CHANGE IN THE SUBCELLULAR LOCALIZATION OF MI-ERIα IS ASSOCIATED WITH BREAST CANCER PROGRESSION

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CHANGE IN THE SUBCELLULAR LOCALIZATION OF MI-ER1α IS ASSOCIATED WITH BREAST CANCER PROGRESSION

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I would like to dedicate this thesis to my father, Patrick J. Hynes (1939-2008).
A man of honor, kindness and a zest for knowledge.
He was a constant source of encouragement and inspiration.
He will be forever missed.
The majority of the work in this thesis has been published in McCarthy et. al. (2008), British Journal of Cancer 99 (4) 639-646.
ABSTRACT

The typical progression of normal breast tissue to an invasive breast carcinoma involves a series of steps that include an increase in the number of breast cells (hyperplasia), an increase in the number of abnormal cells (atypical hyperplasia) and then the development of an in situ carcinoma. An in situ carcinoma means that cancer is present but it still confined to the site where the abnormal growth originally developed, the ducts (ductal carcinoma in situ) or the lobules (lobular carcinoma in situ). The cancer cells that comprise this in situ cancer can then undergo further changes allowing them to become invasive and spread into the surrounding tissues. In situ carcinomas are often referred to as pre-cancerous since individuals who are diagnosed with having such are at an increased risk for developing breast carcinoma.

The incidence of breast cancer is increasing across Canada, which is suspected to be a result of an increase in mammographic screening. This often translates into detection of the carcinoma in its earliest stages when the treatment response is optimal and the prognosis is more favorable. However, for many, the cancer is advanced and therefore may have limited treatment options. Also, there are individuals who have the option of treatment upon diagnosis, and still unfortunately have a high likelihood of recurrence. Yet again, there are individuals for whom the cancer is found in the early stages, but have a short disease-free period following treatment. In such cases, it may be that the tumor(s) is resistant to the treatment they received. For all of these reasons there is a need for improved screening methods, improved prognostic indicators and improved therapeutic regimes.
Estrogen receptor-alpha (ERα) is one of the steroid hormone receptors that plays a role in normal growth and development of the breast; it has also long been implicated in breast tumorigenesis. Many of the current breast cancer treatments target the action of the estrogen receptor (ER) indirectly: by blocking either the ability of the ER ligand, estrogen, from binding to the receptor or the synthesis of estrogen. Hence, ERα expression could potentially guide physicians in predicting prognosis and devising a treatment plan.

In an effort to improve upon current standard treatments or devise new, more efficacious therapies, the molecular pathway of ERα needs to be resolved. Human mesoderm induction early response 1 (hMI-ER1) is a key regulator of this pathway through its interaction with ERα. During investigations into the role of fibroblast growth factors (FGFs) in the development and differentiation of *Xenopus laevis* embryo, MI-ER1 was found to be an initial target of the FGF signal transduction pathway.

The *hmi-er1* gene was cloned and characterized (Paterno et. al., 1998) and shown to have two major protein isoforms, hMI-ER1α and hMI-ER1β. Both isoforms contain a number of motifs characteristic of transcriptional regulators and have been shown to act as repressors of transcription (Paterno, et. al., 1997; Ding, et. al., 2003; Ding, et. al., 2004). The alpha and beta isoforms differ in their C-terminus. hMI-ER1α contains a classic nuclear hormone receptor (NR) co-regulator motif, an LXXLL domain, which is not found within the beta isoform.

MI-ER1α has been shown to interact with ERα in breast cancer cells, in the presence and absence of estrogen. It was also found to reduce ER-mediated breast cell
growth in ER-positive breast cancer cells (McCarthy, et. al., 2008). Theoretically, this data supports a role for MI-ER1α in breast cancer cell proliferation.

My hypothesis is that MI-ER1α might be differentially expressed in normal breast tissue and breast carcinoma. Immunohistochemical analysis of MI-ERα expression pattern and subcellular localization in both normal breast and breast carcinoma was carried out using 156 whole tissue sections and 771 cases from tissue microarrays. While there was no consistent difference in the level of expression between normal cells and tumor cells, there was a striking difference in the subcellular localization. In normal and hyperplastic breast 72% of the cases had nuclear MI-ER1α, whereas in breast diseases only 51% ductal carcinoma in situ (DCIS), 25% invasive lobular carcinoma (ILC) and only 4% invasive ductal carcinoma (IDC) has nuclear MI-ERα1 staining (McCarthy,P. et. al., 2008). This represents a shift in the subcellular localization of MI-ER1α, from nuclear to cytoplasmic, during breast cancer progression. Such a shift in MI-ER1α localization might then be associated with the progression of breast cancer; hence, MI-ER1α might prove useful for prognostic determination. Hence, it is possible that the lack of nuclear MI-ER1α expression in DCIS lesions will serve as a means of identifying women who are at a higher risk for developing invasive breast carcinoma.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

°C Degrees Celius
ADH Atypical Ductal Hyperplasia
ALH Atypical Lobular Hyperplasia
ATM Ataxia-telangiectasia Mutated
BIRADS Breast Imaging Reporting and Data Systems
BRCA1 Breast Cancer 1
BSA Bovine Serum Albumin
BVI Blood Vascular Invasion
CBP CREB Binding Protein
Cdk Cyclin-dependent kinase
CI Confidence Interval
CNB Core Needle Biopsy
DAB 3,3'-Diaminobenzidine
DCIS Ductal Carcinoma in situ
DCIS-LG Ductal Carcinoma in situ-low grade
ERα Estrogen Receptor-alpha
ERβ Estrogen Receptor-beta
ERE Estrogen Response Element
FNAB Fine Needle Aspiration Biopsy
H&E Hematoxylin and eosin
HAT Histone Acetyltransferase
Her-2/neu Human Epidermal growth factor Receptor 2
HIC Human Investigations Committee
HRP Horse Radish Peroxidase
HSC Health Science Center
Hx History
IDC Infiltrating Ductal Carcinoma
IEG Immediate Early Genes
IHC Immunohistochemistry
ILC Infiltrating Lobular Carcinoma
IS Intensity Score
LSAB Labeled Streptavidin Biotin
LBD Ligand Binding Domain
LN Lymph Node
LN Mets LN Metastases
LVI Lymphovascular Invasion
M Distant Metastases
MIER1-α Mesoderm Induction Early Response 1 alpha
MRI Magnetic Resonance Imaging
N Regional Lymph Nodes
n/a not applicable
NR Nuclear Hormone Receptors
NL Newfoundland and Labrador
**List of Abbreviations (continued)**

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<th>Abbreviation</th>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
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<td>RPAC</td>
<td>Research Proposal Approval Committee</td>
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<td>PS</td>
<td>Proportional Score</td>
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<tr>
<td>RT</td>
<td>Room Temperature</td>
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<td>SD</td>
<td>Standard Deviation</td>
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<td>T</td>
<td>Primary Tumor</td>
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<td>TDLU</td>
<td>Terminal Duct Lobular Unit</td>
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<td>TMA</td>
<td>Tissue Microarray</td>
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<tr>
<td>w/v</td>
<td>Weight per Volume</td>
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<td>Whole Tissue Sections</td>
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1. **INTRODUCTION**

1.1 General Introduction

1.1.1 Canadian Cancer Incidence and Mortality Rates

It is estimated that 166,400 Canadians will be diagnosed with some type of cancer in 2008, with approximately 73,800 Canadian deaths due to cancer. The 3 major types of cancer that constitute the new cases are prostate, lung and colorectal for males and breast, lung and colorectal for females (Canadian Cancer Society & National Cancer Institute of Canada, 2008).

While lung cancer continues to be the leading cause of cancer mortality for Canadian women, breast cancer leads with respect to cancer incidence. Atlantic Canada and Quebec have both the highest incidence and mortality rates for all cancers. This year in Canada, 22,400 new cases of breast cancer and 5300 deaths as a result of breast cancer are estimated. Of those, 360 new cases of breast cancer are estimated for Newfoundland and Labrador (NL) and 100 of the total NL individuals with breast cancer are estimated to die from breast cancer in 2008 (Canadian Cancer Society & National Cancer Institute of Canada, 2008).

Breast cancer incidence rates have increased steadily from 1979-1999, which is thought to be largely due to an increase in mammography screening (Canadian Cancer Society & National Cancer Institute of Canada, 2008). This increase in screening has lead to earlier detection of breast cancer and resulting in a better treatment response and a decrease in the national mortality rates as a result (Canadian Cancer Society & National Cancer Institute of Canada, 2008; Guarneri & Conte, 2004). There is a positive correlation between an increase in breast cancer incidence and an increase in women’s
age; as such, its incidence rate tends to be higher among post-menopausal women. A woman with a first-degree relative (mother, sister or daughter) who has had breast cancer, has an increased risk of developing breast cancer. In fact, their relative risk is 1.5-2.5 times higher than someone not having a first degree relative who has had breast cancer.

1.1.2 Cell Cycle Regulation and Cell Proliferation

The cell cycle is an event that results in either cell growth or in the division of a single cell, through a series of sequential steps or stages referred to as the cell cycle, to give rise to two new daughter cells (Norbury & Nurse, 1992). Specific elements of the cell cycle vary from organism to organism and within a single organism. This essential process may also occur at different times points throughout an organism’s lifetime. However, there are some parts of the cell cycle that are known to be universal.

The progressional phases within the cell cycle are G\textsubscript{1} phase (Gap\textsubscript{1} phase), S phase (Synthesis phase), G\textsubscript{2} phase (Gap\textsubscript{2} phase), M phase (Mitotic phase) (Figure 1) (Vermeulen, Van Bockstaele, & Berneman, 2003). The first three of these, the G\textsubscript{1} phase, the S phase and the G\textsubscript{2} phase, as well as the G\textsubscript{0} phase comprise what is known as the interphase of the cell cycle. For actively dividing cells, interphase can last from 12 to 24 hours and is the fraction of the cell cycle during which the cell synthesizes its RNA, makes protein and grows in size. The interphase is microvisually inactive; however, on a molecular level it is very active (Murray & Hunt, 1993).
Cells undergoing normal cell division, either G₀ (quiescent cells) or newly formed cells from M phase (mitotic phase), progress sequentially from a period that permits growth, RNA and protein synthesis in G₁ (Gap1), to the S-phase (Synthesis phase) in which the cells genome duplicates, to a second Gap phase (G₂) and then the Mitotic phase (M-phase). During the mitotic phase the cell’s nucleus divides in half (karyokinesis), followed by a physical division of the cell (cytokinesis) into two new daughter cells. The specific junctures of the cell cycle at which the activated Cdk/cyclin complexes exert their influences are indicated (Vermeulen et. al., 2003).
1.1.2 Cell Cycle Regulation and Cell Proliferation (continued)

One complete transition through all phases of the cell cycle is that which involves both one cycle of DNA synthesis and one round of chromosomal segregation followed by division to give rise to two new daughter cells. Cells which are non-dividing, quiescent cells, are those which are not actively progressing through the cell cycle and are classified as being in G₀ (Gap 0). This is the period when the cell has either left the cell cycle on a permanent basis and is no longer dividing or is in a temporary rest state (Lodish, 2004; Norbury & Nurse, 1992). The cell cycle begins when one of the new cells generated in the M phase or a cell which was in G₀ receives a growth or mitogenic signal and enters the cell cycle at G₁ (Lodish, 2004; Vermeulen et al., 2003). During G₁ the cell grows and prepares for DNA replication in S phase. This replication of the genome allows for each new daughter cell to have its own copy. Once the cell passes through G₂, the cell cycle will culminate following two microvisual events, the division of the nucleus (karyokinesis) and a physical division of cell (cytokinesis) (Murray & Hunt, 1993; Norbury & Nurse, 1992). Since the entry into each phase is dependent on its success in the precedent one; a means of monitoring the accuracy exists. Before and after DNA synthesis, the Gap phases (G₁ and G₂) permit time for the cell to grow and synthesize both RNA and proteins. In addition, these two junctures serve as cell cycle checkpoints and is regarded as the decision-making period for the cell (Figure 2) (Gorbsky, 1997; Hartwell & Weinert, 1989; Russell, 1998). In that, G₁ and G₂, are the
intervals when the cell determines if it is ready to proceed to into the S phase and the M phase or if the cell will remain there to allow further growth or to repair DNA damages through activation of the DNA damage checkpoint.
Figure 2  Check points that occur during normal cell cycle progression

At various timepoints during normal cell cycle progression, namely, G1/S phase transition, during S-phase, G2/M phase and during M-phase, there exists several checkpoints (indicated by arrows) to ensure the fidelity of DNA replication and spindle formation, for example. (Adapted from http://homepage.mac.com/enognog/checkpoint.htm)
1.1.2 Cell Cycle Regulation and Cell Proliferation (continued)

Aside from these two main checkpoints, G₁ and G₂, two other critical checks occur during cell cycle progression. An intra-S phase checkpoint in the M-phase verifies the accuracy of replication as it proceeds and the correctness of spindle formation, respectively (Figure 2) (Nyberg, Michelson, Putnam, & Weinert, 2002). The latter of which is critical during mitosis to allow for equal chromosome segregation.

Enzymes from the Cyclin-dependent kinase (Cdk) family are those which predominantly direct normal progression of the cell cycle. At precise time points during the cell cycle, specific activities are tightly regulated through the formation of a heterodimer between a Cdk, which possess catalytic power and its cognate regulatory unit, the cyclin (Figure 1) (Collins & Garrett, 2005; Lodish, 2004; Vermeulen et al., 2003). As such, these heterodimers can be regarded as switches that allow for the transition from one cell cycle juncture to another. Each of the Cdks become activated in the presence of a Cdk-activating kinase (CAK), which phosphorylates the cdk, thus inducing in it a conformational change that increases binding efficiency to cyclins (Carnero, 2002; Morgan, 1997; Vermeulen et al., 2003). A complex of Cdk4 or Cdk6 and one of the D-type cyclins (D1, D2, or D3) are responsible for entry into G₁ phase of the cell cycle (Morgan, 1997). The Cdk2/cyclin E complex regulates transition from the latter phase into the S-phase of the cell cycle, whereas Cdk2 complexed with cyclin A is necessary for progression through the S-phase (Woo & Poon, 2003). Cdk1 complexed with cyclin A and then cyclin B is required for the transition from G₂ into the M-phase and during the M-phase, respectively. Once activated, a G₁ phase Cdk/cyclin complex
such as Cdk 4/cyclin D phosphorylates its substrate, the retinoblastoma protein (RB) (Grana, Garriga, & Mayol, 1998; Vermeulen et al., 2003). Phosphorylated RB protein disrupts a complex that results in release of E2F, a potent regulator of gene transcription. In that, free E2F can then drive transcription of genes whose products are essential for advancement to and progression through the S-phase (Vermeulen et al., 2003).

1.1.3 Cell Cycle Control and Cancer Development

The term cancer can be used to define, in basic terms, a disease in which an abnormal population of cells (tumor) has arisen that interferes with the normal function of a tissue and often the system as a whole (Hanahan & Weinberg, 2000); hence, it is the reason why so many individuals eventually succumb to this disease.

The three dominant features of cancerous growth include uncontrolled cell proliferation, an invasive phenotype (has broken through the confines of initial site of origin) and the potential to become metastatic (spread to another organ(s) through the blood and/or lymphatic system) (Hanahan & Weinberg, 2000). In contrast, a benign tumor is not characterized by the latter two attributes; it is a growth arising from an increased proliferation, but it is non-invasive and non-metastatic. However, a benign tumor can still impose serious health consequences (Cifone, 1982). In addition to the three features mentioned above, there are two other characteristics that also define an abnormal growth as tumorigenic, one is its ability to both promote vascularization and the other is its ability to escape programmed cell death (apoptosis) (Garrett, 2001; Hanahan & Weinberg, 2000).
Perturbation of the normal cell cycle, and hence an increased probability of cancer development, can occur through activation of a proto-oncogene or inactivation of a tumor suppressor gene. A proto-oncogene is a normal gene, but when mutated or over-expressed can give rise to a product that is tumor-promoting and is then referred to as an oncogene. Therefore, an oncogene has the potential, if it is inappropriately expressed, to influence the transformation of a cell from one that is normal to one that is cancerous. Tumor suppressor genes code for proteins that repress cell growth and/or promote cell death. However, when such genes are inactivated they can cause an increase in the rate of cell growth and cell division.

