal end of the arm. Compression bandages and garments are the foundation of maintenance therapy after intensive CDT is completed[38]. A recent study conducted by King et al. (2011) examined the difference in outcomes when using compression bandages compared to compression garments. The results of the study indicated that use of compression bandaging may be more effective in leading to greater limb volume reduction while compression garments may result in a better functionality or range of motion for the upper extremity[40].

Patients are encouraged to remain physically active as exercise can improve both their range of motion and restore upper extremity function[41]. The internal pressure changes created by exercising promote the flow of lymph and lymph drainage. Shoulder exercises as well as weight lifting and strengthening exercises lead to a more rapid recovery of mobility and decrease symptoms associated with lymphedema[42, 43]. Patients who began exercise therapy 6-26 weeks after surgery improved their range of motion without increasing their lymphedema[44]. It had been previously accepted that women should avoid and limit the use of their affected arm after breast cancer surgery to decrease the risk of lymphedema[45]. More recent data indicates that well controlled exercise programs provide benefit to many women. There are exercise guidelines available for patients; however, these guidelines may be modified to fit an individual's needs determined by a baseline assessment prior to surgery[27]. Postoperative exercises can improve mobility, shoulder function, overall physical fitness, body composition, self-esteem, and quality of life. Early physiotherapy, initiated for at least one year prior to breast cancer surgery, is effective in the prevention of secondary lymphedema onset[36].

The final component of CDT includes integration of skin care[27, 38]. Proper skin care education promotes daily application of moisturizer as well as appropriate management of nail care. Individuals are cautioned to avoid obtaining any open wounds via cuts, scratches, insect bites, etc. Through patient education of proper skin care the goal is to prevent infection since data suggests there is an increased risk of infection and lymphedema associated with skin trauma.

In addition to CDT, many other treatment and management options are available to patients including pharmacologic treatment, pneumatic pumps, low level laser therapy, and

surgery[3, 6, 46]. The goal of pharmacological therapy by use of benzopryones is to increase lymph flow by lowering vascular permeability and decreasing accumulation of protein in lymph fluid. Some substances involved in the pharmacologic treatment of lymphedema remain controversial as the use of agents, such as coumarin, can induce hepatic dysfunction[6]. Pneumatic pumps may consist of single or multiple chambers which inflate and deflate at varying levels of pressure[46]. The goal of pneumatic pumps is to promote muscle movement and thereby drainage of fluid from the limb. Low level laser therapy (LLLT) has been used in the United States as an accepted form of breast cancer related secondary lymphedema therapy since 2007[47]. LLLT uses a low level carbon dioxide laser to decrease the amount of excess protein and fluid thus increasing lymphatic flow. When considering surgical treatment for lymphedema there are four main options: debulking, liposuction, omental pedicles and myocutaneous flap construction, and lymphatic microsurgical preventive healing approach (LYMPHA)[6, 48]. Debulking surgery removes excessive skin and tissue and is usually performed when there are vast changes in limb size. Liposuction involves the removal of excess adipose tissue. Omental pedicles and myocutaneous flap interposition allows for the creation of a lymphatic bridge which may function in the reestablishment of lymphatic flow[49]. A recently developed surgical treatment for lymphedema, LYMPHA, is most appropriate for individuals receiving axillary lymph node dissection (ALND)[48]. The LYMPHA procedure includes lymphatico-venous anastomoses (LVA) at the time of an ALND procedure[50]. This procedure aids in preventing the development of secondary lymphedema. With all surgery there are associated risks of complication and therefore most women choose more conservative approaches to management. The majority of women who pursue surgical means to treatment are

those which have severe presentation and do not gain improvement or benefit from the conservative measures.

1.5 PREDISPOSITION

1.5.1 Risk Factors

Since there is currently no c ure for lymphedema, the most effective means to decrease the incidence of this condition is through early detection and risk reduction intervention. Several studies have investigated risk assessment related to multiple predisposing factors involved in the development of lymphedema. Such factors examined include cancer diagnosis and treatment, health and behavioral features, as well as patient characteristics.

It is generally accepted that more advanced cancer typically requiring more extensive surgery and exposure to radiation is associated with increased predisposition to lymphedema[34, 51-53]. A study by Hayes et al. reported a six-fold increase in odds for development of lymphedema with extensive surgery[34]. Associations between positive lymph node findings and onset of lymphedema have also been reported. In the same study by Hayes et al. a four-fold increase in odds for development of lymphedema was noted when more than twenty lymph nodes were removed during surgery. No confirmed associations have been reported with regard to tumor stage, chemotherapy, or surgery of the patient's dominant side[34, 53, 54].

A high body mass index (BMI) indicating obesity is one of the most commonly accepted health and behavioral characteristics to have an association with increased risk of lymphedema[52, 53]. Both obesity present at the time of diagnosis and weight gain post treatment is believed to be contributory[52]. Obesity results in an increased risk for lymphedema onset due to the increased stress on the lymphatic system. In addition to obesity, infection and limb injury[51-53] as well as the subjection to lower pressure during air travel[51] have also been associated with increased risk of lymphedema onset. Circulatory diseases, such as hypertension, may also play a role in increasing risk for lymphedema; however, the data supporting this hypothesis is inconsistent[51, 52].

In regards to patient characteristics, age is one of the most studied factors with many studies reporting mixed findings[34, 52]. Different reports indicate a greater association with women of a young age (<50 years), older age (> 50 years), and some with no association at all. However, in the majority of studies the consensus is that age does not play a contributory role in lymphedema onset. In a study by Hayes, et al. women with young children and of lower socioeconomic status were found to have a decreased risk in which the odds of lymphedema development were reduced by five- to ten-fold[34]. From the authors' data, this risk reduction is based on the idea that such women find themselves using their treated side more often when caring for young children and that lower socioeconomic status can be associated with occupations involving more manual labor. These findings support the theory that physical activity and use of an individual's treated side does not increase risk of lymphedema but rather improves arm symptoms.