Since many cancers occur as a result of a deregulated cell cycle, each of the elements involved are often the target for cancer therapeutics (Carnero, 2002; Hennighausen & Robinson, 2005). Down-regulation or mutation of several tumor suppressor genes, including p53 and RB have been implicated in the development of breast cancer and are associated with a poor prognosis (Oliveira, Ross, & Fletcher, 2005).

A cell can acquire genetic errors by inheriting a genetic mutation(s) and/or through exposure to damaging environmental agents. In the first instance, an individual can have an inherited genetic pattern whereby a specific segment of the genome, a gene, is already altered at birth. In this regard, he/she has an innate or a genetic predisposition to cancer. This alteration is such that it increases that individual’s lifetime risk to developing cancer (Hodgson, 2008). Secondly, DNA is constantly exposed to environmental agents that create insults or mutations in the genetic code. Such damaging agents include, but are not limited to, exposure to both ultraviolet rays and genotoxic chemicals such as tobacco.
smoke, as well as free radicals that are generated during cellular metabolism (Cadet, Berger, Douki, & Ravanat, 1997; Hoeijmakers, 2001).

Under normal circumstances these genetic errors will be resolved by the cell's DNA machinery upon detection during cell cycle checkpoints (Fantes & Brooks, 1993; Stein, 1998). However, both inherited or environmental mutations can occur in genes whose products participate in the cell cycle’s DNA maintenance checkpoints. These include the DNA damage checkpoint and DNA replication checkpoint (Ashwell & Zabludoff, 2008; Nyberg et al., 2002). When a mutation occurs in either a protein that initiates a DNA damage checkpoint or in a DNA repair protein itself, such as ataxia-telangiectasia mutated (ATM) or breast cancer 1 (BRCA1), respectively, then the mutation can go undetected and unrepaired. Hence, in spite of the cell containing a genetic alteration, it continues to proliferate (Brumer & Shakhnovich, 2004; Christmann, Tomicic, Roos, & Kaina, 2003; McGowan & Russell, 2004). A cancerous growth then, by definition, will develop through accumulation of such abnormal cells.

1.2 Normal Breast and Breast Carcinoma

1.2.1 Development of the Normal Female Mammary Gland

The mammary gland, defined as a modified form of the apocrine gland, is first recognizable, in the development of mammals and specifically humans, in the embryo at approximately 5 weeks gestation (Tavassoli, 1999). It first appears as an epidermally-derived pair of bands referred to as ectodermal thickenings, milklines or milk ridges (Figure 3) (Thor & Osunkoya, 2008). These primitive milk ridges extend from the axilla
Figure 3  Earliest evidence of the presence of the mammary gland in the human embryo

The presence of milk ridges within the embryo is first visible at approximately week 5 of gestation. These structures extend from the axilla to the groin area. (Brokaw, J.J.; Condon, K.; Swantz, D.R., 2008)
1.2.1 Development of the Normal Female Mammary Gland (continued)

down to the groin area (Tavassoli, Devilee, International Agency for Research on Cancer, & World Health Organization, 2003). Shortly after these have developed, the majority of these milk ridge atrophies and the remnant of such remains in the thorax region. By week 7 of gestation, the remaining portion of the ridge proliferates and forms a primary milk bud that invades below the epidermis, into the dermal layer, and at week 10 it begins to branch into solid epithelial structures. Such cellular structures become more defined by week 12 and are termed secondary milk buds. Between weeks 12 and 16 the areola and nipple will begin to emerge from the mesenchyme (Tavassoli, 1999). Throughout the remainder of the gestational period, the secondary milk buds will continue to lengthen and branch giving rise to the first emergence of lumens by week 20 (Revis, 2006). The lengthened secondary buds will eventually canalize and give rise to the lactiferous ducts.

At birth, the mammary gland is composed of 15-20 lobes which drain into the lactiferous ducts (Revis, 2006). Prior to the onset of puberty, the lobules do not contain any glandular structures; rather there exists only a few ducts. Before menarche, the ducts lengthen and become more defined as the terminal buds begin to materialize. Simultaneously, the amount of fat and connective tissue increases (Walker, 2000). Although mammary gland development begins embryonically, it continues into both the neonatal, where the gland is undeveloped, and pubertal stages and is considered complete postpartum in the adult female or during the childbearing years (Figure 4) (Revis, 2006; Watson & Khaled, 2008).
Figure 4  Mammary gland development between the stages of in utero to birth

Early stages of the primary bud formation and secondary bud development (A and B, respectively). Emergence of ducts (C). Beginnings of nipple development (D). Further elongation and branching of the ducts and areola development (E). (Tavassoli, F.A. 1999)
1.2.2 Anatomy and Histology of the Adult Female Breast

The adult female breast consists of 3 principal structures: the skin, subcutaneous tissue and the breast tissue which is divided into the parenchyma (functional unit of the breast) and the supportive tissue of the breast, known as the stroma. The breast tissue itself is comprised of 3 types of tissue, namely, glandular, fibrous and adipose tissue (Thor & Osunkoya, 2008). Young adolescent females generally have large amount of fibrous tissue and less adipose tissue, in contrast post-menopausal women usually have larger amounts of fatty breast tissue. The stratified squamous epithelium (thin, flattened cells arranged in layers) which overlies the skin, nipple and areola continues into the beginning of the ductal system, but then immediately changes to a double layered cuboidal epithelium (Lester, 2005).

The breast, which rests on the pectoralis muscles of the upper chest wall, consists of 15-20 segments or lobes which are inter-connected. The lobe is the functional portion of the breast and it is the site of milk production. Each lobe drains into the lactiferous and collecting ducts, which converge at the nipple (Figure 5).
Figure 5  Anatomy of the adult female breast

The structure of the adult female breast. In humans, a pair of mammary glands are juxtaposed to the pectoralis muscle (2), which is adjacent to the upper chest wall and the ribs (1, 9). The site of milk production occurs within the lobes (3), which drain into the lactiferous ducts (6). This lobules are surrounded by fatty tissue (7). The nipple (4), which consists of fibrous tissue and smooth muscle, is surrounded by areola (5). The areola is more heavily pigmented than the surrounding skin (8). (Adapted from: Image: Breast anatomy normal scheme.png - Wikimedia Commons)
1.2.2. Anatomy and Histology of the Adult Female Breast *(continued)*

The secretory units of the breast, the lobules, consist of a variable number of glands (acini). The interior of each acinus, those which are immediately adjacent to luminal side of the gland is lined with secretory epithelial cells. As such, they are the sites of milk production during latter part of pregnancy and post-partum. These luminal cells are surrounded by a flattened, discontinous layer of myoepithelial cells which have contractile ability. These two cell layers are then bounded by a basement membrane, a thin membranous layer of connective tissue (stroma) separating the duct from the rest of the lobular connective tissue (Watson & Khaled, 2008). Immediately, surrounding the acini is an area of gelatinous connective tissue, known as intralobular stroma, which is specific to the breast and responsive to hormones. (Figure 6) (Walker, 2000).

Six to ten major ducts extend posteriorly from the nipple. Each of which diverge many times before terminating in the glandular structures, the lobules. Should a lesion occur within the ductal system, each ductal branch has been assigned a name to aid in the communication of the site of a lesion (Tavassoli, 1999). Within the lobule, the contents of each acini empties into an intralobular duct; the latter of which is the portion of the breast ductal system that is physically within the confines of the lobule and is named as such (Figure 6) (Elston & Ellis, 1998). The secretory epithelial cells of the acinus are those that have differentiated from the non-secretory cells of intralobular duct (Lester, 2005). This intralobular duct crosses through the lobular connective tissue and continues into what is known as the extralobular duct; together these two portions of the intricate duct system are referred to as terminal ducts (Figure 6, Figure 7, Figure 8A and 8B).
Figure 6  Illustration of the components within an adult female breast lobule

The glands, or the acini, are the site of breast milk production. These acini are composed of two cell types. The secretory epithelial cells on the luminal side of the gland are surrounded by a layer of flattened myoepithelial cells which have contractile ability. These cell layers are surrounded by a thin layer of stromal connective tissue. (Walker, 2000)
Figure 7  Illustration of the anatomical unit of the breast

A portion of the breast stained with hematoxylin. The secretions from the lobules (L1-L4) drain into the intralobular ducts and then into the extralobular terminal ducts (ETD). The ETDs converge and its contents then spill into larger ducts (D), including the major duct and the lactiferous ducts. L1 together with its ETD make up the functional unit of the breast, the terminal ductal lobular unit (TDLU). (Adapted from tgmouse.compmef.ucdavis.edu/.../anatomy3.html)
A is an illustration of the terminological breakdown of the adult female breast ductal system within a lobe (A) and a lobule (B). The contents of the acini or the terminal ducts of a lobule (DTL) filters into the intralobular terminal duct (ITD), these identities, including the intralobular stroma gives rise to a lobule (L). Within a single lobe, B, the sequential convergence of the ducts begins with the terminal (intra and then extra) ducts coming together to form the major ducts and merge to give rise to the lactiferous ducts. From there the lobule secretions are emptied into and stored in the lactiferous sinuses. (Adapted from IMAGES OF LECTURE: 1-16 - srwimg1.htm and Tavassoli, 1999)
1.2.2. Anatomy and Histology of the Adult Female Breast (continued)

In the adult breast, each lobule is associated with a single extralobular duct, which originates immediately outside the lobule (Figure 7, Figure 8A). The acini and its surrounding intralobular stroma, the intralobular ducts and the extralobular ducts constitute what has come to be known as the Terminal Ductal Lobular Unit (TDLU) (Figure 9) (Walker, 2000; Woolf, 1998). Each lobule is surrounded by a dense interlobular connective tissue, in contrast to the more loose intralobular stroma (Figure 10) (Tavassoli, 1999).

The extralobular ducts that extend from each lobule converge and are known as the subsegmental duct or the major duct. The secretions from all glands within each lobe come through the major ducts and into the lactiferous ducts. The milk is then stored in the lactiferous sinus; a dilated portion of the lactiferous duct (Figure 8B) (Tavassoli, 1999).
Figure 9  Hematoxylin and Eosin stain of a section containing a terminal duct lobular unit (TDLU)

A H&E stain of a portion of normal adult female breast. Note the dense interlobular stroma (ELS) that surrounds the lobules which contain glands/acini that are embedded in the loose intralobular stroma (ILS). The acini, intralobular stroma, intralobular terminal ducts (ITD) and the extralobular terminal ducts (ETD) make up the functional unit of the breast the TDLU. (Adapted from Thor, 2008)
Figure 10  Intralobular stroma verses the interlobular stroma within the normal adult female mammary gland

A H&E stained section of normal breast tissue illustrating the interlobular stroma, which is a dense network of connective tissue and is intermingled with adipose tissue (fat). In contrast, the intralobular stroma is a loose network of connective tissue. (40X magnification) (Adapted from Tavassoli, F.A. 1999)
1.2.3 Hormonal Regulation in the Development of the Adult Female Breast

A coordinated hormonal (systemic and local) action is required for female mammary gland development. Hormones have either a passive or active role in its development. Specific hormones influence the development of specific portions of the breast; prolactin, and two steroid hormones, namely, estrogen and progesterone are 3 such hormones (LaMarca & Rosen, 2008). While estrogen primarily contributes to the growth and development of the ducts, progesterone influences the growth and development of the lobular units (McManus & Welsch, 1984; Tavassoli, 1999). The function of both estrogen and progesterone are dependent upon the presence of prolactin; a hormone that is released from the pituitary gland. Estrogen stimulates the epithelial cells to divide, but this will only proceed if prolactin, insulin and other growth hormones are present. All of these hormones exploit their actions throughout all stages of mammary gland development from the embryonic stage to neonatal development, puberty and into the reproductive years of an adult female (Harris, 2000; Reyniak, 1979; Tavassoli, 1999). With the commencement of menstruation, both the TDLUs and the epithelial cells will continue to develop and differentiate, respectively. The mammary glands will continue to respond to a cyclic hormonal environment that corresponds with menses (Thor & Osunkoya, 2008).

1.2.4 Diagnosis of Breast Abnormalities and Invasive Breast Carcinomas

There are several different anomalies of the female breast. Some are classified as normal physiological changes and are not associated with an increased risk for development of breast carcinoma. Many clinicopathological criteria have been established
to aid in distinguishing these normal physiological changes from more precarious pathological conditions, both benign and malignant.

Most breast conditions are identified through routine mammograms in women who are asymptomatic; women less frequently present with symptoms such as pain, skin changes, nipple discharge, palpable mass or lumpiness (Lester, 2005). Pain is the most common symptom, however a low percentage of these cases prevail as a result of breast carcinoma. Majority of such masses are associated with either menses or they are simply random and non-cyclical and can be managed using hormonal therapy. Causes of pain can include trauma, infection or a ruptured cyst (Barton, Elmore, & Fletcher, 1999; Lester, 2005). The second most common breast condition symptom, a palpable mass, may be associated with normal cyclical changes of the breast or it can be a neoplastic lesion; this warrants further investigation to distinguish between the two. As a result of decreased hormone levels with increasing age, the breasts’ stromal tissue becomes more dense and there is a thickening of the basement membrane surrounding the glandular structures. This can occur within the lobules at an uneven rate and produce palpable lumps (Walker, 2000).

Characteristic features of breast lumps are used, in combination with other investigative methods, to help distinguish between those which are benign from those which are carcinomas. These include the circumscription of the mass (well- or poorly-defined), the number of masses, the density (soft or dense), and the mobility of the mass (mobile or fixed) (Walker, 2000).

Diagnostic investigations used to examine breast lesions may include mammography, magnetic resonance imaging (MRI), ultrasound, fine-needle aspiration,
biopsy and or frozen sections of samples obtained during surgery. Mammography and breast MRI are used both as a screening tool (usually performed annually or once every two years) and to further characterize palpable masses.

Screening mammographies were introduced in 1980 with the goal of detecting early, carcinomas that are non-palpable in asymptomatic individuals. Women who are asymptomatic are encouraged to have an annual screening mammogram once they reach the age of 40. However, screening mammograms can also be requested for women who are less than 40 years but are at risk for developing breast cancer. Many institutions also use breast MRI as part of their standard care for individuals who are at increased risk for developing breast cancer. Leach (2009), reported that there is a substantial amount of evidence which supports the recommendation to use breast MRI when screening individuals who are at increased risk for developing breast cancer. In his comparative study review, Leach (2009) stated that several studies report that MRI has twice the sensitivity of x-ray mammography for screening women who are at increased risk. Individuals who are carriers of BRAC1 or breast cancer 2 (BRAC2) mutations or those who have E-cadherin mutations are at an increased risk for developing breast cancer. Mutations in the gene coding for the cell-cell adhesion protein, E-cadherin, has been linked to the development of breast carcinoma by increasing cell proliferation, invasion and/or metastasis (Berx, Becker, Hofler, & van Roy, 1998). A woman presenting with a palpable mass is usually first sent to have a diagnostic mammogram and an ultrasound so that the location, size, and number of masses can be recorded. A diagnostic mammogram cannot distinguish between lesions that are cystic from those that are solid, but an ultrasound can which is why both are used in the investigation. Other details that maybe
identified from a mammogram are the mass density and presence or absence of calcifications. Most tumors are solid and will show up as a dense body on radiographs. They can also be detected through a mammogram long before they become clinically palpable (Walker, 2000). The deposition of calcium salts, calcification, is often associated with secretory or necrotic material (morphological characteristics associated with cell death).

There are two types of calcification, macro and micro. Macro is a large collection of calcium deposits and not usually associated with breast carcinoma, in contrast, microcalcifications are non-clustered trace amounts of calcium and is often found in association with DCIS or invasive breast carcinoma.

Following a mammogram the radiologists reads the image and writes a report detailing the image and describing his/her medical opinion on what should be done next in regards to treatment and follow-up. The American College of Radiologists devised a set of standards for assessing mammograms, which are collectively known as Breast Imaging Reporting and Data System (BIRADS) (Eberl, Fox, Edge, Carter & Mahoney, 2006). Within BIRADS framework there are 7 categories, 0-6, with categories 3-6 requiring some form of follow-up. The absence or presence of calcifications are noted in the report and, if present, are analyzed based on the size, shape, number and distribution. More specifically, calcifications that are large, round or oval in shape and uniform in size are more likely to be associated with benign lesions. However, calcifications that are heterogenous in size, polymorphic and/or branching have a higher probability of being associated with malignant lesions. Assessment of calcifications are included in the BIRADS framework and are classified into 3 groups; those which are benign (1), those of
intermediate concern (2) and those that are more likely to be associated with a malignant tumor (Eberl et al., 2006).

The particulars noted in the results of a mammogram and/or an ultrasound are used by clinicians to determine the next course of action; which may be a follow-up mammogram, a needle-core biopsy or fine-needle aspiration (Miltenburg & Speights, 2008).

Fine-needle aspiration biopsy (FNAB) is a technique whereby a thin needle is inserted into the suspicious area, using ultrasound or mammography as a guide for non-palpable lesions, cells are extracted, smeared onto a glass slide, stained and then examined by a pathologist. It is considered to be a minimally invasive procedure that is relatively painless and can be carried out without the need for general anesthetic. FNAB is a less traumatic event than the larger, tissue biopsy procedure and samples can be processed relatively quick. The concern is, which is often regarded as a disadvantage, that the cells from the suspicious area will be missed and therefore give a false-negative result. Another disadvantage is that FNAB cannot always distinguish between invasive lesions from those which are not invasive. For this reason, FNAB should only be performed by experts in cytopathology. Studies looking into the use of FNAB for breast lesions have shown that the accuracy of reading FNAB results improve with experience and should be used only in combination with core needle biopsies (Feoli, Paesmans, & Van Eeckhout, 2008; Nggada et al., 2007).

Core needle biopsy (CNB) involves the removal of approximately 6-8 cores of tissue from the lesion using a needle that is inserted percutaneously. As for FNAB, this technique can be carried out in using image-guidance for non-palpable masses. This
procedure is considered to be more advantageous than FNAB because it is usually more accurate and it can remove a large enough sample that contains surrounding tissue which can be compared to the morphology of the lesion. It is also more absolute than FNAB in identifying lesions which are borderline and it can allow for the distinction between IDC and ILC to be made; all of which are particularly important in regards to the devise of a treatment plan (Houssami, Cuzick, & Dixon, 2006; Oyama, Koibuchi, & McKee, 2004). The disadvantages include the need for anesthetic, overnight processing and since it removes only a small sample of the lesion it can miss morphological features indicative of a more serious disease (Walker, 2000).

On occasion, some masses are highly suspicious but cannot be confirmed though radiographical or biopsy measures. In these situations lesions are diagnosed intraoperatively using frozen sections. For this, samples of the lesion and surrounding tissue are snap frozen, sectioned, cut and stained and microscopically evaluated by pathologists within a relatively short period of time (Walker, 2000). However, this procedure is not routinely carried out as a number of cases diagnosed through this method have been identified as false-positives. In addition, this procedure may disadvantageous for the patient since before surgery they are unaware of the extent of surgery. Ideally, the mass is removed through a relatively simple procedure called a lumpectomy. However in some cases the mass is large or there are multiple lesions, both of which may require a mastectomy. Therefore, before a patient goes in for surgery she may not know if a lumpectomy or a mastectomy will be performed. In most cases, core biopsy (not FNAB) is done for suspicious lesions, followed by lumpectomy and axillary lymph node dissection. Alternatively, the patient could have a modified radical mastectomy.
1.2.5 Pathological Characteristics of Benign and Malignant Breast Conditions

All non-infiltrating breast lesions are pathologically categorized into 1 of the 4 following groups (Table 1) (Croce, Bretz-Grenier, & Mathelin, 2008; Lester, 2005; Walker, 2000):

1. Non-proliferative breast changes
2. Proliferative without atypia
3. Proliferative disease with atypia
4. Carcinoma in situ

1.2.5.1 Non-proliferative Breast Changes

This group of breast changes is often referred to as fibrocystic change, but Lester et. al. (2005) classified them as non-proliferative since they are conditions for which there is not an increased risk for future development of breast carcinoma. Many of these non-proliferative breast changes can arise clinically due to the presence of a palpable lump or areas of calcification that show up during a routine mammogram.
Table 1  Pathologic Lesions and Relative Risk for Breast Cancer Development\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>Pathologic Lesion</th>
<th>Relative Risk</th>
<th>Breast at Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-proliferative Breast changes</td>
<td>1.0</td>
<td>Neither</td>
</tr>
<tr>
<td>Duct ectasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysts</td>
<td></td>
<td></td>
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<tr>
<td>Apocrine change</td>
<td></td>
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<tr>
<td>Mild Hyperplasia</td>
<td></td>
<td></td>
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<tr>
<td>Adenosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroadenoma without complex features</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative Disease without Atypia</td>
<td>1.5-2.0</td>
<td>Both breasts</td>
</tr>
<tr>
<td>Moderate or florid hyperplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sclerosing adenosis</td>
<td></td>
<td></td>
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<tr>
<td>Papilloma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex sclerosing lesion (radial scar)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroadenoma with complex features</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative Disease with Atypia</td>
<td>4.0-5.0</td>
<td>Both breasts</td>
</tr>
<tr>
<td>Atypical ductal hyperplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atypical lobular hyperplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma in Situ</td>
<td>8.0-10.0</td>
<td>Both breasts</td>
</tr>
<tr>
<td>Lobular Carcinoma in Situ</td>
<td></td>
<td>Ipsilateral breast</td>
</tr>
<tr>
<td>Ductal Carcinoma in Situ</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} Adapted from Lester, S.C., 2005
\textsuperscript{2} Relative Risk values are appropriate for patients without a family history of breast cancer
1.2.5.1 Non-proliferative Breast Changes (continued)

When patients manifest such clinical observations it warrants further investigation to be certain that the change is indeed non-threatening. Some of the conditions included in this group are duct ectasia, cysts, apocrine change, adenosis, fibroadenomas and hyperplasia. There are different grades of hyperplasia; mild, moderate and florid (severe). The latter two types of hyperplasia are those which confer a 1.5-2.0 fold increase in developing breast carcinoma (Lester, 2005; Rosai & Ackerman, 2004; Thor & Osunkoya, 2008; Walker, 2000). However, mild hyperplasia is not associated with an increased risk for developing breast carcinoma. Please refer below for a description of hyperplasia, for all other conditions please refer to appendix A.

As previously mentioned, normal breast acini consist of a single layer of luminal epithelial cells which are surrounded by a layer of myoepithelial cells. Epithelial hyperplasia is when there are more than 2 cell layers; more specifically there is an increase in the proliferation of the epithelial cell layer. An increased cellular proliferation that may occur as a result of a shift in the balance of between apoptosis and cell proliferation in favor of the latter (Bai et al., 2001). An example of normal hyperplastic growth would be the increase in the number of glandular cells during pregnancy. Although hyperplastic cells can give rise to a grossly enlarged lesion, a result of a response to specific stimuli, in many cases they still have a physiological response to normal cell control mechanisms (Lester, 2005; Rosen, 2001). It is considered mild hyperplasia when there are less than 4 cell layers, this can affect the whole duct or just a segment of a duct. This increase in the epithelial layer can be evenly distributed around the duct or can slightly project into the lumen of the duct and give rise to what is known
as papillary structures. **Moderate hyperplasia** is characterized by 5 or more cell layers and can have papillary structures that will either extend into the lumen or may occupy the entire lumen. **Florid hyperplasia** is easily distinguishable from moderate hyperplasia since the ducts are significantly increased in size compared to non-hyperplastic ducts and the proliferative epithelium often fills the ducts (Lester, 2005; Rosai & Ackerman, 2004; Thor & Osunkoya, 2008; Walker, 2000). A previous study had also found a link between the expression ratio of two isoforms (α and β) of Estrogen Receptor (ER), hyperplasia and risk for subsequent development of breast cancer. It was found that ERβ expressed at a lower level compared to ERα, hence a high ERα:ERβ ratio, is associated with hyperplastic cases that are likely to develop breast carcinoma (Shaaban et al., 2005).

### 1.2.5.2 Proliferative Disease without Atypia

These group of proliferative breast diseases often do not present as palpable lumps but rather they are often detected during routine mammograms or biopies that were ordered by physicians for other reasons. Diseases which are categorized under this group include moderate and florid hyperplasia, sclerosing adenosis, papillomas, complex sclerosing lesions and fibroadenomas with complex features. For all of these changes, the increased the risk for developing breast carcinoma is 1.5-2.0 fold (Lester, 2005). A more detailed description for each of these conditions can be found in Appendix A.

### 1.2.5.3 Proliferative disease with atypia

Atypical ductal hyperplasia (ADH) and atypical lobular hyperplasia (ALH) often resembles DCIS and LCIS, respectively, but lack certain features. In ADH the
number of lesions per specimen fall short of that observed for DCIS and the cells do not completely fill the duct. Peripheral cells of an ADH lesion are columnar and the nuclei of cells that are more central to the lesion are more rounded. Since the cells of ADH do not completely fill the duct, rounded spaces may be noted throughout the lesion. ALH involves at least half of the acini with the cells demonstrating little variation in their size or their shape. ALH holds a more aggressive phenotype when it advances into the ducts and is therefore associated with a higher risk for future breast cancer development (Page, Dupont, & Rogers, 1988). The risk for carcinoma is 4-5 times higher; which increases to 10 times if the patient has a first degree relative that has had breast cancer (Table 1) (Page, Dupont, Rogers, & Rados, 1985).

1.2.5.4 Ductal Carcinoma In Situ and Lobular Carcinoma In Situ

The TDLU, previously described, is the site where most of the non-proliferative breast changes and proliferative breast diseases arise. However, it is typically within the larger ducts that most papillomas and papillary carcinomas manifest. Breast carcinomas are divided into two main categories, those that are non-invasive and those that are invasive.

Non-invasive breast carcinoma is used to describe neoplasms which are confined to the duct or lobule and has not advanced beyond the confines of the basement membrane; as such it does not have the potential to metastasize to other sites. Two main groups of in situ breast cancers are Ductal Carcinoma In Situ (DCIS) and Lobular Carcinoma In Situ.
DCIS, also referred to as intraductal carcinoma, is a population of cells that, individually, appear morphologically similar to cells of invasive carcinomas. These abnormal cells cumulate within the ducts and although the basement membrane and the myoepithelial cell layer is in tact, the number of cells that comprise the latter may have lessened. However, disruption of both the basement membrane and the myoepithelial cells layer are a pre-requisite for progression to an invasive carcinoma (Shekhar, Tait, Pauley, Wu, Santner, & Nangia-Makker, 2008).

There are several different histological subtypes of DCIS and often a pathologist may notice several types within a single specimen. These different types include solid, cribriform, comedocarcinoma, papillary and micropapillary (Lester, 2005). Solid DCIS is used to describe lesions in which the duct is completely filled with neoplastic cells (Figure 11). In contrast, papillary (Figure 11) and micropapillary DCIS have fibrovascular cores and cancerous papillae that project into the lumen, respectively. The micropapillae have another histological distinguishing feature, in that, the base through which the projections are attached to the duct wall is constricted. Cribriform DCIS has rounded spaces that exist between the cancer cells which are usually filled with calcified secretions (Figure 11) (Figure12) (Burstein, Polyak, Wong, Lester, & Kaelin, 2004). Comedocarcinoma is a type of DCIS that is characterized by ducts completely filled with cells that contain a central necrotic area having calcified material within it; it is this feature that is often detected on mammograms (Fisher et al., 1999). The necrotic debris build-up arises due to both apoptosis and passive cell death or oncosis (Moinfar, Mannion, Man, & Tavassoli, 2000). Comedocarcinoma is considered to be a more aggressive (faster growing) form of DCIS, with approximately 40% progressing to
an invasive carcinoma. Pathologists are often very cautious when reviewing cases of comedocarcinomas, as there have been reported cases of invasive ductal carcinomas resembling comedocarcinoma with the presence of necrotic debris. This is an issue that is usually resolved and confirmed using routine laboratory immunohistochemical staining (Pervez & Khan, 2007).

Usually DCIS does not form a palpable lump and is habitually detected through mammography. Even though the cancer is contained, the relative risk of an in situ recurrence and subsequent development of breast carcinoma following treatment for DCIS is substantially higher (Thor & Osunkoya, 2008). The risk of future development of invasive carcinoma is about 10 times that of a person who has never had breast carcinoma, but this is largely influenced by the course of treatment. Due to this high risk, women are referred for appropriate treatment and should be followed by a screening program (Sanders, Schuyler, Dupont, & Page, 2005).
Figure 11  An illustration of the histological appearances of 3 types of DCIS

Illustration of three different types of DCIS. In solid DCIS, the ducts are filled with carcinogenic cells that are surrounded by a basement membrane (A). Cribriform DCIS is characterized by cancer cells which do not completely fill the lumen of duct and spaces/pockets which exist in between the cancer cells (B). In papillary carcinoma, the cancer grows into the lumen as "finger-like" projections (C). The malignant cells and basement membrane and lumen of the duct are marked by an A, B and C, respectively. (Adapted from www.breastcancer.org)
A: Solid DCIS

B: Cribriform DCIS

C: Papillary DCIS
Figure 12  Cribriform DCIS, Comedo DCIS, Comedo DCIS with early infiltrate

H & E staining of two common forms of DCIS, Cribriform and Comedo. Cribriform has pockets of spaces between the cancer cells (A), whereas Comedo has a central area of necrosis (B and C). In each of A and B the cancer is contained within the basement membrane, however in C, there is evidence of early infiltrate (arrow) in the presence of comedo DCIS. (Magnification 100X) (Burstein, 2004)
1.2.5.4 Ductal Carcinoma *In Situ* and Lobular Carcinoma *In Situ* (continued)

*In Situ* carcinomas are assigned a grade of I, II or III by a pathologist to reflect the morphology of the carcinoma in relation to normal breast tissue. A grade of I through to III is assigned based on how closely they resemble the normal cells. How well the neoplastic cells resemble the normal is defined by parameters such as the tubule formation, mitotic count, and the nuclear pleomorphism. Also an important consideration when assigning a grade to an *in situ* lesion is the extent of central necrosis (Silverstein et al., 1996; Wells, Carney, Eliassen, Grove, & Tosteson, 2000). Grade I (well-differentiated) lesions are those which closely resemble the normal cells and are often slow-growing, whereas grade II (moderately differentiated) lesions are those which do not resemble the normal cells and appear to be growing faster than that of normal cells. Grade III (poorly differentiated) are those cells which are faster-growing and morphologically have no resemblance to normal cells. For example, those that are low grade (well-differentiated) would demonstrate well-defined tubule formation, low mitotic count and small uniform cells. DCIS can arise in several areas of the ductal system (multifocal) and this is often found to be associated with grade I type of DCIS lesions (Foschini et al., 2007). The grade of DCIS can influence the type and the aggressiveness of the treatment plan, however the validity of its application has been challenged (Boland, Chan, Knox, Roberts, & Bundred, 2003; MacAusland et al., 2007; Silverstein et al., 1996). In one study, it was found that for the low grade DCIS group, it took decades before an invasive phenotype developed when not treated by surgical means (Sanders et al., 2005).