1.5.2 Genetic Etiology

Several studies have examined the genetic etiology of primary and secondary lymphedema development and function. Lymphedema is a heterogeneous condition which can exist as part of a genetic syndrome or as an isolated occurrence. Disease causing mutations account for few lymphedema cases; however, continued study of genetic etiology may reveal new insights and findings of lymphatic disease[55].

Milroy's disease is an autosomal dominant condition characterized by congenital lymphedema[55]. Mutations in vascular endothelial growth factor C receptor (VEGFR3; FLT4) are responsible for Milroy's Disease[56]. Milroy's disease has variable expressivity and typically presents as a bilateral manifestation affecting the lower limbs. Approximately 80-90% of individuals with an identified VEGFR3 mutation develop lymphedema before the age of three[57].

Hypotrichosis-lymphedema-telangiectasia syndrome is characterized by lower limb lymphedema, telangiectasias, and hypotrichosis. Mutations in SRY-box 18 (SOX18) are responsible for this condition[55, 56]. Hypotrichosis-lymphedema-telangiectasia syndrome has been observed with both autosomal dominant and autosomal recessive forms of inheritance[58].

Lymphedema-distichiasis syndrome is an autosomal dominant condition characterized by lower limb lymphedema and distichiasis and is caused by mutations in FOXC2 (MFH1)[55]. FOXC2 is believed to play a critical role in the institution of a smooth muscle cell-free lymphatic network. Truncating FOXC2 mutations disrupt activation of gene transcription and have been shown to result in broad phenotypic variability within families[59]. FOXC2 mutations are

primarily responsible for lymphedema-distichiasis; however, based on this broad phenotypic observation, mutations in this gene are also believed to contribute to other lymphedema phenotypes.

Hennekam syndrome is an autosomal recessive condition characterized by severe congenital lymphedema, intestinal lymphangiectasia, seizures, growth retardation, and facial anomalies[60, 61]. Within Hennekam syndrome, lymphedema is most commonly gradual and progressive primarily affecting the limbs and bowels but can also be observed in the face and genitals[62]. Additional defects associated with this syndrome include congenital glaucoma, congenital heart defects, vascular anomalies, craniosynostosis, renal malformations, and hearing loss. In several individuals with Hennekam syndrome homozygous and compound heterozygous mutations have been identified in the CCBE1 gene located at chromosome 18q21[63]. The CCBE1 (collagen and calcium-binding EGF-domain-1) gene is involved in the extracellular matrix and is not expressed in endothelial lymph vessel cells. It is believed that CCBE1 plays a regulatory role in the early migratory patterns of lymphatic development since *ccbe1* is expressed in the migratory routes of endothelial cells destined for lymphatic vessels in zebrafish.

Emberger syndrome is an autosomal dominant disorder characterized by primary lymphedema with myelodysplasia[64, 65]. Myelodysplasia predisposes affected individuals to the development of acute myeloid leukemia. Mutations in GATA2 are responsible for Emberger syndrome. GATA2 is a transcription factor responsible for the control and management of hematopoietic differentiation and vascular development.

Microcephaly-Lymphedema-Chorioretinopathy is an autosomal dominant condition presenting with a wide spectrum of CNS and ocular findings[66]. Microcephaly is considered

the critical component of this syndrome and can range from mild to severe. Lymphedema associated with this syndrome presents as the congenital form and typically affects the dorsa of the feet. Variable visual deficiencies have been observed as a result of chorioretinal dysplasia[67]. Mutations in the KLF11 gene are found to be causal of Microcephaly-Lymphedema-Chorioretinopathy[66]. The KLF11 gene encodes EG5 which is a kinesin motor known to contribute to spindle formation. Mutations in the KLF11 gene are expected to disrupt protein function underlining the importance of spindle assembly in CNS development and function.

Ferrell et al. investigated 25 candidate genes in families with primary lymphedema[56]. Results showed causative mutations in the genes FABP4, NRP2, SOX17, and VACM1. In addition, Finegold et al. identified mutations in HGF (hepatocyte growth factor) and MET (high affinity hepatocyte growth factor receptor) in individuals with primary and secondary lymphedema[68]. HGF/MET is highly expressed in lymphatic endothelial cells and has a wide range of biological functions including roles in cell growth, mobility, differentiation, and intracellular junctions. Truncating and missense mutations were reported in both primary and secondary lymphedema cases.

Mutations in the connexin 47 (GJC2) gene were also observed in several primary lymphedema families as well as women with secondary lymphedema following breast cancer therapy. T he role of the connexin genes involves the formation of gap junctions which are believed to contribute to the progression of lymphatic flow and function. In primary lymphedema families, a total of 6 out of 150 probands were identified with a unique connexin 47 missense mutation[69]. Two of the probands were part of large families in which segregation of

the mutation with the associated lymphedema phenotype was observed. In a case-control study designed to investigate secondary lymphedema, 188 women diagnosed with breast cancer were screened for mutations in connexin 47 (GJC2), FOXC2, HGF, MET, and FLT4 (VEGFR3)[70]. Connexin 47 mutations were identified in 4 women with secondary lymphedema following breast cancer treatment.

The finding of HGF and MET mutations first raised the possibility of a genetic predisposition to secondary lymphedema and with the recent finding of connexin 47 mutations a genetic predisposition to the development of secondary lymphedema was confirmed. Previously identified genes contribute to our understanding of genetic risk factors involved with lymphedema; however, these genes only account for a few secondary lymphedema cases. Continuing advances can aid in the effort for early detection and prevention; in turn, further studies examining genetic etiology are warranted.

2.0 SPECIFIC AIM OF THE STUDY

The goal of this study is to further investigate the significance of additional connexin genes, specifically connexin 40, in the development of secondary lymphedema. Connexins 37, 40, 43 and 47 are all expressed in the lymphatics and thus provide rationale for investigating other connexins in lymphedema. Mutations in the connexin 47 (GJC2) gene have been previously identified as a causal and/or predisposing factor in the development of primary and secondary lymphedema[69, 70]. The exact mechanism by which connexin 47 mutations increase susceptibility to lymphedema is unknown; however, it is proposed that such mutations disrupt gap junction communication resulting in impaired lymphatic flow. The previously identified mutations support genetic susceptibility to secondary lymphedema onset and warrant further consideration of additional connexin genes. Overall, there are twenty different connexins which can form gap junctions[71]. Gap junctions function as intercellular communication channels between cells allowing for the passage of substances between cell membranes. Expression of connexins is highly regulated and the mechanism by which regulation is maintained is not clearly understood. Gap junctions are involved in a variety of different tissues and are required for many developmental and physiologic processes[72].

Aim: The aim of this study was to determine if individuals with secondary lymphedema following breast cancer treatment have mutations in the connexin 40 (GJA5) gene.

Hypothesis: The hypothesis for this study is that other connexin genes, in addition to connexin 47, play a contributory role in lymphedema development and that in a subset of women treated for breast cancer who developed secondary lymphedema, inherent mutations in the connexin 40 (GJA5) gene will be identified by gene sequence analysis.

3.0 MATERIALS AND METHODS

3.1 STUDY DESIGN

The breast cancer secondary lymphedema research study was initially submitted to and approved by the Institutional Review Board of the University of Pittsburgh. This case-control study is a comparison of the occurrence of lymphedema in women with and without a diagnosis of secondary lymphedema following breast cancer treatment.

Women were recruited and screened to determine eligibility. Screening questions were asked by telephone prior to enrollment. All appointments for enrolled and consented patients were held in the Clinical Translational Research Center (CTRC) at Magee Women's Hospital of the University of Pittsburgh Medical Center (UPMC). Women clinically diagnosed with lymphedema by a physician or physical therapist are defined as cases and women who have not developed lymphedema and are at risk for development are defined as controls.

Within the study there are two components: a retrospective analysis and a prospective analysis. The retrospective study received IRB approval September 5th, 2000 under IRB#960639 and is no longer accruing patients. The cohort of individuals enrolled in the retrospective study received breast cancer treatment between the years 1988 and 1999. The prospective study received IRB approval March 8th, 2007 (PRO06080011) and is currently open to enrollment with

the goal of accruing 750 participants. The individuals enrolled in the prospective study received breast cancer treatment within the past several years. Women from the prospective study were recontacted annually to determine if their lymphedema status had changed since the time of initial enrollment in the study. If a woman developed lymphedema since the initial time of contact and enrollment, she was appropriately reclassified as a case. DNA samples were obtained from all individuals in both the retrospective and prospective groups.

3.2 SUBJECT RECRUITMENT

As described in a master's thesis by Roxanne Miller, 2003, potential participants in the retrospective study were recruited via letters mailed from Magee Women's Hospital and contacted by phone[73]. Interested individuals were eligible for enrollment if they were diagnosed with breast cancer between the years 1988 and 1999. One hundred and sixty potential cases and five hundred potential controls were contacted through the Physical Therapy and Medical Records Department at Magee Women's Hospital. A total of 64 cases and 63 controls were successfully recruited, enrolled, and consented to participate in the retrospective study. Participants completed a medical history questionnaire verifying their breast cancer diagnosis and lymphedema diagnosis (if applicable). Family history was also obtained. A DNA sample was collected through a 30cc blood sample or buccal swab. Sequencing of the connexin 40 (GJA5) gene was analyzed for 52 cases and 52 controls in the retrospective study.

Potential participants in the prospective study were recruited from the University of Pittsburgh Breast Cancer Program from flyers and brochures located in Magee Women's Hospital, referral from the genetics of post mastectomy pain study directed by Inna Belfer, M.D., and direct contact in the medical oncology and lymphedema clinics. The potential participants directly contacted in clinic were first approached by a physician or nurse to gain permission for a researcher to speak with them in regards to the study. Individuals who gave permission were provided details of the research study by the researcher. Participants were asked to inform family members of the study and those who initiated contact were given the opportunity to participate as well. A total of 185 individuals enrolled and consented to participate in the study including 43 cases, 120 controls, 8 unsure, and 14 family members. Participants classified as unsure reported having variable degrees of swelling post breast cancer treatment; however, at the present time it was uncertain if the swelling was lymphedema or related to post-surgical swelling. None of the participants in the unsure group had an official diagnosis of lymphedema by a health care professional. A standardized medical questionnaire was completed and family history obtained for all individuals. Participants also underwent a series of three measurement analyses using circumferential measurement of the limbs, BIS, and Perometry. All participants had blood samples drawn for DNA analysis. In the event a blood sample could not be obtained, an alternative method using an Oragene DNA® saliva kit was utilized. Sequencing of the connexin 40 (GJA5) gene was analyzed for 39 cases and 116 controls in the prospective study.

3.3 LABORATORY PROCEDURES

DNA was isolated from ETDA anti-coagulated peripheral blood by the salting out procedure described by Miller et al. [74]. DNA extraction from the saliva samples was performed using an Oragene DNA® kit. Amplification and sequencing primers were synthesized based on reported human connexin 40 (GJA5) cDNA sequence NM_005266. The sequence was downloaded from GenBank and flanking primers were designed to amplify the target sequence. The following primers were used to screen for the connexin 40 (GJA5) gene:

2F, 5'-CCATTGGATGGATGGATC-3'

2R, 5'-CCGTAGATGAAGTACTGG-3' (54°C T_a; 1.5 mM Mg^{++})

2F2, 5'-GGAAGGGAATGGAAGGAT-3'

2R3, 5'-CAGTTCAGAAGGGACACG-3' (54°C T_a; 1.5 mM Mg^{++})

Sequences were amplified using the previously listed primers by a polymerase chain reaction. The PCR technique utilized an Invitrogen Taq at an annealing temperature of 54°C. Several templates were amplified using a QIAGEN Multiplex PCR kit. The QIAGEN kit provided greater sensitivity and specificity with a built-in hot start and more efficient amplification of G-C rich regions. The initial denaturing step was followed by 94 cycles at 30 seconds each, 54 cycles at 30 seconds each, and 72 cycles at 45 seconds each.