DCIS is 4 times more common than LCIS, which is a form of *in situ* cancer that develops in the lobules, more specifically within the acini of the lobules. LCIS is thought
to incur a higher risk of a recurrence or developing invasive breast carcinoma in the future; a risk that is about 8-10 times that of someone who has never had breast cancer (Chuba et al., 2005). The risk for developing infiltrating breast carcinoma is higher for the ipsilateral breast, which is independent of the amount of LCIS that was present initially (Li, Malone, Saltzman, & Daling, 2006). If an invasive cancer does develop in the future it can be lobular or ductal. It is an uncommon breast abnormality and it is not usually detected through mammograms but rather biopsies of the breast performed for other reasons. LCIS histology appears as cells which are loosely cohesive and completely filling the acini. However, the overall outline of the lobule is still apparent.

E-cadherin is a transmembrane protein involved in calcium-dependent cell-to-cell adhesion. Since there is loss of E-cadherin in LCIS, but not DCIS, staining of paraffin sections with E-caherin can aid in the distinction between the two (Stein, Zisman, Rapelyea, Schwartz, Abell & Brem, 2005).

Treatment for DCIS and LCIS typically involves surgery combined with some adjuvant therapy. The extent of the surgery can be either breast conserving (lumpectomy), or breast removing surgery (mastectomy), which largely depends on the size, number and grade of the lesion(s). When lesions are removed, the margins are painted with an ink so that when it is processed and sectioned the margin of excision of the removed tissue can be identified. The pathologists will note the distance between the cancer cells and the edge of the removed tissue. This aids in determining a treatment plan. For example, if the cancer cells are close to the edge additional surgery may be warranted. If the lesion is confined to one area and can be removed with clear margins of resection, the recommended form of treatment is lumpectomy followed by radiation. Radiation
following lumpectomy has been shown to significantly reduce ipsilateral recurrence of breast carcinoma in those patients. As well, administration of another adjuvant therapy, Tamoxifen, has been shown to inhibit increased breast cell proliferation and reduce the recurrence of breast cancer in ER positive cases (Cunnick & Mokbel, 2004; Mokbel, 2005). Hence, Tamoxifen is often factored into the treatment plan for breast cancer patients. Fisher et al. (1999) demonstrated that if Tamoxifen is added to the treatment regime there is a reduction in the breast cancer events by year 5 from 13.4% to 8.2% ($P=0.0009$). If there is more than one lesion and they are located in different regions of the breast and if the grade of the lesion is high then a mastectomy may be proposed.

1.2.5.5 Invasive Lobular and Ductal Carcinomas

Invasive breast cancer indicates that it has spread beyond the milk duct(s) or acini. In that, the cancerous cells has broken through the basement membrane and invaded the surrounding normal tissues via the vascular or lymphatic system. Of all the invasive breast cancers, about 80% are ductal carcinomas and approximately 5% are lobular carcinomas, with the remaining cases being special or rare forms of breast carcinoma. Most women diagnosed with breast cancer who have not been screened through mammography, present with a palpable mass. Unfortunately, the majority of these cancers have already metastasized to the axillary (armpit region) lymph nodes (Lester, 2005).

While it is still unclear as to how an invasive breast lesion develops from an in situ carcinoma, many theories have been proposed. It has also been difficult to identify
which gene(s) are specifically involved in the invasion step (Porter et al., 2005). However, it has been proposed that 7 changes must occur in normal cells to become hyperplastic and give rise to an in situ carcinoma and invasive breast carcinoma (Figure 13) (Lester, 2005). Early in the process, there needs to be an increase in genomic instability and/or loss of both growth inhibition and apoptosis. In the case of hereditary breast cancers, these changes may occur because of mutation in genes involved in those particular pathways. In addition, the cell must gain the ability to supply its own growth signals (“self-sufficient growth”) and they must be capable of initiating vascularity which will provide a route for nutrients and other growth promoting molecules. As well, the cells must continue to proceed through the cell cycle without interruption. Several theories have been suggested as to how cancer cells can invade through the basement membrane, myoepithelial cell layer and into the stroma. It is proposed that the myoepithelial cells and stroma undergo changes as opposed to the malignant cells acquiring the ability to invade these layers (Lester, 2005). In contrast, Tait et al. (2007) has proposed that it is the malignant cells which recruit or initiate the development of a stroma that permits its invasion.

Histologically, invasive ductal carcinoma varies from those that form well-defined tubules that have monomorphic cells to those that have sheet of cells, with no evidence of tubules, with variation in size and shape (Figure 14). The neoplastic cells are also notably larger than those of normal epithelium. In the case of lobular carcinoma, the cells tend to be small in comparison to that of invasive ductal carcinoma and infiltrate as a single file of cells or in a targetoid pattern (Figure 15).
Figure 13  Pathobiologic events that are associated with the progression from normal to breast carcinoma

H&E stained breast tissue sections indicating the progressional steps in going from a normal cell to non-infiltrating lesions such as hyperplasia or DCIS to invasive ductal carcinoma. Genetic changes occur in genes that regulate cell proliferation and apoptosis, such that cells can evade control of the normal cell growth and proliferation. In some cases, an individual may inherit a mutation, germline mutation, in such critical genes putting them at high risk for developing breast carcinoma. As a lesion becomes more invasive, it will develop a blood supply which serves a dual role. One it will allow the tumor to receive nutrients and other growth factors so that it can continue to grow and it serves as a mode for which the cancer cells can travel to other organs. (Adapted from Burstein, 2004)
Figure 14  Histological differences in grade of invasive ductal carcinoma

Some invasive ductal carcinomas have well-defined tubules formed from cells that are of the same size and similar shape (A: Well-differentiated). However, many invasive carcinomas do not have any tubules, but rather a network of cells of varying size and shape (B: Poorly-differentiated). (H&E stain) (Adapted from Tavassoli, F.A., 1999)
Figure 15  H&E stained sections of two areas of lobular carcinoma with different morphologies

Lobular carcinoma can have many morphological patterns. The most common seen are those which form solid pockets of cells with a targetoid pattern, where cells appear to wrap themselves about one nest of cells (A). Another common pattern is when the cells infiltrate in a single file (B).

(adapted from www.med-ed.virginia.edu/courses/path/gyn/breast6.cfm)
1.2.5.5 Invasive Lobular and Ductal Carcinomas (continued)

Some of the hormonal risk factors are directly related to an increase in exposure time to the steroid hormone estrogen; these include early menarche, late menopause, nulliparity (a women who has never given birth) and women taking hormone replacement medications to treat menopausal symptoms. A risk factor that is related to amount of estrogen in the body, is obesity; a risk that also correlates with poor prognosis (Carmichael, 2006). In obese patients the amount of estrogen is often higher than non-obese patients; a risk factor that also puts them at an increased risk for endometrial cancer.

Whether invasive ductal and lobular carcinomas differ in regards to prognosis and biology has been a controversial issue for some time. Some believe that lobular carcinoma is associated with a prognosis that is similar to that of ductal carcinoma (Viale et al., 2008). Pestaozzi, et al. (2008) compared and contrasted the prognosis and biology of IDC and ILC in 12,206 breast cancer patients. The results of this study demonstrate that the 6th year mark, following the diagnosis of lobular carcinoma, is associated with a better prognosis in comparison to ductal carcinoma. However, at the 10th year the prognosis is worse for ILC than that for ductal carcinoma.
1.2.6 Malignant Conditions of the Adult Female Breast: Stage and Grade

Staging is a procedure that is not only used to predict a patient's survival and treatment plan, it also a universal system that permits clinicians worldwide to communicate about the disease. Using information gathered from radiographs, surgery and pathological investigations of the tumor tissue, clinicians classify each breast tumor into 1 of the 4 possible stages. Each of these stages is associated with a similar outcome; tumors that are stage I, for example, have approximately the same prognosis. The size of the primary tumor (T), the number regional lymph nodes involved (N) and the presence or absence of distant metastases (M) are the 3 determinants used in the classification (TNM classification) of breast cancer staging (Sobin & Wittekind 1997). In other words, the assessment will reveal if the cancer has spread to other areas of the body, number of lymph nodes and if so, where and how many, as well as the primary tumor size. See Table 2 for further dissection of the TNM classification. Together, the scores for T, N, and M are used to determine which stage the cancer is in for each patient. Stage 0 is used to describe those tumors that are non-invasive such as DCIS or LCIS. There is no evidence that the tumor has left the site in which it has occurred and is not present in another area of the body that was assessed. Stage I is when the tumor is 2cm or less in greatest dimension, there is no lymph node involvement and no metastases. Stage II is divided into 2 subcategories, stage IIA and stage IIB. Stage IIA has 3 possibilities which include, those in which no tumor is found in the breast but cancer is present in the ipsilateral (same side) axillary lymph nodes, tumor is 2cm or less and is also present in the axillary lymph nodes or the tumor is between 2 and 5cm and there is no cancer in the axillary lymph nodes. If a tumor is between 2 and 5cm in diameter and involves the axillary

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lymph nodes then it is stage IIIB. However, a tumor can also be stage IIIB if it is larger than 5cm in diameter but it has not spread to the axillary lymph nodes. Stage IIIA characterizes tumors that are not in the breast tissue but rather are detected in the axillary lymph nodes which are clumped together or are adhered to other structures. Another group of tumors that are also classified as stage IIIA are those that have a diameter of either 5cm or less or those which are greater than 5cm and are associated with spread to axillary lymph nodes that are clumped together or attached to other structures. A tumor of any size with spread into the chest wall and/or the skin of the breast, with or without spread to lymph nodes that have clumped together or attached to other structures, is classified as stage IIIB. In Stage IIIC, the tumor may be of any size, there is spread to the chest wall and/or skin and the cancer has also metastasized to lymph nodes above and below the collar bone. Metastases at presentation, or Stage IV, means that the cancer has advanced beyond the breast and regional lymph nodes and is now present in other organs such as the lungs, liver, bone or brain. Also taken into consideration when staging a breast tumor(s) is the presence or absence of ER, PR and Her2/neu. Her-2/neu is a proto-oncogene that codes for a receptor protein involved in signal transduction pathways that give rise to cell growth and differentiation. Her-2/neu is amplified in about 10-20% of breast cancers and can lead to an increase in the amount of Her-2/neu protein. An increase in this protein receptor is often associated with increased breast disease recurrence and poor prognosis (Moelans, de Weger, Ezendam & van Diest, 2009).
<table>
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<th>Code</th>
<th>Description</th>
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</thead>
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<tr>
<td>TX</td>
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<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor is 2cm or less in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor is more than 2cm but not more than 5cm in greatest dimension</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor is greater than 5cm in greatest dimension</td>
</tr>
<tr>
<td>T4</td>
<td>Tumor of any size with extension to the chest wall or skin</td>
</tr>
<tr>
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<tr>
<td>N0</td>
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<td>Metastases in movable ipsilateral axillary lymph nodes</td>
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<td>N3</td>
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<tr>
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<sup>1</sup> Adapted from the “December 2004 Revision, TNM 6<sup>th</sup> Edition: Disease Staging Form” utilized by the Cancer Care Program within the Dr. H. Bliss Murphy Cancer Centre, St. John’s, NL.

<sup>2</sup> Tumor size T1 is further classified into Tmic, T1a, T1b, T1c to represent tumor sizes between 0.1cm (microinvasion) to 2cm

<sup>3</sup> Regional Lymph Node N2 and N3 is further classified into N2a, N2b and N3a, N3b, N3c
Table 2 continued  

**TNM Classification used for Breast Cancer Staging**

<table>
<thead>
<tr>
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<th>T</th>
<th>N</th>
<th>M</th>
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<td>M0</td>
</tr>
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<td>M0</td>
</tr>
<tr>
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<td>M0</td>
</tr>
<tr>
<td></td>
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<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>T2</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>T0</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N2</td>
<td>M0</td>
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<td>N0, N1, N2</td>
<td>M0</td>
</tr>
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<td>any T</td>
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<tr>
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<td>any N</td>
<td>M1</td>
</tr>
</tbody>
</table>

1 Adapted from the “December 2004 Revision, TNM 6th Edition: Disease Staging Form” utilized by the Cancer Care Program within the Dr. H. Bliss Murphy Cancer Centre, St. John’s, NL.
1.2.6 Malignant Conditions of the Adult Female Breast: Stage and Grade
(continued)

Histological grade is also a strong predictor of survival for breast cancer patients and some researchers believe it should be included in the staging system (Rakha et al., 2008). In 1950, H.J.G.Bloom proposed a means of assessing the grade of a tumor that was to be based on 3 factors: (1) extent of tubule formation, (2) nuclear pleomorphism, (3) nuclear hyperchromasia and mitotic activity (Bloom, 1950; Tavassoli, 1999). Since this time, the grading system has been modified many times and as such has evolved into one that is currently more appropriate. This modified version is referred to as the Modified-Bloom-Richardson Grading System. Currently, it is a system whereby a score of 1 to 3 is assigned for each of the above factors and the total of these 3 scores (ranging from 3-9) is used to categorize the tumor as low, moderate or high grade. A total score of 3-5 is a low grade, well-differentiated tumor, 6-7 is a moderately differentiated tumor and a score of 8-9 is a high grade breast tumor. Of these, it is the high grade tumors that are associated with a worse prognosis.

The following criteria are used in the compilation of the score:

**Tubule formation:**

Tubules that are well defined (present in greater than 75% of the lesion) get a score of 1 and those with little, those that have a moderate degree of tubule formation (10-75% of the lesion) are given a score of 2 and those with no evidence of tubule formation (less than 10% of the lesion) receives a score of 3.
**Nuclear Pleomorphism:**

Breast cancer specimens with very little nuclear pleomorphism are assigned a score of 1 and those that have a moderate variation in nuclear size and shape get a score of 2. Tumor in which the cells are extensively pleomorphic are given a score of 3.

**Mitotic Activity**

The number of mitotic figures, that are located in the periphery of the tumor or in the highest mitotically active area of the lesion, in 10 high power fields are counted to determine the mitotically activity (Tavassoli, 1999). Tumor specimens that have few or absent mitotic activity get a score of 1 and those with a lot of mitotic activity are assigned a score of 3.

It has been determined that if one were to digress from this well established scoring method by doing just a “quick-scan” for mitotic figures, this would affect the prognostic estimation and would negatively influence the choice of treatment (Skaland, van Diest, Janssen, Gudlaugsson, & Baak, 2008). Other studies suggest that using this method of grading is non-reproducible and should be replaced with one which measures the proliferative rate (Meyer et al., 2005; Tawfik et al., 2007). Both Ignatiadis et. al. (2008) and Sotiriou et al. (2006) suggest that the category for moderately differentiated, grade II, tumors should be removed from the system. In this then, tumors would be classified as either low grade (good prognosis) or high grade (poor prognosis). The current grading system has been challenged in many respects, however, it is thought that when a system is used consistently and universally, then with increased experience and training the system will become more reproducible.
1.3  **Estrogen and the Estrogen Receptor**

As mentioned earlier, estrogen and progesterone are steroid hormones which are critical for a number of physiological processes, including mammary gland growth and development (Anderson, 2002). Estrogen is produced through a process known as steroidogenesis, with cholesterol as its starting molecule. These hormones are produced primarily by the ovary and the fetoplacental unit. To a lesser extent they are produced in the adrenal gland, liver and mammary glands.

1.3.1  **Estrogen Receptor: A Nuclear Hormone Receptor**

Estrogens have low solubility in aqueous solutions and exist in circulation as biologically inactive complexes or as free (unbound) and active (Nussey & Whitehead, 2001). 17β-Estradiol (E₂) is one of the three major estrogens and has the most influence on the growth and development of the breast. It travels by way of the circulatory system to its peripheral target tissues, it is within these tissues that the hormone interacts with it cognate receptor (estrogen receptor) thereby stimulating a series of biological events. In some cases estrogen is produced locally within the target tissue; breast tissue is one such example. In this case, steroid precursors are first recruited from circulation and it is within the breast tissue that estrogen synthesis is completed (Foster, 2008).

There are two forms of the estrogen receptor, Estrogen Receptor-alpha (ERα) and Estrogen Receptor-beta (ERβ), which are encoded by separate genes. E₂ can interact with either ERα or ERβ. Estrogen receptors belong to a family of receptors known as Nuclear Hormone Receptors (NRs) which are intracellular proteins that regulate, once
bound to a ligand such as E₂, the transcription of target genes. Hence, they are frequently referred to as ligand-inducible transcription factors (Pearce & Jordan, 2004).