A shrimp alkaline phosphatase and Exonuclease I were used to treat the amplimers. The amplimers were 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. Sequences were aligned using Sequencher V5.0 software (Gene Codes Corp., Ann Arbor, Michigan 48108).

4.0 RESULTS

4.1 GENE SEQUENCING ANALYSIS

Sequence analysis of the connexin 40 (GJA5) gene was completed for 52 cases and 52 controls in the retrospective study and 39 cases and 116 controls in the prospective study. The connexin 40 (GJA5) gene is located on chromosome 1q21.1 encoding 358 amino acids. The gene is comprised of 2 exons in which exon 1 is noncoding.

Sequencing analysis identified a common single nucleotide variant in a noncoding region of the gene. This variant was confirmed in 1 control in the retrospective study and 3 controls in the prospective study. The variant, located at base pair 15,268 is a G → A nucleotide change. This variant is validated and catalogued as rs16192141 in the National Center for Biotechnology Information (NCBI) database.

No other variants or mutations were identified in any of the cases or controls screened for connexin 40 (GJA5).

4.2 DESCRIPTIVE STATISTICS

Demographic and risk factor exposure information was collected from the medical history questionnaires completed by all participants in both retrospective and prospective groups. Demographic information includes age at enrollment, age at breast cancer diagnosis, age at lymphedema diagnosis, BMI, and ethnicity. The timing of onset of lymphedema after a breast cancer diagnosis was also examined in all cases. Risk factor information contains treatment variables of mastectomy and radiation therapy in addition to self-reported exposures including blood draw, blood pressure measurement, cat scratch, cut, insect bite, manicure, and sunburn. Statistical analysis was performed using SPSS Statistics 20 and SAS 9.3 for Windows.

4.2.1 Demographic Analysis

Table 1 and Table 2 contain the respective demographic information for ethnicity, age, age at breast cancer diagnosis, age at lymphedema diagnosis, and BMI for retrospective and prospective groups.

According to the National Heart, Lung, and Blood Institute, classification of BMI (kg/m^2) measurements less than 18.5 is underweight, between 18.5-24.9 is normal, between 25.0-29.9 is overweight, and greater than or equal to 30.0 is obese[75]. The average BMI for cases in the retrospective group is 28.8 and in the prospective group is 28.7 which are both classified within the upper limit of the overweight category.

Table 1. Retrospective Group: Demographic Variable Means and Standard Deviations

	Cases N=64	Controls N=63
Ethnicity (% Caucasian)	56/64 (87.5%)	62/63 (98.4%)
Age (range)	59.4 (38-85) SD=10.39	57.0 (33-78) SD=8.53
Age at Breast Cancer Diagnosis (range)	52.6 (30-74) SD=10.01	51.4 (29-74) SD=8.83
Age at Lymphedema Diagnosis (range)	55.0 (32-82) SD=10.42	-
BMI (range)	28.8 (19.6-48.4) SD=6.25	27.05 (19.2-41.6) SD=5.00

Table 2. Prospective Group: Demographic Variable Means and Standard Deviations

	Cases N=43	Controls N=120	Unsure N=8
Ethnicity (% Caucasian)	41/43 (95.3%)	110/120 (91.7%)	8/8 (100%)
Age (range)	58.3 (38-93) SD=12.85	53.8 (22-76) SD=9.77	57.8 (40-75) SD=12.89
Age at Breast Cancer Diagnosis (range)	53.5 (34-77) SD=11.03	52.0 (20-75) SD=9.69	56.9 (39-71) SD=12.51
Age at Lymphedema Diagnosis (range)	56.0 (37-78) SD=11.70	-	-
BMI (range)	28.7 (19.7-44.5) SD=6.33	28.1 (17.5-48.7) SD=5.80	30.0 (24.1-37.4) SD=4.55

Independent sample t-tests were carried out to investigate the differences in demographic variables between cases and controls. The cases and controls from both the retrospective and prospective groups were combined to examine differences between the total number of cases and controls involved in the study. Independent t-tests shown in Table 3 reveal a significant finding between the age of cases and controls at the time of enrollment. With a p-value of $p=0.002$ ($p<0.05$, two-tailed) there is a significant difference between the ages of cases and controls with

an older age being observed in the cases compared to the controls. Differences between cases and controls for age at breast cancer diagnosis and BMI are not significant ($p>0.05$, two-tailed).

Table 3. Retrospective and Prospective Groups Combined: Independent Samples T-Test for Equality of Means of Demographic Variables

		N	Mean	Standard Deviation	t	p
Age	*Cases* *Controls*	107 183	59.0 54.9	11.40 9.46	-3.120	0.002*
Age at Breast Cancer Diagnosis	*Cases* *Controls*	107 183	53.0 51.8	10.39 9.38	-1.002	0.317
BMI	*Cases* *Controls*	107 183	28.8 27.8	6.28 5.56	-1.482	0.139

The timing of secondary lymphedema onset after breast cancer diagnosis was examined. The cases from the retrospective group (N=64) and the prospective group (N=43) were combined to determine the distribution of onset. The majority of women in this study reported lymphedema onset occurring within 1-2 years following a diagnosis of breast cancer. It is known that lymphedema can occur weeks to years after surgery. Many controls involved in the prospective study were enrolled within 1 year of their breast cancer surgery; therefore, they are still at risk to develop lymphedema. Over time, controls who develop lymphedema will be reclassified as cases thus altering the distribution analysis.

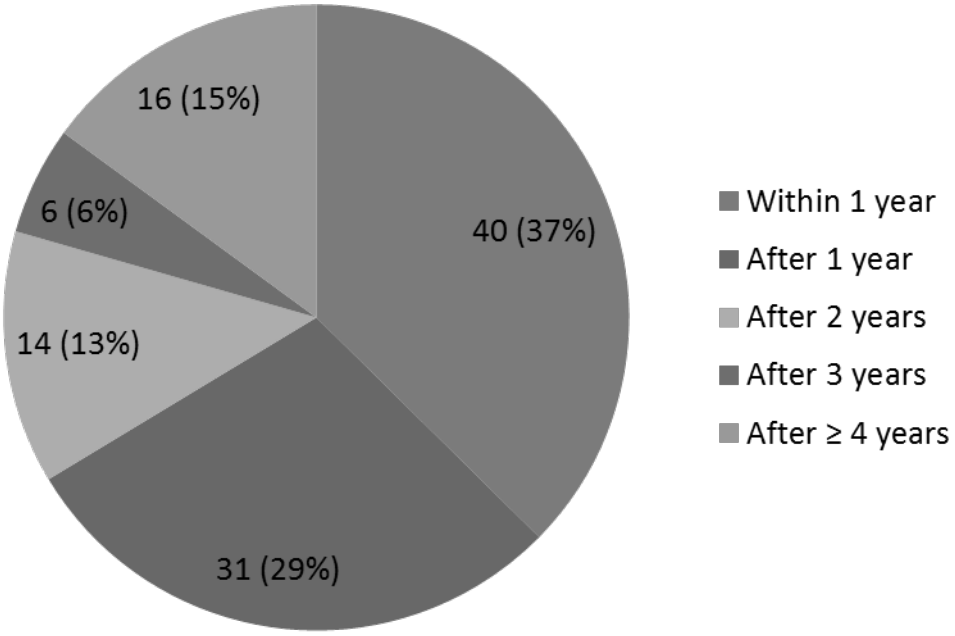

Figure 1. Timing of Lymphedema Onset After Breast Cancer Diagnosis

(Retrospective and Prospective Cases, N=107)

4.2.2 Exposures and Risk Factor Analysis

Self-reported exposures and risk factors for cases and controls are listed in table 4 for the retrospective group and in table 5 for the prospective group. Participants were asked to indicate which type of breast cancer treatment they had undergone and which risk factors they had exposure to after surgery. Fisher exact tests were carried out to investigate differences between cases and controls in the retrospective group and between cases, controls, and those classified as unsure in the prospective group.

In table 4 there is a significant difference between retrospective cases and controls and reported manicure exposure. In this group, 13/64 (20%) cases and 25/63 (40%) controls reported having a manicure. With a p-value of p=0.021 there is a significant difference ($p<0.05$, two-tailed) in that the control group has a higher reported number of manicure exposures.

In table 5 there is a significant difference between prospective cases, controls, and those classified as unsure for reported radiation exposure and insect bite exposure. In this group 33/43 (77%) cases, 64/120 (53%) controls, and 4/8 (50%) unsure reported having radiation therapy. With a p-value of p=0.019 there is a significant difference ($p<0.05$, two-tailed) with cases having a higher number of participants receiving radiation therapy. In addition, 15/43 (35%) cases, 22/120 (18%) controls, 0/8 (0%) unsure reported having an insect bite exposure. With a p-value of p=0.029 there is a significant difference ($p<0.05$, two-tailed) with cases having a higher number of reported insect bite exposures. Both radiation therapy and insect bite exposure are known risk factors for lymphedema development.

Table 4. Retrospective Group: Self-Reported Risk Factor Exposure

	Cases N=64	Controls N=63	p
Mastectomy	27	23	0.587
Radiation	52	46	0.297
Blood Draw	8	5	0.560
Blood Pressure	7	6	1.00
Cat Scratch	6	11	0.203
Cut	20	23	0.577
Insect Bite	17	22	0.340
Manicure	13	25	0.021*
Sunburn	12	17	0.297

Table 5. Prospective Group: Self-Reported Risk Factor Exposure

	Cases N=43	Controls N=120	Unsure N=8	p
Mastectomy	19	46	5	0.339
Radiation	33	64	4	0.019*
Blood Draw	4	24	0	0.138
Blood Pressure	5	25	0	0.205
Cat Scratch	5	14	0	0.828
Cut	16	33	1	0.309
Insect Bite	15	22	0	0.029*
Manicure	10	26	0	0.412
Sunburn	5	12	0	0.812

The total number of reported exposures was investigated using an analysis of variance (ANOVA). ANOVA analysis was carried out using the total number of reported exposures in the retrospective and prospective groups. There are no statistically significant differences ($p>0.05$, two-tailed) in the average number of exposures between cases and controls in the retrospective group and between cases, controls, and those classified as unsure in the prospective group.

Table 6. Retrospective Group: Analysis of Total Number of Risk Factor Exposures Reported

	N	Mean	Standard Deviation	F	p
Cases	64	2.53	1.583	1.097	0.297
Controls	63	2.83	1.582		

Table 7. Prospective Group: Analysis of Total Number of Risk Factor Exposures Reported

	N	Mean	Standard Deviation	F	p
Cases	43	2.60	1.591	2.761	0.066
Controls	120	2.22	1.583		
Unsure	8	1.25	0.886		

5.0 DISCUSSION

With advances in breast cancer detection and treatment over time, the life expectancy of many women has improved raising the importance of quality of life post-surgery. The exact incidence of secondary lymphedema is unclear due to the lack of universally accepted clinical criteria defining a lymphedema diagnosis and multiple methods of measurement reported in medical literature[16]. Lymphedema being a common complication following breast cancer treatment can result in physical impairment as well as psychological morbidity[11]. Women who develop secondary lymphedema may experience variable degrees of functional impairment, pain, weakness, stiffness, numbness, and increased risk of infection. Psychological distress may also accompany lymphedema onset in many affected women. Several studies have shown that women with lymphedema develop higher levels of social and psychological morbidity than women who do not develop secondary lymphedema[76]. Anxiety, depression, social avoidance, and self-consciousness may all contribute to the psychological distress experienced by many of these women.