While ERα has been shown to be distributed among fewer tissues within the body than ERβ, both receptors are expressed within the normal mammary gland (Cordera & Jordan, 2006). Within the mammary gland its localization is predominantly within the nucleus and to a lesser extent within the cytoplasm, mitochondria and the cell membrane (J. Q. Chen, Delannoy, Cooke, & Yager, 2004; Levin, 2002; Yang, Barnes, & Kumar, 2004). Iwao et. al. (2000) demonstrated that ERα is over expressed; in contrast Roger et. al. (2001) found that ERβ is down-regulated in breast carcinomas.

The domain structures of ERα and ERβ have significant sequence homology (Figure 16). Of the 5 domains (A/B, C, D, E and F) within the ER, it is the A/B domain that contains an activation function 1 (AF-1) which elicits the ligand-independent transcriptional activation function of the ERα and permits many protein-protein interactions. Juxtaposed to the A/B domain is the DNA-binding domain or the C-domain. It is through this portion of the receptor that it can bind to specific sequences, known as estrogen response elements (EREs), within target genes and activate gene transcription. Adjacent to this domain are the D and E domains, which are the hinge region and ligand-binding domain (LBD), respectively. The LBD allows for ligand interaction and is responsible for nuclear translocation, interaction with heat shock proteins (some of which keep the receptor in its inactive form) and homo- or heterodimerization. In addition, the LBD (E-domain), in contrast to the AF-1 region, can induce ligand-dependent transactivation of target genes through its activation function 2 (AF-2) region (Cordera & Jordan, 2006; Foster, 2008). The role of the F domain, located within the C-terminus of
the ER, is still uncertain. However, a recent study suggests that it inhibits ligand-induced receptor dimerization (Yang, Singleton, Shaughnessy, & Khan, 2008) (Figure 16).
Figure 16  Structural domains of Estrogen Receptor α (ERα)

The ER protein has 5 domains; A/B, C, D, E and F. Its ligand-independent transcriptional activity occurs through the activation function 1 (AF-1) region within the A/B domain. The DBD allows the protein to recognize and bind to specific target gene sequences known as estrogen response elements (EREs) and induce gene transcription. The D domain is the hinge region and adjacent to this domain is the LBD that houses numerous responsibilities such as ligand-dependent transcriptional activation, dimerization and nuclear translocation. The F-domain is a variable region. (Pearce, S. T., & Jordan, V. C., 2004)
1.3.2 ER Models of Function

Estrogen can exert its transcriptional activation capabilities through both genomic (classical) and non-genomic (non-classical) processes. Being a steroid hormone, estrogen is lipophillic, thus permitting it to cross the lipid bilayer of the cell membrane. Once inside the cell it can bind to the ER within the cytoplasm or within the nucleus.

In the classical model (Figure 17A), the estrogen receptor undergoes a conformation change upon binding to a ligand such as \( E_2 \). After which, it then forms a homodimer and translocates to the nucleus. In this mode of action, the homodimer will bind to the EREs located within the target gene(s) and recruit co-activators, which is subsequently followed by initiation of transcription.

The explanation described above is a simplistic one since what actually takes place in the classical model involves many protein-protein interactions between the ER and other agonist and antagonists. When not bound to either of these, the ER will interact with a co-repressor, which will act to prevent the ER from initiating transcription (Nilsson et. al., 2001).

ER can also act through non-classical modes of action. In that, it is capable of eliciting a growth or proliferative response without directly interacting with the DNA. Initially, it was thought that the only way in which ER elicited transactivation was through direct interaction with DNA through ERE. However, it is now known that the ER can activate transcription of target genes indirectly. In that, activated ERs can interact with other transcription factors and hence participate in the regulation of target gene transcription.
Figure 17   Modes through which ER can exert its gene transactivation capabilities

Classical mode of action whereby the ER will interact with ERE of target genes, upon binding of its cognate ligand (Estradiol, E₂), to induce their transcription (A). The ER also acts to induce transcription of target genes without directly binding to DNA, rather it interacts with other proteins (Sp1 (B), Ap1 (C), NFκB (D)), in addition to E₂, to influence gene transcription. (Nilsson S., et. al. 2001)
1.3.2 ER Models of Function (continued)

For example, ERα can interact with the c-rel subunit of the NFκB complex and prevent NFκB from binding to target gene promoters such as Interleukin-6 (IL-6) and hence its transactivation (Nilsson et al., 2001).

Both types of the estrogen receptor can interact with Sp1; following translocation to the nucleus the E$_2$/ERα complex binds to Sp1 which in turn binds to specific sequences in the promoter regions to initiate transcription of target genes such as RARα1, Hsp27, E2F1, cyclin D1, Bcl-2, VEGF (Figure 17B) (Nilsson et al., 2001; Safe & Kim, 2008). RXRα1 is known to have an increased expression in ERα-positive breast cancer cells and play a role in increased cell proliferation of such cells. It is also a target of many drug therapies designed to combat E$_2$/ERα/β-induced breast cell proliferation (Sun, Porter, & Safe, 1998; Zou et al., 1999). Hsp27 has a cytoprotective role and has been shown to enhance chemoresistance in some cancers (McCollum, Teneyck, Sauer, Toft, & Erlichman, 2006). CyclinD1 is a positive regulator of cell cycle progression and Bcl-2, as previously mentioned, is an anti-apoptotic protein. Finally, VEGF is a signaling protein that initiates new blood vessel growth, allowing neoplasms to grow in size and a method of traveling to other sites within the body. Hence, ER-Sp1 transcriptional pathway is associated with breast cell growth, chemoresistance and metastases.

The ERα and ERβ can induce gene transcription via interactions with the specific proteins, fos and jun, that are part of the larger Ap1 complex (Figure 17C). The interaction of ERα or ERβ is one which enhances Ap1 activity in the presence of E$_2$. E$_2$/ERα complexes enhance gene transcription through recruitment of Ap1 co-activators (p160 family), whereas E$_2$/ERβ enhances Ap1 activity through release of co-repressors.
from the Ap1 complex (Paech et al., 1997; Philips, Chalbos, & Rochefort, 1993; Webb et al., 1999).

ER also acts to inhibit transcription of IL-6, thus negatively regulating cell proliferation, through interaction with the c-Rel portion of the Rel/NFκB complex (Figure 17D) (Galien & Garcia, 1997). The inactive form of the Rel/NFκB complex within the cytoplasm (through its interactions with cytoplasmic inhibitory proteins) becomes active upon both through degradation/release of such restrictive proteins and translocation to the nucleus. This subcellular localization then allows Rel/NFκB to activate transcription of target genes such as the pro-tumorgenic IL-6; an action which is inhibited by the interaction of ER with the Rel portion of the complex. IL-6 acts to decrease tumor cell apoptosis and increase their angiogenic potential, both of which contribute to cancer cell growth and metastases (Lukaszewicz, Mroczko, & Szmitkowski, 2007). In this circumstance, ER acts to prevent the formation of neoplasms.

ER can also be activated through a number of other ligand-independent, signal transduction pathways, such as the mitogen activated protein kinase (MAPK) pathway. Once activated, through overexpression of HER2 (Human Epidermal Growth Factor Receptor 2, erb2 or HER2), the MAPK can in turn activate the ER through phosphorylation. The ER can then initiate gene transcription (Clarke, Anderson, & Howell, 2004; Kato et al., 1995; Thomas, Sarwar, Phoenix, Coombes, & Ali, 2008).

Song, et. al. (2007) also report ER involvement in a Membrane-initiated steroid signaling (MISS) pathway. This is regarded as an extranuclear ER role, in which the receptor forms a rapid and transient complex with many signaling molecules. In response
to E₂, this complex leads to activation of the MAPK and akt pathways; signaling which gives rise to cell growth.

1.3.3 ER-alpha and Breast cancer

It is well established that many breast carcinomas are hormone-dependent, in that, their development arises, largely, due to the presence of genomic/non-genomic growth promoting activities of nuclear hormones and nuclear hormone receptors such as estrogen and the estrogen receptor, respectively. There is a differential expression of ERα in normal breast tissue and breast carcinomas. In normal tissue, ERα is expressed at low levels in non-proliferating epithelial cells, but in higher amounts in proliferating breast tumors (Fowler & Alarid, 2007; Holst et al., 2007). It is also known that benign, proliferative breast diseases and invasive breast carcinomas have a higher ERα expression compared to non-proliferative disease. There are many mechanisms which might account for such increases including amplification of the ERα gene, ESRI (Fowler & Alarid, 2007; Holst et al., 2007).

The E₂/ERα complex can, in part, lead to the development of a breast neoplasm through stimulating the transcription of genes that are responsible for increased cell growth and differentiation. More specifically, the complex can drive the transcription of genes that give rise to growth promoting proteins (Perillo, Sasso, Abbondanza, & Palumbo, 2000; Savicky, 2004).

As discussed in the previous section, Bcl-2, VEGF, cyclinD1 are common transcriptional gene targets of the E₂/ERα complex. Transcription of the PR gene can also be induced through ERα-dependent pathways or through E₂/ER-independent pathways.
(Lee & Gorski, 1996; Nardulli, Greene, O'Malley, & Katzenellenbogen, 1988). The role of PR in the development of breast cancer is still controversial (Lange et al., 2008), but many agree that it promotes increased cell cycle progression (Kariagina, Aupperlee, & Haslam, 2008). Two other proteins that contribute, under normal circumstances, to the advancement of breast cells from G₁ to S phase transition of the cell cycle are cyclin D₁ (discussed previously) and c-Myc. However, when either these are overexpressed as a result of increased ER-dependent gene transcription, cells will progress through the cell cycle at an abnormal rate. This may result in an increase in the number of genetic mutations and development of malignant growth (Butt et al., 2008).

There are a number of cofactors that associate with the ER to either foster ER-dependent transcription (coactivators) and inhibit it (corepressors). SRC-3 and TRAP220 are ERα coactivators, whereas NCoR (Nuclear Receptor Corepressor) is a corepressor (McKenna, Lanz, & O'Malley, 1999; Nilsson et al., 2001). A change in the level or pattern of expression of such cofactors can give rise to a malignant breast tumor through deregulated ERα-dependent transcription.

Many therapies have been designed to combat breast cell growth with goal of either reducing the tumor burden or as a preventative measure for recurrences. Such therapies include those that alter the level of estrogen (aromatase inhibitors) and those which alter ERα by inhibiting the binding of its ligand and/or its co-activators. The latter of which are collectively known as Selective Estrogen Receptor Modulators (SERMs) (Lo & Vogel, 2004). Other studies suggest that resistance to SERMs maybe due to Akt or MAPK pathway aberration and cross-talk with components ER pathways. Hence,
targeting such pathways may be a suitable target for those who develop a resistance to the traditional treatment regimes (Sengupta & Jordan, 2008).

In summary, the last two sections explained how ER acts to induces gene transcription and how deregulation of such can promote mammary carcinogenesis. The ER-dependent transcriptional pathways have a number of regulatory processes such as phosphorylation of ERα, binding of corepressors and/or coactivators as well as crosstalk with other pathways. It has been established that breast carcinomas, which occur under the influence of E₂ and ERα, arise through a cumulation of deregulated regulatory events.

Aside from the endogenous effects of E₂ and the ER, a study by Rohan et. al. (2008) of 16,608 post-menopausal women found that those who take synthetic estrogen and progesterone were at an increased risk for benign proliferative breast disease and subsequent development of invasive breast carcinoma. The result of such studies has brought about a public awareness that often deters women from taking medications that are designed to curb menopausal effects.

1.4 MI-ER1α

Mesoderm Induction Early Response 1 (MI-ER1) was found to be an immediate-early gene, or an initial target of signal transduction, during Fibroblast Growth Factor (FGF) signal transduction. MI-ER1 is a novel protein that was discovered during the investigations into the role of FGF in *Xenopus laevis* embryonic cell differentiation (Paterno, L. I., Luchman, Ryan, & Gillespie, 1997). During these investigations, Paterno et. al. (1997) demonstrated, using *Xenopus* explants, that MI-ER1 levels are increased following addition of FGF.
In an effort to characterize human \textit{mi-er} (\textit{hmi-er/}), the human orthologue of \textit{xmi-er/} was cloned by Paterno et. al. (1998, 2002) and was found to be a 63kb, single copy gene located on chromosome 1.

1.4.1 MI-ER1 Structure

The \textit{hmi-er} gene gives rise to 2 major protein isoforms $\alpha$ and $\beta$ (Figure 18) (Paterno et al., 2002). The $\alpha$ C-terminus is 23 amino acids long, in contrast the $\beta$ C-terminus is 102 amino acids in length and includes the facultative intron 15 (Paterno et al., 2002).

It was noted when comparing hMI-ER1 with the XMI-ER1 that there was 91% sequence similarity overall, with some regions displaying 100% identity, indicating a highly conserved protein.
Figure 18 Structure of MI-ER1

The two hMI-ER1 isoforms hMI-ER1α and hMI-ER1β (A). The acidic activation domain, ELM2 and the SANT domains are a part of a common internal region to both protein isoforms. The amino acid sequences for the two alternate C-termini of MI-ER1, α and β (B).
1.4.2 MI-ER1 Functional Domains

Both the structure and sequences of MI-ER1 were compared to other known proteins to see there were any functional domains from which one could infer MI-ER1’s function. Indeed, several domains that are common to transcriptional regulators were identified (Figure 18).

A: Acidic activation domain: This is a domain that is, by definition, rich in acidic amino acids and in the MI-ER1 protein is located near the N-terminus. Paterno et. al. (1997) identified four stretches that were rich in acidic amino acid residues. Acidic activation domains are commonly known to participate in the recruitment of transcriptional machinery and therefore initiate gene transcription (Melcher, 2000).

B: ELM2 domain: The EML2 domain immediately adjacent and downstream from the acidic activation domain. It is a domain that has been identified in other proteins that function as transcriptional regulators; specifically, this domain permits proteins to recruit and bind to other proteins that act to suppress gene transcription (Ding, Gillespie, & Paterno, 2003). Recruitment of the transcriptional repressor Histone Deacetylase 1 (HDAC1) to MI-ER1 occurs through the ELM2 domain. Histones are proteins around which DNA winds, compacting the genome and thus permitting it to fit within the small confines of a cell’s nucleus. HDAC is an enzyme with an ability to remove acetyl groups from lysine residues of histone proteins. When this occurs the chromatin, that was once uncoiled, becomes more tightly wound onto the histone protein, inhibiting the transcriptional machinery, hence inhibiting gene transcription.

CREB-binding protein (CBP) is a transcriptional co-activator that has intrinsic histone acetyltransferase (HAT) activity. HATs add acetyl groups to lysine residues of
histone proteins and allowing for the uncoiling of DNA, thus enabling gene transcription. The addition of acetyl groups neutralize the positive charged associated with the lysine residues which therefore reduces the affinity that the negatively charged DNA has for the histone proteins. Recently, it was found the MI-ER1 interacts with CBP through both its acidic activation domain and its ELM2 domain and prevents CBP from exerting its HAT activity. Hence, this represents another pathway through which MI-ER1 is able to act as a transcriptional repressor (Blackmore, Mercer, Paterno, & Gillespie, 2008).

C: **SANT domain:** C-terminal to the ELM2 domain within MI-ER1 is the SANT domain, which was so named following its initial identification in SWI3, ADA2, NCoR and TFIIB transcriptional factors. SANT domains allow for DNA binding and protein-protein interactions, including HDACs (Aasland, Stewart, & Gibson, 1996). This domain was also found in transcription regulatory molecules and complexes, such as SMRT (Ordentlich et al., 1999) and MTA-1 (Toh et al., 2000), respectively.

Spl is a transcription factor that recognizes and binds to GC rich areas within the promoters of target genes, recruits the TFIID complex and together initiate gene transcription (Kolell & Crawford, 2002; Song et al., 2003). Ding et al. (2004) found that the SANT domain within MI-ER1 allows for its interaction with Spl; an interaction that prevents Spl from binding to the promoters of target genes and thereby inhibits its transactivation capabilities.

D: **Proline rich region:** Many signaling proteins bind to proline-rich region within other proteins through SH3 domains. A small protein motif is defined by the sequence PXXP, with P representing a Proline and X representing an amino acid. Such a region has been identified in both XMI-ER1 and hMI-ER1 and Teplitsky et al. (2003) demonstrated
that only proline 365 is imperative to the role that MI-ER1 plays in Xenopus embryo development and mesoderm induction.

**E:** **NLS signal:** MI-ER1β contains a nuclear localization signal (NLS) within the C-terminus (Post, Gillespie, & Paterno, 2001). NLSs are short segments of amino acids located on the surface of certain proteins that are recognized by cytosolic nuclear transport receptors which mediate the transport of the protein into the nucleus. In contrast, MI-ER1α does not contain a NLS and therefore relies on binding to other proteins that do contain such sequences in order to move into the nucleus.

**F:** **LXXL motif:** Many nuclear hormone co-activators (e.g. SRC-1 and CBP) and co-repressors (e.g. NCoR) contain a domain, known as a NR box or a LXXL domain (where L represents a leucine residue and X represents any amino acid), that permits it to bind to NRs. Such a domain has been identified within the C-terminus of MI-ER1α.

LXXL domains can confer both co-activator and co-repressor activities within a single protein (Sauve et al., 2001). MIcoA (MTA1-interacting coactivator) binds to the ER, through the interaction of the LXXL domain of the MIcoA protein and the AF-2 region of the ER, to stimulate ER-driven transcription. Repression of this MIcoA-mediated ER transactivation can occur when MTA binds to the LXXL domain of MIcoA, thus preventing it from binding to the ER and acting as a coactivator (Mishra et al., 2003).

**1.4.3. Previous work: MI-ER1α**

Human embryonic kidney cells (HEK293), transfected with both mi-er1α and era, were used to determine if MI-ER1α and ERα physically interact with one another.
Co-immunoprecipitation assays revealed these proteins do indeed interact in the presence and absence of its ligand E₂ and this interaction is stronger in the absence of E₂ (McCarthy et al., 2008) (See Appendix B, Figure 1A).

Extracts from an MCF-7 cell line, ER positive breast carcinoma cells, were used to investigate the endogenous interaction between MI-ER1α and ERα. Supporting the results of the transfected assays, endogenous MI-ER1α and ERα were shown to interact (See appendix B, Figure 1B). Again, this interaction was observed to be stronger in the absence of E₂ (McCarthy et al., 2008).

Anchorage-independent growth is an acquired characteristic of many tumor cells which provides them with the ability to grow without adhering to the substratum: a characteristic which distinguishes cancer cell proliferation from normal cell proliferation. As previously stated MI-ER1α was shown to possess domains that are characteristic of co-activators and co-repressors, hence investigations into the ability of MI-ER1α to repress ER stimulated cancer cell anchorage-independent growth was carried out using soft agar assays. Using a doxycycline-inducible MI-ER1α T47D breast cancer cell line, overexpression of MI-ER1α was shown to suppress ERα-induced colony growth in soft agar (See Appendix B, Figure 2) (McCarthy et al., 2008).
1.5 Hypothesis and objectives

Elucidation of all steps, as well as the regulatory factors, within the ERα transactivation pathway is essential for the development of early detection/screening markers and tests, prognostic indicators and more effective treatment strategies. It is now known that MI-ER1α interacts with ERα and negatively influences breast carcinoma cell proliferation. In particular, it has been shown to suppress ERα activity in a variety of breast carcinoma cell lines. It is also known that MI-ER1α contains an LXXLL domain that is common to many NR co-activators and co-repressors.

From this, it is hypothesized that MI-ER1α is a key factor in the regulating the activity of ERα and hence ERα-dependent tumor growth. Before identifying MI-ER1α as a potential therapeutic agent or its use as a prognostic indicator, its expression in human normal and neoplastic breast tissue first needs to be determined. The specific aims of this study were:

1. To determine the expression of MI-ER1α in whole tissue sections of human breast carcinoma and adjacent normal cells. More specifically, we wanted to determine the average level of expression and the proportion of cells expressing MI-ER1α in both the tumor and the adjacent normal cells. In addition, we wanted to determine the expression of MI-ER1α in normal breast tissue; breast tissue from patients not having had breast carcinoma or having a family history of such.

2. To compare MI-ER1α expression level in human breast carcinoma tissue to that of adjacent normal breast tissue to determine if a differential in the expression exists between the two.
3. To determine if there is a correlation between the expression level and clinicopathological parameters such as tumor size, grade, stage, and hormone receptor positivity.

4. To determine the subcellular localization of MI-ER1α in whole tissue sections of both human normal breast tissue and human breast carcinoma. As well as examination of such in tissue microarrays (TMAs) of human normal breast tissue, breast carcinoma and various breast pre-invasive subtypes.
2. **MATERIALS AND METHODS**

2.1 **Patient Selection**

As outlined in the objectives for this project, Section 1.5, an investigation of: (1) the expression in normal breast tissue (from cases having no history of cancer) and (2) a comparison of expression between tumor cells and adjacent normal ductal or lobular epithelium within breast tumor samples was to be carried out. The normal breast samples were archived samples of tissue removed from patients who had a reduction mammoplasty. Both the criteria and the process of selection for the tumor samples differ from that for the normal samples, as such they are discussed separately in the pages to follow. Sections 2.1.3 - 2.1.5 detail the selection process for the tumor samples and 2.1.6-2.1.8 explain the selection process for the normal samples. This study was approved by both HIC (HIC approval No. 05.56) and RPAC.

2.1.1 **Time Period and Hospital Selection - Normal and the Tumor Samples**

Formalin-fixed paraffin embedded breast tissue specimens archived at St. Clare’s Mercy Hospital and the Health Science Center (HSC), St. John’s, Newfoundland and Labrador were chosen for this study.

The time period chosen for this study was 2005 – 2006, inclusively. Specimens within this time period were the most recently fixed and processed at the time of selection and therefore any concerns regarding tissue degradation were minimized. Secondly, for the years of 2005 onward, the protocol for fixation and processing had been identified as consistent for both of the aforementioned hospitals. In that, St. Clare’s and the Health
Sciences Center were using the same protocol for fixation and processing of the samples. Therefore, differences observed would not be attributed to differences in fixation and processing.

2.1.2 Database and Pathology Reports- Breast Tumor Samples

A database of all breast cancer cases diagnosed at either the St. Clare’s Mercy Hospital or the HSC between January 1st, 2005 and December 31st, 2006 was obtained from the NL Eastern Health Cancer Registry. All of the invasive ductal carcinoma (IDC) cases were extracted from this database and pathology reports were retrieved for each of these cases. Pathology reports for each case were reviewed to ensure:

I. That all of the cases were an IDC and not a mixed tumor having a morphological appearance of both lobular and ductal carcinoma.

II. That the archived specimen under review was the primary cancer and not a recurrence of breast cancer or a metastatic cancer.

III. That this report is associated with whole tissue sections of the lesion in question and not a biopsy sample. In that, these samples were a resection specimen and not a needle core biopsy sample.

IV. That these cases were accessible from either St. Clare’s Mercy Hospital or the HSC and were not associated with a referral from an outside hospital.
V. That cases were only selected if the pathology report had stated that the
standard test for ER/PR was carried out at Mount Sinai Hospital, Toronto,
Ontario.¹

Any cases not meeting the above criteria during this review process were excluded
from the study.

2.1.3 Criteria for Specimen Selection-Breast Tumor Samples

Prior to selection of the cases that were to be used for this study, four criteria were
established:

I. That upon review of the hematoxylin and eosin (H & E) stained slides, which
contained a 4 or 5μm thick representative of archived, paraffin-embedded breast
tumor tissue, only those which were both adequately fixed and processed prior to
being embedded in paraffin were selected. Adequate fixation and processing is
based upon the morphological appearance of the cells. One sign of poor fixation is
when the cells appear to be detached from the connective tissue and surrounding
cells. Appropriate inclusion of each sample, based on this criteria, was determined
by pathologist Dr. Bev Carter.

II. To compare the expression between tumor cells and that of the adjacent normal
cells, only cases with ample amounts of both tumor cells and adjacent normal
ductal epithelium were selected.

¹ Due to the Estrogen Receptor (ER) and Progesterone Receptor (PR) retesting of breast
carcinoma cases here in NL, for a time period that includes 2005, Mount Sinai’s
pathology department was the selected hospital for retesting until quality control within
the pathology department of Eastern Health was established.
III. Only blocks that contained copious amounts of breast tissue were selected; which was done as a means to avoid exhaustion of the sample. In this regard, samples were preserved for future clinical purposes.

2.1.4 Number of Cases used in the Study-Breast Tumor Samples

This was a retrospective study in which an initial 129 IDC cases were identified from the Cancer Registry database. Pathology reports for all 129 cases were reviewed and cases not meeting criteria I-IV, as identified in section 2.1.2, were excluded from the study. One hundred and nineteen cases were identified after review of the reports. For each of the 119 cases selected up to this point, H & E stained slides were retrieved and reviewed with pathologist, Dr. Beverly Carter of Eastern Health. Only those cases that fulfilled the specimen selection criteria identified in section 2.1.3 were included in the study; the study cohort for this project consisted of 110 women diagnosed with primary IDC. For each case, one archived, formalin-fixed, paraffin-embedded block of breast tumor tissue was selected and retrieved from storage at either St. Clare’s Mercy Hospital or the HSC (Figure 19).

2.1.5 Database and Pathology Reports- Normal Breast Tissue Samples

To investigate the expression in normal breast tissue with no history of breast cancer, a list of patients having had a reduction mammoplasty between January 1st, 2005 and December 31st, 2006 were obtained from the Pathology department. Pathology reports for all these cases were retrieved and reviewed; cases meeting all of the following criteria were added to a database:
I. Only cases that had archived, normal breast tissue as a result of having a reduction mammoplasty were selected. In that, those having archived normal tissue as a result of a mastectomy due to an increased risk for breast cancer were excluded from the study.

II. Only patients with no history of hyperplasia, Ductal Carcinoma in Situ (DCIS) or breast carcinoma were selected.

III. Cases with no family history of breast carcinoma were selected.

IV. Microscopic findings detailed in the pathology report must not describe any of the following proliferative breast abnormalities as detailed in Table 1:

   a. Proliferative diseases without atypia
   b. Proliferative disease with atypia
   c. In Situ lesions

Ensuring that there was no history of that listed above was done as a means to increase the validity of the results; that any analysis of the normal tissue were not due to the influence of other cellular changes within the breast tissue.

2.1.6 Criteria for Specimen Selection-Normal Breast Samples

Before selecting the normal breast specimens to be used in this study, three criteria were established:

1. That upon review of the hematoxylin and eosin (H & E) stained slides, which contained a 4µm thick representative of archived, paraffin-embedded normal breast tissue, only those which were both adequately fixed and processed prior to...
II To determine the expression pattern in normal breast tissue, specimens which contained sufficient amount of and only normal ductal epithelium were selected.

III Only blocks that contained copious amounts of breast tissue were selected; which was done as a means to avoid exhaustion of the sample. In this regard, samples were preserved for future clinical and research purposes.

2.1.7 Number of Cases used in the Study-Normal Breast Tissue

All cases that satisfied the criteria outlined in sections 2.1.5 were subsequently added to a database. From this database 60 cases were randomly selected using a random integer generator located on the World Wide Web (http://www.random.org/integers/).

The H & E stained slides for each case were reviewed with pathologist, Dr. Beverly Carter of Eastern Health. During the review of these slides, the diagnosis and microscopic descriptions detailed in the pathology report were confirmed and one archived, formalin-fixed, paraffin-embedded block of normal breast tissue was selected. As a consequence of the selection criteria delineated in section 2.1.6, 14 cases were excluded from the study. For each of the 46 cases, one archived formalin-fixed, paraffin-embedded block of normal breast tissue was retrieved from storage at either St. Clare’s Mercy Hospital or the Health Science Center (Figure 19).
Figure 19  Process for selecting cases of normal breast tissue and primary IDC.

Database and pathology reports for all cases between 2005-2006 were reviewed to confirm that normal cases were strictly reduction mammoplasties and the tumor cases were primary IDC cases. It was also confirmed using the reports that there were no other abnormalities. H&E slides were reviewed to confirm adequate fixation and paraffin blocks were subsequently retrieved.
2.2 Tissue Microarrays (TMAs)

2.2.1 Number of Cases Used in the Study-TMAs

Fourteen breast Tissue Microarrays (TMAs) were acquired from BioChain Institute Inc. (Hayward, California, U.S.), US Biomax Inc. (Rockville, Maryland, U.S.) and the Cooperative Human Tissue Network (CHTN) (Columbus, Ohio, U.S.). In total, these TMAs contained 204 normal breast, 78 DCIS, 102 Invasive Lobular Carcinoma (ILC) and 343 IDC cases.

2.2.2 Tissue Microarray Construction

All TMAs used in this study were constructed by the manufacturer. Briefly, a fresh section of the donor block (the original paraffin-embedded tissue) is made and stained with H&E. An optimal area of the tissue block from which to extract the core is selected by a pathologist upon review of the H&E slide; this area is then circled on the corresponding donor block to localize the region (Shergill, Shergill, Arya, & Patel, 2004). A grid, or sector map, which details the location of each core on the TMA is planned and used as a guide to construct and score the TMA (Voduc, Kenney, & Nielsen, 2008). This sector map also provides a link to the donor block and hence, experimental results can be rapidly compared to the clinicopathological characteristics associated with each case (Shergill et al., 2004). A core, ranging in size from 0.6mm to 2.0mm, is removed from a blank paraffin block (recipient block) and is replaced with a core from the target area of the donor block (Figure 20) (Voduc et al., 2008).
Each core of TMA originates from an area of interest located on a donor block after review of the corresponding H&E section. A core of is removed from a blank paraffin block and replaced with a core from the donor block (Shergill, I.S., 2004).
2.3. **Tissue Fixation and Processing**

The most important step in preserving human tissue, is fixation. Fixation is an attempt to preserve the natural state of the tissue as much as possible. Fixation and processing for the WTS is described below in section 2.3.1 and in Appendix C for the TMAs.

2.3.1. **NL Normal and Tumor Breast Tissue**

All paraffin tissues used in this study were fixed and processed by the pathology laboratory staff at either St. Clare’s Mercy Hospital or the HSC according to the “Fixation Procedure for Pathology Specimens”, Section No. PRC-PAT-102, within the HSC Pathology Division’s “Anatomical Pathology/Specimen Collection and Handling” manual, written by the Quality Management Coordinator of the Pathology Division, Catherine Parnell. Briefly, the specimen is placed in a 10% buffered Formalin (a 4% formaldehyde solution) within 30 minutes of removal from the body. The specimen is placed in volume of formalin that is 10 times that of the tissue; for small tissues this incubation is between 3 and 24 hours and 24-48 hours for large tissues (Parnell, 2008). The tissues are then inserted into specimen cassettes and placed into an automatic Tissue Tek VIP 5 Vacuum Infiltration Processor (Sakura Finetek USA, Inc.). Cassettes are placed into a retort where reagents and molten paraffin move in and out sequentially via vacuum and pressure. This processor has a closed system design, which allows for reduced air exposure to the tissues. Processing of the tissues involves dehydrating, clearing and then infiltration of the tissues samples. First is the dehydration step, whereby water is gradually removed from the tissue through immersion in various grades of
alcohol and finally replaced with 100% alcohol. Secondly, the alcohol is cleared in Xylene and replaced with a solvent that is miscible with wax. The tissue is then infused with a paraffin wax which removes the clearing reagent and results in a hardened tissue that facilitates cutting of the tissue into sections. Finally, the tissue, which is engrossed in a paraffin wax within a tissue cassette, is then sectioned and stained with H&E prior to being archived and stored for future use. The H&E slides are examined by pathologists during their diagnostic procedures, following which, the slides are archived along with the paraffin tissue blocks.