Unfortunately, there is no cure for lymphedema. Several management options exist to help diminish arm symptoms; however, not all women find benefit from the available treatments. Efforts towards the treatment and management of lymphedema are most effective when

implemented early[11]. It is therefore important to clarify predisposing risk factors and to better understand genetic etiology in the effort for early detection and efficient intervention.

5.1 GENE SEQUENCING

Initial genetic studies contributed to the current understanding of primary lymphedema while more recent discoveries have expanded our knowledge of the genetic etiology of secondary lymphedema. Mutations in HGF, MET, and connexin 47 have been recently identified in secondary lymphedema patients[68, 70]. Connexins are gap junctions responsible for facilitating cell to cell communication. Connexins are also found to be highly expressed in the lymphatics warranting further investigation of additional connexin genes. The primary aim of this study was to identify mutations in the connexin 40 (GJA5) gene which may increase susceptibility to development of secondary lymphedema in women treated for breast cancer. It was hypothesized that previously unidentified mutations would be found in the connexin 40 (GJA5) gene of women who developed lymphedema post breast cancer treatment. The connexin 40 (GJA5) gene was sequenced in a cohort of 259 (N=91 cases, N=168 controls) women diagnosed with breast cancer with and without secondary lymphedema. A previously recognized single nucleotide variant was identified in the noncoding region of four women with breast cancer and without lymphedema. No previously unidentified mutations were identified in the connexin 40 (GJA5) gene of women treated for breast cancer with secondary lymphedema which is inconsistent with

our hypothesis. Despite the negative findings in this study, further investigation of additional connexin genes is still warranted given their involvement with the lymphatic system.

5.2 DEMOGRAPHIC AND RISK FACTOR INFORMATION ANALYSIS

Demographic and self-reported risk factor information was collected from all participants involved in the study. Statistical analysis of such data revealed several statistically significant differences between cases and controls. Overall, the age at time of enrollment was significantly higher in cases than in the controls (p=0.002). In regards to predisposing risk factors, there was a statistically significant difference between cases and controls for which reported radiation exposure (p=0.019) and insect bite exposure (p=0.029) was greater in cases compared to controls. Radiation therapy and insect bite exposure are risk factors reported in the literature associated with increased risk for development of lymphedema. A study by Niwinski et al. reported a lymphedema incidence of 10% among women receiving radiation therapy in breast-conserving surgery while another study by Bani et al. reported a lymphedema incidence of 30% among women treated with radiation therapy as part of breast cancer treatment[27, 77]. In our study a total of 85 out of 199 (42.7%) women who reported receiving radiation treatment developed lymphedema. In regards to the insect bite exposure, breast cancer patients are advised to protect their skin and to avoid cutaneous skin trauma (cuts, insect bites, etc.) as a prevention measure[78]. In our study, a total of 32 out of 76 (42.1%) women who reported having an insect bite exposure developed lymphedema.

The timing of lymphedema onset after breast cancer diagnosis was also evaluated. It is known that lymphedema onset can occur weeks to years after breast cancer therapy. A study of 282 patients found the average timing of lymphedema onset was 14 months after treatment with a range of 2 to 92 months[79]. As reported in the literature the onset can be gradual or rapid and the majority of patients experience lymphedema onset within the first three years after breast cancer surgery[80, 81]. It is estimated that 75% of secondary lymphedema cases develop lymphedema within 2 years of breast cancer treatment and 90% within 3 years[82]. In our study, out of a total of 107 enrolled cases 40 (37%) women experienced lymphedema onset within the same year as their diagnosis, 31 (29%) experienced onset after the second year following a cancer diagnosis, and 14 (13%) experienced onset after the third year following a cancer diagnosis.

5.3 LIMITATIONS OF STUDY AND FUTURE CONSIDERATIONS

Within this study controls are still considered to be at risk for development of lymphedema since onset can occur multiple years after surgery. It is possible that in the future current controls may develop lymphedema and would be reclassified as cases thus altering the current analyses. In addition, within the prospective cohort several individuals had arm symptoms but did not have a clinical diagnosis of lymphedema. These individuals were classified in an unsure group. With proper evaluation and future follow-up we would expect to reclassify each subject as either a case or a control.

There is presently no standard clinical definition of lymphedema; therefore, for the purposes of this study a clinical diagnosis of lymphedema by a healthcare professional was used to define lymphedema cases. Measurement analyses had been performed on all subjects involved in the study. The measurement analyses obtained were unfortunately inconsistent and inaccurate. In the future, with the obtainment of reliable measurements such analyses will aid in the appropriate classification of cases and controls.

Additionally, risk factor exposure information was subject to recall bias. Recall bias occurs when information is differentially misclassified between cases and controls. With recall bias individuals who have experienced disease may tend to think about possible "causes" of the outcome which can lead to differential recall. All risk factor exposures (blood draw, blood pressure, cut, cat scratch, insect bite, and sunburn) were collected based on patient provided information and therefore subject to recall bias.

Secondary lymphedema is not only a problem for patients treated for breast cancer but also for patients treated for other cancers such as malignant melanoma and gynecological malignancies. Research on lymphedema development secondary to additional malignancies is limited compared to breast cancer. The prevalence of secondary lymphedema following gynecological malignancy treatment ranges from 1-49%[83]. Post treatment edema of the lower limb is believed to occur at a similar frequency compared to lymphedema onset post breast cancer therapy. Research also suggests risk factors related to lower limb secondary lymphedema are similar to those reported in upper extremity secondary lymphedema[84]. Genetic variation may also be a contributing factor to the development of lower limb edema secondary to gynecological malignancy. Gynecological malignancy patients have recently been added to the

IRB approved protocol for this study. In future studies, gynecologic malignancy subjects will be sequenced for candidate genes to identify underlying genetic variation responsible for or predisposing to secondary lymphedema development.