2.4 Slide Preparation and IHC Pre-treatment

A NL Normal and Tumor Breast Tissue

Four 4μm think sections were cut (by P. McCarthy) from each of the selected blocks and transferred to positively charged slides (Fisher); such tissue sections are referred to as Whole Tissue Sections (WTS). Most animal tissues carry an overall net negative charge due to a small excess of acidic amino acids over basic amino acids in the structural proteins. Hence, tissue sections can be expected to adhere well to a surface that has been treated in such a way as to make it positively charged (Lloyd & DeLellis, 2001). Slides are left overnight at room temperature (RT) and placed at 58°C for one hour prior to the start of IHC. This is done to aid in the removal of the paraffin and hence antigen exposure.
B TMAs

TMAs were constructed as described in section 2.2.2. The TMA block was sectioned, by the manufacturer, 5µm thick and transferred to positively charged slides. As was the case for the whole tissue sections, TMA slides from Biochain and CHTN were placed at 58°C immediately before the commencement of IHC. TMA slides from Biomax are wrapped in a thin layer of paraffin and supplied in this manner in an effort to prevent oxidation and decay. As a result of this paraffin layer, slides needed to be baked at 60°C for two hours (as per the manufacturer’s recommendation) prior to the start of the IHC protocol.

2.5 Immunohistochemistry

2.5.1 Primary Antibody

The anti-MI-ER1α antibody used in this study generated through the immunization of rabbits with a short, synthetic peptide (amino acids 413 - 426) of the MI-ER1 protein sequence (Paterno et al., 2002). The peptide sequence used was \(^{413}\text{CQLLPVHFSAISSR}^{426}\). A Melon Gel IgG purification kit (Pierce) was used (by Dr. Laura Gillespie) to purify MI-ER1 IgG from both pre-immune and immune rabbit serum.

Specificity of the MI-ER1α antibody was verified through an antibody pre-absorption assay using the alpha-specific peptide used to generate the MI-ER1α, as well as an unrelated (control) peptide. Whole tissue sections of breast carcinoma were stained concurrently with either pre-immune IgG, anti-MI-ER1α IgG, anti-MI-ER1α IgG pre-absorbed with 0.1µg of the α-specific peptide or anti-MI-ER1α IgG pre-absorbed with 0.1µg of an unrelated peptide (amino acids 466-480 of the MI-ER1β C-terminus).
Sections were first deparaffinized, hydrated and antigen retrieval was carried out as detailed below in Section 2.6.3. One hundred ng of each the specific and unrelated peptides were dissolved in a 1% Bovine serum albumin/Phosphate buffered saline (BSA/PBS) solution (Sigma-Aldrich, Canada, see Appendix A for recipe). The anti-MI-ER1α IgG was then diluted in this solution to 1:800; the same dilution used for the experimental sections. The IgG was incubated with each of the peptides on ice, for 30 minutes, prior to its application on the tissue sections.

2.5.2 Staining Kit used for WTS and TMAs

A Universal LSAB+ System-HRP kit (DakoCytomation) was utilized in the immunohistochemical staining procedure for both WTS and TMAs. Please see Appendix D for a more detail description of this kit.

2.5.3 Immunohistochemical Protocol

Slides were removed from the oven and deparaffinized in two changes of xylene for 5 minutes each and hydrated through an alcohol gradient; two changes of 100% alcohol for three minutes each, one change of 95% alcohol for one minute and 70% alcohol for one minute. Slides were then rinsed in running distilled water for 5 minutes to complete the hydration process and to remove any residual alcohol. To quench endogenous peroxidase activity slides were immersed in 3% hydrogen peroxide for 10 minutes. To remove any excess hydrogen peroxide, slides were rinsed in running distilled water for five minutes.
Frequently antigens become masked during fixation and processing of the tissues; in an effort to retrieve the antigens slides were immersed in a 10mM citrate buffer, pH 6.0 (1.92g citric acid, 1L deionized water, adjust pH using 1N NaOH). Optimum antigen retrieval time was empirically determined to be 40 minutes for WTS and 30 minutes for TMAs. First, the citrate buffer was pre-heated to 95°C in plastic coplin jars and then slides were placed into the coplin jars and incubated in a 95°C water bath for 40 minutes (WTS) or 30 minutes (TMAs).

Following antigen retrieval, the slides were removed from the water bath, retained in citrate buffer and cooled to room temperature. Slides were then rinsed in a 1X phosphate buffer saline (PBS) (Sigma) solution, pH 7.4 (see Appendix E for recipe), for 5 minutes in a humidifying chamber to prevent evaporation.

To inhibit non-specific labeling, sections were incubated in a serum-free protein block (DakoCytomation) for 10 minutes. Slides were then incubated in the humidifying chamber overnight at 4°C with the MIERα antibody, previously described, diluted to 1:800 in a 0.01M PBS containing 1% weight per volume (w/v) Bovine Serum Albumin (BSA), pH 7.4 (Sigma Cat. No. P3688).

Immunohistochemical staining was continued the following day using a streptavidin-biotin immunoperoxidase method according to the protocol specified for the Universal LSAB+ System-HRP kit (DakoCytomation). First, sections were rinsed in 1X PBS for 5 minutes to remove excess primary antibody. Sections were then incubated sequentially with a biotinylated link antibody and a peroxidase-labeled streptavidin for 15 minutes each; with one 5 minute rinse in 1X PBS between each incubation. After addition of Streptavidin, slides were again rinsed for 5 minutes in 1X PBS. Slides were incubated
with a Liquid DAB+ Substrate Chromogen (3,3'-diaminobenzidine chromogen solution within an imidazole-HCl substrate buffer, pH 7.5) (DakoCytomation) for 5 minutes and then rinsed in running distilled water for 5 minutes.

Sections were then counterstained in hematoxylin (Fisher) (see Appendix E for recipe) for three minutes and rinsed in running distilled water for 5 minutes. To accentuate the details of the tissue, excess dye is removed through a process known as differentiation by immersion for approximately three seconds in a 1% acid alcohol solution (see Appendix E for recipe). Next, slides were rinsed in running distilled water for 5 minutes, blued in Scott’s tap water (see Appendix A for recipe) and rinsed again for 5 minutes in running distilled water.

To complete the staining, slides were dehydrated in an alcohol gradient: 1 minute in 70% alcohol, 1 minute in 95% alcohol, then 2 changes for 3 minutes each in 100% alcohol. Sections were then cleared in 2 changes of xylene for 5 minutes each. Slides were mounted using Permount media (Fisher). After allowing the Permount to dry overnight, sections were reviewed using a compound light microscope (Olympus BH-2) and pictures were taken with a coolsnap digital camera.

2.5.4 Controls

Included in each batch of staining, was a section of breast tissue stained with the pre-immune serum. This pre-immune serum was applied to the tissue at the same concentration and in the same diluent (1% BSA/PBS) as the primary antibody. All steps of the IHC procedure were identical with the exception that a pre-immune serum was applied to the tissue instead of the primary antibody.
Tissue sections known, from previous experiments, to give positive immunostaining results can be used to verify the performance of IHC reagents and the basic conditions of the experimental procedure (DakoCytomation, 2006). Positive and negative tissue controls suitable for use in this study were identified following review of murine tissue sections stained with an anti-M1-ER1α antibody in a study by Thorne et. al. (2008). Normal sections of the ileum from the small intestine and normal adrenal gland were selected as a negative and positive control, respectively. In the previously mentioned study, staining was barely detectable in, and limited to, the mucosal columnar epithelium of the mouse small intestine; in contrast, the mouse adrenal gland was intensely positive. Normal human tissue sections of such were obtained from US Biomax Inc..

Staining of positive and negative tissue controls ensure that both the target antigen retrieval and staining procedures were properly carried out. It is favorable when the positive control contains a spectrum of weak to strong staining. If the negative tissue control has positive staining it indicates that either the antibody is not specific for the target antigen or there is non-specific binding. If both the positive and negative tissues do not present as expected, results from the experimental specimens should be considered invalid (DakoCytomation, 2006).

For this study, whole tissue, paraffin-embedded tissue sections of human adrenal gland and small intestine (US Biomax Inc.) were used as a positive and negative control, respectively.
2.7 Grading of Whole Tissue Sections and Tissue Microarrays

All of the specimens, WTS and TMAs, were graded 24 hours after the slides were mounted. Grading of WTS was carried out by Dr. B. Carter and myself and the TMA sections were graded by Dr. L. Gillespie and myself; any cores of a questionable pathology were then reviewed by Dr. B. Carter. Staining was evaluated on three levels; the protein expression pattern, subcellular localization and the Allred scoring system. The protein expression pattern and subcellular localization were assessed in the TMAs and in both tumor and normal WTS.

IHC is one tool used by physicians to evaluate the ER expression within breast tumors. It is done as a means of estimating a patient’s potential response to endocrine treatment (predictive factor) and their expected clinical outcome or survival (prognosis factor) (Allred et al., 1993; Cleator et al., 2006; Harvey, Clark, Osborne, & Allred, 1999; Kurosumi, 2007). The Allred Scoring System is one such means of assessing the ER expression (ER status), in breast carcinoma tissue and has been widely adapted among the clinical pathology community (Allred et al., 1993; Harvey et al. 1999). This scoring system has been validated for several makers, such as ER, PR, Her2/neu and p53, and has been widely adapted for the use in the clinical assessment of breast tumor specimens (O’Malley et al., 2001).

In this study, staining of WTS of breast tumor were assessed using this semi-quantitative scoring system developed by Allred et al. (1993). In accordance with this scoring system, all specimens were reviewed to assign a proportional score (PS) and an intensity score (IS), which represent the estimated proportion of positively-stained cells and the average intensity of positively-stained cells, respectively (Figure 21).
Representative samples of normal breast and IDC stained with the anti-MI-ER1α antibody and assessed using the Allred Scoring system can be seen in Figure 22. This figure illustrates the variability in the intensity of MI-ER1α within normal and tumor breast tissue and the corresponding IS assigned.

Both the tumor and adjacent normal within the WTS were each assigned a PS and an IS. The PS and the IS for each of the tumor and the normal areas within the WTS were then added together to give an overall Allred Score; ultimately giving rise to an Allred Score for the tumor and an Allred Score for the adjacent normal. See Table 3 for the equivalent of each score within the Allred scoring system. The Allred Score for the tumor was compared with that of the normal tissue and then grouped in to one of the following: tumor score is less than the normal score, tumor score is equal to the normal score or tumor score is greater than the normal score.

WTS and TMAs were also graded to assess the subcellular localization of MI-ER1α and then assigned to one of the three following groups; nuclear staining only, cytoplasmic staining only or nuclear and cytoplasmic staining.

Prior to the commencement of the localization assessment, a cutoff point was determined for the consideration of positively-stained nuclei. In that, samples were only scored positive if greater than or equal to 5% of nuclei were positively-stained.
Scoring Immunostained Slides

Proportion Score (PS)

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1/100</td>
</tr>
<tr>
<td>2</td>
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<td>1/3</td>
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<tr>
<td>4</td>
<td>2/3</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
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</table>

Intensity Score (IS)

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>intermed</td>
</tr>
<tr>
<td>3</td>
<td>strong</td>
</tr>
</tbody>
</table>

Total Score (TS) = PS + IS (range 0-8)

Figure 21 Illustration of the Allred Scoring System

Grading of the WTS, using the Allred Scoring System, involves review of the whole section to first determine the estimated proportion of positively-stained cells and then the average intensity level for the positively-stained cells using the above illustration as a guide (Kurosumi, 2007).
Figure 22  Variable staining intensity within the cytoplasm of both normal ductal epithelium and breast tumor tissue

Samples of IDC and normal breast tissue from tissue microarrays stained with anti-MI-ER1α. Variable cytoplasmic staining intensity is observable in separate cases of normal breast tissue are illustrated in panels A-C (50X) and D-F (100X). Representatives of individual cases of IDC, also illustrating the variability in the intensity of cytoplasmic staining, can be seen in panels G-I (50X) and J-L (100X). Intensity Score (IS) for each of the representative samples are as follows: negative/weak cytoplasmic staining = IS of 1, moderate cytoplasmic staining = IS of 2, strong cytoplasmic staining = IS of 3. Scale bar for panels A-C and G-I is 50μm and 25μm for panels D-F and J-L.
Table 3  Allred Scoring System used in the assessment of MI-ER1α staining of breast carcinoma WTSs

<table>
<thead>
<tr>
<th>Intensity Score (IS)</th>
<th>Average Intensity Level: Positively-stained cells</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proportion Score (PS)</td>
</tr>
<tr>
<td>0</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Weak</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Strong</td>
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</tr>
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<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

1 For each case an IS and a PS was assigned; when added together it gives rise to an overall Allred Score within a range of 0-8.
2.8 **Statistical Analysis**

Chi-squared analysis (Pearson's test, two-tailed, 95% confidence interval (CI)), using SPSS v.13 software, was used to determine if a significant correlation existed between various clinicopathological parameters and both the intensity and frequency of MI-ER1α expression within breast carcinoma WTS. Using the two-sided Fisher's exact test within the InStat v.3 software program (GraphPad Software, San Diego, USA), the percent nuclear staining within samples of the breast hyperplasia, DCIS, ILC, LN metastases and IDC was compared to that of the normal breast samples.
3. **RESULTS**

For this study, immunohistochemistry was used in an effort to determine if there was a differential in both the pattern of staining and subcellular localization of MI-ER1α between normal breast tissue and breast cancer tissue.

3.1 **The anti-MI-ER1α Antibody is Specific for the MI-ER1α Protein**

Paterno et al. (2002) demonstrated that the anti-MI-ER1α antibody specifically recognizes MIER1α through immunoprecipitation and western blotting. However, for this study it was also important to demonstrate that this specificity extends to histological sections. To verify the specificity of the purified anti-MI-ER1α antibody for the MI-ER1α protein within human tissue, an antibody pre-absorption assay was carried out using WTS of human breast carcinoma sections. Sections were stained in parallel with one of the following: pre-immune IgG, anti-MI-ER1α antibody, the anti-MI-ER1α antibody that was pre-absorbed with the α peptide used to generate the antibody or the anti-MI-ER1α antibody pre-incubated with the control peptide. This assay was performed in duplicate. As shown in Figure 23, staining is blocked when the antibody is pre-absorbed with the α peptide (Figure 23C) and results are very similar to that obtain when the section is incubated with the pre-immune IgG (Figure 23A). Positive staining was observed in sections incubated with the anti-MI-ER1α antibody pre-incubated with the control peptide (Figure 23D) and staining was identical to that seen with the MI-ER1α antibody (Figure 23B). The lack of staining observed in sections incubated with the pre-immune IgG is as expected since this is serum obtained prior to injection of the rabbits with the MI-ER1α specific peptide. In other words, this is serum which, by definition, is obtained prior to
induction of an immune response and therefore should not contain any IgG's that would
give rise to immunostaining. Knowing that it is the C-terminus which distinguishes the
MI-ER1α isoform from the MI-ER1β isoform, that it is a peptide from the α C-terminus
that is used to generate the antibody and that the α-peptide alone (not the control peptide)
prevents the anti-MI-ER1α antibody from binding to the tissue, indicates that the anti-MI­
ER1α antibody specifically detects the MI-ER1α protein in histological sections.
Figure 23  Antibody pre-absorption assay using breast carcinoma tissue
The MI-ER1α pre-absorption assay was performed by staining whole tissue sections of human breast tumor with pre-immune IgG (A), anti-MIER1α IgG (B), anti-MI-ER1α IgG that was pre-incubated with the α-specific peptide (C), or anti-MI-ER1α that had been pre-incubated with a control peptide (D). Scale bar is 50μm for A-D (50X).
3.2 Positive and Negative Tissue Controls

Staining of the human positive (adrenal gland) and negative tissue (small intestine) controls gave similar results to that observed in the murine samples (Figure 24). Strong staining was observed in the zona glomerulosa and the zona fasciculata of the adrenal cortex (Figure 24A and 24C). However, immunoreactivity was absent in the ileum of the small intestine (Figure 24B and 24D).