Given the evidence suggesting connexins play an important role in lymphatic regulation as well as the previously identified connexin 47 mutations in secondary lymphedema cases; studies of additional connexin genes are warranted. Additional studies and genetic findings will further support that genetic variation within specific genes is involved in secondary lymphedema onset.

5.4 PUBLIC HEALTH SIGNIFICANCE

Lymphedema has become one of the most feared long term complications after breast cancer treatment[11]. Secondary lymphedema is an incurable, progressive, and debilitating disease affecting approximately 2-3 million people in the United States of which 600,000 are women who received breast cancer treatment[8, 85]. It is estimated that out of two million women with breast cancer in the United States, at least one in every four women is likely to develop lymphedema within 11 years[16]. Our understanding of disease etiology is unclear and further complicated by inconsistent reports of incidence as well as conflicting evidence regarding personal and cancer treatment related risk factors. Finding heritable components to the development of secondary lymphedema following cancer therapy will allow early identification of women who are at risk for developing lymphedema post treatment. Currently, there is no

model to successfully predict which women are at high risk for lymphedema onset. With the identification of women predisposed to developing this complication, earlier diagnosis and management can be offered with the anticipation of an increased benefit from treatment and even prevention.

5.5 CONCLUSIONS

Secondary lymphedema is a chronic condition that occurs worldwide in breast cancer patients. This condition broadly impacts function and quality of life of many cancer survivors. Previous studies have identified mutations in the HGF, MET, and Connexin 47 (GJA5) genes which are believed to be causal and/or predisposing factors in the development of secondary lymphedema. This study further investigated the role connexins play in lymphedema by sequencing of the connexin 40 gene in a case-control study. Despite the fact that we did not find previously unidentified mutations in the connexin 40 gene within this population, further investigation of additional connexin genes is warranted due to their previously identified role in lymphatics. Studying the genetics of lymphedema will aid in the effort to better predict who is at risk for lymphedema onset after cancer treatment. We expect that such discoveries will contribute to an earlier diagnosis, improved treatment, and possible prevention.

APPENDIX A

BREAST CANCER SECONDARY LYMPHEDEMA IRB APPROVAL LETTER: PROSPECTIVE STUDY

University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Ground Level
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
http://www.irb.pitt.edu

Memorandum

To: DAVID FINEGOLD, MD

From: MARGARET HSIEH, MD, Vice Chair

Date: 3/8/2007

IRB#: PRO06080011

Subject: Familial susceptibility for lymphedema secondary to breast cancer therapy.

Your research study was approved by the Institutional Review Board, **Committee B**, which met on **2/13/2007**.

Please note the following information:

Approval Date: 3/8/2007
Expiration Date: 2/12/2008

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month** prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

APPENDIX B

BREAST CANCER SECONDARY LYMPHEDEMA STUDY QUESTIONNAIRE

QUESTIONNAIRE: SECONDARY LYMPHEDEMA STUDY

Name: _____
Address: _____

_____

Phone (Home) _____ (Work) _____

(Cell) _____

E-Mail: _____

Gender: ☐ Male ☐ Female Birth Date:____/____/

What is your: Height? _____ Weight? _____

What is your ethnic background/ancestry? (examples: German, French, Cuban, Japanese, Ashkenazi Jewish):

_____

I. Cancer, Surgical, and Medical History

(For women with breast cancer)
When were you diagnosed with breast cancer? Year: _____ Age at diagnosis: _____

Was the cancer in your: ☐ Left Breast ☐ Right Breast ☐ Both breasts?

Did it recur? ☐ Yes ☐ No If recurred, what year?_____

What treatments have you had for breast cancer? (Check all that apply, even if it was after a recurrence):

☐ Lumpectomy ☐ Lymph Node Removal

☐ Chemotherapy ☐ Radiation

☐ Unilateral mastectomy ☐ Bilateral mastectomy ☐ Breast Reconstruction

☐ Tamoxifen,or other hormonal therapy Please list

How many lymph nodes were removed? _____

(For all participants)

Have you ever been diagnosed with any other type of cancer? ☐ Yes ☐ No

If Yes, Type of cancer: _____ Age at diagnosis: _____
Location of cancer:_____Treatment: _____

Have you ever had vascular surgery? (surgery on blood or lymphatic vessels) ☐ Yes ☐ No ☐ Not Sure

If Yes: a) Why did you have surgery? _____
b) What kind of surgery?_____
c) How old were you? _____

Have you ever had any other type of surgery not listed above? ☐ Yes ☐ No ☐ Not Sure

If Yes: a) Why did you have surgery? _____

b) What kind of surgery? _____

d) How old were you? _____

Please check (and give age at diagnosis) if you have you ever been diagnosed with any of the following conditions:

☐ Diabetes (Age at Diagnosis: _____) ☐ Congestive Heart Failure (Age at Diagnosis: _____)

☐ Varicose Veins (Age at Diagnosis: _____) ☐ Rheumatoid Arthritis (Age at Diagnosis: _____)

☐ Phlebitis (Age at Diagnosis: _____) ☐ Liver Disease or Hepatitis (Age at Diagnosis: _____)

Have you had any other diseases, major illnesses, health problems or hospitalizations not listed?

☐ Yes ☐ No ☐ Not Sure

If yes, please list each disease and the age at which it was diagnosed.

a) _____

b) _____

c) _____

(if female)

How old were you when your menstrual periods began? _____

How many pregnancies have you had? _____ ☐ N/A

How many children do you have? _____ ☐ N/A

How old were you, at the time of each birth? _____ ☐ N/A

How old were you when you underwent menopause? _____ ☐ N/A

Was menopause surgically induced (example: hysterectomy)? ☐ Yes ☐ No ☐ Not Sure ☐ N/A

(For women with breast cancer)

Before you developed breast cancer, how did your level of physical activity **compare to other individuals your age**?

☐ Much Less Active ☐ Little Less Active ☐ About as Active ☐ Little More Active ☐ Much More Active

After your breast cancer treatments, how did your level of physical activity **compare to your activity level before you developed cancer**?

☐ Much Less Active ☐ Little Less Active ☐ About as Active ☐ Little More Active ☐ Much More Active

After your breast cancer treatments, did you ever have any of the following on the arm/hand on the SAME side that you had surgery for breast cancer? (Please check all that apply)

☐ Blood pressure reading ☐ Blood draw ☐ Wear a prosthesis ☐ Manicure
☐ Sunburn or other burn ☐ Cut ☐ Insect bite ☐ Cat scratch

Since your cancer treatment(s), how many round-trip airplane flights have you taken? _____

On how many of those flights did you wear a compression sleeve on your arm? _____ ☐ N/A

II. Lymphedema is swelling, usually of an arm or leg, due to an accumulation of fluid under the skin and caused by a poorly functioning lymphatic system. Lymphedema can be inherited (primary) or acquired (secondary). For the following questions, please check all answers that apply, and give the closest estimate of your age whenever applicable. Please note that Before Surgery does NOT include swelling in the breast from treatment for cancer, but is trying to determine if you had lymphedema before developing breast cancer or having surgery. Please answer the applicable questions if you are a relative of a woman with breast cancer.

Do any of your family members have lymphedema? ☐ Yes ☐ No ☐ Not Sure

If yes, please list how they are related to you and the age at which they first developed lymphedema. Also note if the lymphedema is **Primary** (genetic or unknown) or **Secondary** (result of surgery, injury, etc.).

Relationship to you:	Age symptoms began:	Cause of lymphedema:
_____	_____	☐ Primary ☐ Secondary ☐ Not Sure
_____	_____	☐ Primary ☐ Secondary ☐ Not Sure
_____	_____	☐ Primary ☐ Secondary ☐ Not Sure

Do you have lymphedema? ☐ Yes ☐ No Longer ☐ Never ☐ Not Sure

If yes ,age at the **first** sign of swelling: _____

Has your lymphedema been diagnosed by a doctor or physical therapist? ☐ Yes ☐ No

Did you ever have swelling or were you ever diagnosed with lymphedema **BEFORE** you developed breast cancer?

☐ Yes ☐ No ☐ Not Sure; If Yes, At what age did symptoms first begin?

Age at Diagnosis: _____; Please describe your symptoms: _____

_____

Did you ever have swelling or were you ever diagnosed with lymphedema **AFTER** you developed breast cancer?

☐ Post-Surgical Only ☐ Yes ☐ No ☐ Not Sure If Yes:

At what age did symptoms first begin? _____ Age at Diagnosis: _____;

Please describe your symptoms:

_____;

How long after surgery did symptoms begin? ☐ 0-3 Months ☐ 3-12 Months ☐ >1 Year ☐ Not Sure;

Did swelling disappear? ☐ Yes ☐ No ☐ Not Sure; How long did it last?_____

;

Did swelling return? ☐ Yes ☐ No ☐ Not Sure; How old were you?_____

Have you ever had swelling of your arms, hands, or fingers? If Yes, at what age? _____

Did it develop:

☐ before Surgery ☐ 0-3 Months after Surgery ☐ >3 Months after Surgery ☐ Never ☐ Not Sure?

Have you ever had swelling of your legs, feet, or toes ? If Yes, at what age? _____

Did it develop:

☐ before Surgery ☐ 0-3 Months after Surgery ☐ >3 Months after Surgery ☐ Following an accident

☐ unrelated to cancer or treatment ☐ Never ☐ Not Sure?

Do you have a history of recurring skin infections or sores involving your arms, hands, fingers, legs, feet, or toes? (example: cellulitis, erysipelas) If so, age when this **first** occurred? _____;

Did it develop:

☐ before Surgery ☐ 0-3 Months after Surgery ☐ >3 Months after Surgery ☐ Following an accident

☐ unrelated to cancer or treatment ☐ Never ☐ Not Sure

Have you ever been hospitalized for an infection? If Yes, at what age? _____;

Did it develop:

☐ before Surgery ☐ 0-3 Months after Surgery ☐ >3 Months after Surgery ☐ Following an accident

☐ unrelated to cancer or treatment ☐ Never ☐ Not Sure

III. If you answered Yes to any of the questions in Section II, please complete this section; Otherwise, please skip to Section IV.

Please indicate which areas of your body are, or were, affected by swelling (check all that apply):

☐ Left Hand ☐ Left Arm ☐ Left leg ☐ Neck ☐ Chest ☐ Buttocks
☐ Right Hand ☐ Right Arm ☐ Right Leg ☐ Face ☐ Abdomen ☐ Genitals
☐ Left Foot ☐ Right Foot

Was the onset of your swelling: ☐ Gradual (slow) ☐ Rapid (acute) ☐ Not Sure?

Is there a specific event which you feel brought on your first episode of swelling? ☐ Yes ☐ No ☐ Not Sure
(examples: puberty, pregnancy, injury, infection, sunburn, insect bite, surgery, cancer, radiation therapy, long flight)

If yes, what was it and how old were you? _____

Is your swelling, or has it ever been, painful? ☐ Yes ☐ No ☐ Not Sure
Please describe: _____

Have you ever had treatment for lymphedema? ☐ Yes ☐ No ☐ Not Sure

Please check all that apply:

☐ CDP/MLD ☐ Bandaging ☐ Compression Garments ☐ Pump
☐ Surgery ☐ Acupuncture ☐ Herbs/Supplements:
☐ Other:

Please describe each treatment, the age(s) at which you had it, and if it was successful:

a) _____
b) _____
c) _____

Have you found any factors that **increase** or **worsen** your swelling? ☐ Yes ☐ No ☐ Not Sure

If Yes: a) _____
b) _____

Have you found any factors that **decrease** your swelling? ☐ Yes ☐ No ☐ Not Sure

If Yes: a) _____
b) _____

IV. COMMENTS: Please use the space below to add any information, make comments, or ask any questions.

_____
_____
_____

Last Updated: 1/5/2010

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