These results demonstrate the IHC procedure was satisfactory for use on these paraffin embedded sections and that the IHC reagents are of acceptable quality.
Figure 24 Immunohistochemical staining of the positive (adrenal) and negative (ileum) controls with anti-MI-ER1-α

Positive and negative tissue controls included sections of adrenal gland (A and C) and small intestine (B and D), respectively. Sections were stained with the anti-MI-ER1α antibody. Panels A and C exemplifies MI-ER1α expression within the zona glomerulosa (ZG) and the zona fasciculate (ZF). Whereas panels B and D shows a portion of the small intestine, the ileum, that includes crypts of Lieberkühn “C” and Peyer’s patch “PP” (lymphoid tissue). Scale bar is 100μm for A and B (25X) and 50μm for C and D (50X).
3.3 **Allred Scoring Assessment of MI-ER1α Staining in Breast Cancer WTSs**

To determine if there was a difference in expression of MI-ER1α between breast tumor and adjacent normal tissue, WTSs of 110 primary invasive ductal breast carcinoma cases stained with the anti-MI-ER1α antibody were semi-quantitatively evaluated using the Allred Scoring System. Once staining was complete the entire section on each slide was reviewed by light microscopy and included in the assessment. An estimate of both the intensity level (Intensity Score, IS) and the proportional of positive cells (Proportional Score, PS) was determined for each the tumor and the adjacent normal tissue. When the scores were compared overall, there was no consistent difference between the tumor and the adjacent normal tissue. Namely, some tumors had lower values than the adjacent normal tissue, while others were equal to or greater than that of the adjacent normal tissue. The distribution of the IS, PS and Allred scores are illustrated in Figure 25. The average intensity, proportional and Allred score, as well as the standard deviation (SD), for each the tumor and adjacent normal are displayed in Table 4. The \( P \) value obtained from comparing the means of the tumor and adjacent normal is also reported in Table 4.

Correlation analysis between these three categories of relative Allred Scores and various clinicopathological parameters was carried out using Pearson's chi-square (\( \chi^2 \)) test in SPSS (v.13) software (Table 5). Clinicopathological parameters included age, tumor size, lymph node status, grade (modified Scarff-Bloom-Richardson), stage, as well as, ER, PR and Her-2/neu status. Age was broken down two ways; the first was based on the average onset age of menopause being 51 years (Cheung, Chaudhry, Kapral, Jackevicius, & Robinson, 2004) and the average age range during which menopause can
occur (ages 45-64) (Blumberg et al., 1996). Results of ER and PR expression assays (usually through IHC), in combination with that of Her-2/neu, are used to predict response to hormonal therapy and adjuvant systemic therapies such as Trastuzumab (Herceptin), respectively (Payne, Bowen, Jones, & Wells, 2008). As such, these 3 markers were also included in the correlation analysis.

A statistically significant correlation was not found between any one of the clinicalpathological parameters and the Allred score groups (Table 5).

The Allred intensity and proportional scores were analyzed separately for correlation with the clinicopathological parameters; again, a statistically significant correlation was not found (Table 6, Table 7).
Figure 25  Distribution of the intensity, proportional and Allred scores for the invasive ductal carcinoma and the adjacent normal tissue

A histogram illustrating the distribution of intensity, proportional and Allred scores for the invasive ductal carcinoma and adjacent normal breast tissue cases used in the Allred scoring. The intensity, proportional and Allred scores range from 0-3, 0-5 and 0-8, respectively. The scores for the tumor are indicated in green and the scores for the adjacent normal are indicated in blue.
Table 4  The average intensity, proportional and Allred score and the standard deviation for each the tumor and adjacent normal

<table>
<thead>
<tr>
<th></th>
<th>Intensity Score</th>
<th>Proportional Score</th>
<th>Allred Score</th>
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<tr>
<td></td>
<td>Normal</td>
<td>Tumor</td>
<td>Normal</td>
</tr>
<tr>
<td>Mean</td>
<td>1.68</td>
<td>2.18</td>
<td>3.56</td>
</tr>
<tr>
<td>±SD</td>
<td>0.787</td>
<td>0.756</td>
<td>0.130</td>
</tr>
<tr>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.681</td>
<td>0.0005</td>
<td>0.0024</td>
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</tbody>
</table>

<sup>a</sup> P value was obtained through an unpaired t-test of the intensity, proportional, Allred score means for each the tumor and adjacent normal. Statistical significance is assumed when $P<0.0001$.  

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Table 5: Correlation analysis between relative MI-ERα staining in breast carcinoma and clinicopathological parameters using the Allred Scoring System

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Less than Normal</th>
<th>Equal to Normal</th>
<th>Greater than Normal</th>
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<th>P-value&lt;sup&gt;b&lt;/sup&gt;</th>
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<td><strong>Age</strong></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>6 (19.4)</td>
<td>4 (12.9)</td>
<td>21 (67.7)</td>
<td>31</td>
<td>P=0.410</td>
</tr>
<tr>
<td>&gt;=50</td>
<td>8 (101.1)</td>
<td>16 (20.3)</td>
<td>55 (69.5)</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;=44</td>
<td>5 (25.0)</td>
<td>3 (15.0)</td>
<td>12 (60.0)</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>45-64</td>
<td>4 (7.7)</td>
<td>9 (17.0)</td>
<td>40 (75.5)</td>
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<td></td>
</tr>
<tr>
<td>&gt;=65</td>
<td>5 (13.9)</td>
<td>8 (19.4)</td>
<td>24 (66.7)</td>
<td>37</td>
<td>P=0.365</td>
</tr>
<tr>
<td><strong>Tumor Size (TNM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>6 (10.2)</td>
<td>11 (18.6)</td>
<td>42 (71.2)</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>7 (16.7)</td>
<td>6 (14.3)</td>
<td>29 (59.0)</td>
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<td></td>
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<td>T3 and T4</td>
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<td>3 (33.3)</td>
<td>5 (55.6)</td>
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<td><strong>Tumor Size (Categorical)</strong></td>
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<td>6 (10.2)</td>
<td>11 (18.6)</td>
<td>42 (71.2)</td>
<td>59</td>
<td></td>
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<tr>
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<td>9 (17.6)</td>
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<td>18 (72.0)</td>
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<td>4 (10.3)</td>
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<tr>
<td>III</td>
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<td>8 (72.7)</td>
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<td>Positive</td>
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<td>63 (70.6)</td>
<td>89</td>
<td>P=0.891</td>
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Table 5 continued: Correlation analysis between relative MI-ERα staining in breast carcinoma and clinicopathological parameters using the Allred Scoring System

<table>
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<tr>
<th>Clinical Parameters</th>
<th>Less than Normal</th>
<th>Equal to Normal</th>
<th>Greater than Normal</th>
<th>Total</th>
<th>P-value&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
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<td>PR</td>
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<td>23 (82.1)</td>
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</tr>
<tr>
<td>Positive</td>
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<td>52 (68.4)</td>
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<td>HER2/neu status&lt;sup&gt;e&lt;/sup&gt;</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
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<tr>
<td>Positive</td>
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<td>0 (0)</td>
<td>14 (93.3)</td>
<td>15</td>
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</tbody>
</table>

<sup>a</sup>Values are listed as number of subjects, with percentage listed in brackets.

<sup>b</sup>P-value; Chi-square analysis (χ²) (Pearson's test, two-tailed, 95% CI), statistical significance is assumed when P < 0.05

<sup>c</sup>Modified Scarff-Bloom-Richardson grading system

<sup>d</sup>FIGO staging system

<sup>e</sup>Her2/neu status was determined through IHC: 0=negative (no staining present), 1+=negative (Weak/barely perceptible membrane staining), 2+=Borderline results/case referred for FISH analysis (weak to moderate complete membrane staining), 3+=positive (strong complete membrane staining)
Table 6: Correlation analysis between relative MI-ERα staining intensity in breast carcinoma and clinicopathological parameters

<table>
<thead>
<tr>
<th>MIER1 Expression: Tumor Intensity Score vs. Normal Intensity Score</th>
<th>Clinical Parameters</th>
<th>Less than Normal</th>
<th>Equal to Normal</th>
<th>Greater than Normal</th>
<th>Total</th>
<th>P-value(^b)</th>
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</thead>
<tbody>
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<td>Age</td>
<td>Less than Normal</td>
<td>Equal to Normal</td>
<td>Greater than Normal</td>
<td>Total</td>
<td>P-value(^b)</td>
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</tr>
<tr>
<td>&lt;50</td>
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<td>6 (19.4)</td>
<td>16 (51.6)</td>
<td>31</td>
<td>P=0.364</td>
<td></td>
</tr>
<tr>
<td>&gt;=50</td>
<td>11 (13.9)</td>
<td>26 (32.9)</td>
<td>42 (53.2)</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Less than Normal</td>
<td>Equal to Normal</td>
<td>Greater than Normal</td>
<td>Total</td>
<td>P-value(^b)</td>
<td></td>
</tr>
<tr>
<td>&lt;=44</td>
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<tr>
<td>45-64</td>
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<td>16 (30.1)</td>
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<td></td>
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<tr>
<td>&gt;=65</td>
<td>6 (16.2)</td>
<td>11 (29.7)</td>
<td>20 (54.1)</td>
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<td></td>
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<tr>
<td>Tumor Size (TNM)</td>
<td>Less than Normal</td>
<td>Equal to Normal</td>
<td>Greater than Normal</td>
<td>Total</td>
<td>P-value(^b)</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>11 (18.7)</td>
<td>17 (28.8)</td>
<td>31 (52.5)</td>
<td>59</td>
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<td></td>
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<tr>
<td>T2</td>
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<td>11 (26.1)</td>
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<td>42</td>
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<td></td>
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<tr>
<td>T3 and T4</td>
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<td>P=0.618</td>
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<tr>
<td>Tumor Size (Categorical)</td>
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<td>Equal to Normal</td>
<td>Greater than Normal</td>
<td>Total</td>
<td>P-value(^b)</td>
<td></td>
</tr>
<tr>
<td>&lt;=2 cm</td>
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<td>17 (28.8)</td>
<td>31 (52.6)</td>
<td>59</td>
<td>P=0.861</td>
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</tr>
<tr>
<td>&gt;2 cm</td>
<td>9 (17.6)</td>
<td>15 (29.4)</td>
<td>27 (52.9)</td>
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<tr>
<td>Lymph Node Involvement</td>
<td>Less than Normal</td>
<td>Equal to Normal</td>
<td>Greater than Normal</td>
<td>Total</td>
<td>P-value(^b)</td>
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</tr>
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<td>9 (28.1)</td>
<td>19 (59.4)</td>
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<td>Lymph Node Status (TNM)</td>
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<td>Total</td>
<td>P-value(^b)</td>
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<td>20 (46.5)</td>
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<td>6 (100.0)</td>
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<td>0 (0)</td>
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<td></td>
</tr>
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<td>Grade(^c)</td>
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<td>Greater than Normal</td>
<td>Total</td>
<td>P-value(^b)</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>3 (8.1)</td>
<td>13 (35.1)</td>
<td>21 (56.8)</td>
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<td></td>
</tr>
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<td>Staged(^d)</td>
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<td>Greater than Normal</td>
<td>Total</td>
<td>P-value(^b)</td>
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</tr>
<tr>
<td>I</td>
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<td>9 (36.0)</td>
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<td></td>
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<tr>
<td>III</td>
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<td>3 (27.2)</td>
<td>8 (72.8)</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>Less than Normal</td>
<td>Equal to Normal</td>
<td>Greater than Normal</td>
<td>Total</td>
<td>P-value(^b)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
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<td>10 (62.5)</td>
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<td>P=0.194</td>
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<td>17 (19.1)</td>
<td>25 (28.0)</td>
<td>47 (52.9)</td>
<td>89</td>
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<td></td>
</tr>
</tbody>
</table>

\(^a\) Expressed as a relative intensity score.
\(^b\) For Fisher's exact test.
\(^c\) Grade of tumor differentiation.
\(^d\) Stage of disease.
Table 6 continued: Correlation analysis between relative MI-ERα staining intensity in breast carcinoma and clinicopathological parameters

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Less than Normal</th>
<th>Equal to Normal</th>
<th>Greater than Normal</th>
<th>Total</th>
<th>P-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>3 (10.7)</td>
<td>6 (21.4)</td>
<td>19 (67.9)</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>16 (21.0)</td>
<td>22 (28.9)</td>
<td>38 (50.0)</td>
<td>76</td>
<td>P=0.125</td>
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<tr>
<td>HER2/neu status&lt;sup&gt;c&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>17 (23.0)</td>
<td>22 (29.7)</td>
<td>35 (47.3)</td>
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<td>P=0.388</td>
</tr>
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<td>Positive</td>
<td>1 (6.6)</td>
<td>4 (26.7)</td>
<td>10 (66.7)</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are listed as number of subjects, with percentage listed in brackets.

<sup>b</sup>P-value; Chi-square analysis (χ²) (Pearson's test, two-tailed, 95% CI), statistical significance is assumed when P < 0.05

<sup>c</sup>Modified Scarff-Bloom-Richardson grading system

<sup>d</sup>FIGO staging system

<sup>e</sup>Her2/neu status was determined through IHC: 0=negative (no staining present), 1+=negative (Weak/barely perceptible membrane staining), 2+=Borderline results/case referred for FISH analysis (weak to moderate complete membrane staining), 3+=positive (strong complete membrane staining)
Table 7: Correlation analysis between the proportion of Mi-ERα positively-stained cells in breast carcinoma and clinicopathological parameters

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>MIER1 Expression: a Tumor Proportional Score vs. Normal Proportional Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Less than Normal</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>5 (16.1)</td>
</tr>
<tr>
<td>&gt;=50</td>
<td>9 (11.4)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;=44</td>
<td>4 (20.0)</td>
</tr>
<tr>
<td>45-64</td>
<td>6 (11.3)</td>
</tr>
<tr>
<td>&gt;=65</td>
<td>4 (10.8)</td>
</tr>
<tr>
<td>Tumor Size (TNM)</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>6 (10.2)</td>
</tr>
<tr>
<td>T2</td>
<td>6 (14.3)</td>
</tr>
<tr>
<td>T3 and T4</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>Tumor Size (Categorical)</td>
<td></td>
</tr>
<tr>
<td>&lt;=2 cm</td>
<td>6 (10.1)</td>
</tr>
<tr>
<td>&gt;2 cm</td>
<td>8 (15.7)</td>
</tr>
<tr>
<td>Lymph Node Involvement</td>
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</tr>
<tr>
<td>No</td>
<td>4 (9.3)</td>
</tr>
<tr>
<td>Yes</td>
<td>5 (15.6)</td>
</tr>
<tr>
<td>Lymph Node Status (TNM)</td>
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</tr>
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<td>NO</td>
<td>4 (9.3)</td>
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<tr>
<td>N1</td>
<td>4 (16.0)</td>
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<tr>
<td>N2</td>
<td>1 (16.7)</td>
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<tr>
<td>N3</td>
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<td>Grade c</td>
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<td>6 (20.0)</td>
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<tr>
<td>2</td>
<td>5 (11.9)</td>
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<tr>
<td>3</td>
<td>3 (8.1)</td>
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<td>Stage d</td>
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<td>I</td>
<td>1 (4.0)</td>
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<tr>
<td>II</td>
<td>5 (12.8)</td>
</tr>
<tr>
<td>III</td>
<td>3 (27.2)</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
</tr>
<tr>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>3 (18.7)</td>
</tr>
<tr>
<td>Positive</td>
<td>9 (10.1)</td>
</tr>
</tbody>
</table>
Table 7 continued: Correlation analysis between the proportion of MI-ERα positively-stained cells in breast carcinoma and clinicopathological parameters

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Less than Normal</th>
<th>Equal to Normal</th>
<th>Greater than Normal</th>
<th>Total</th>
<th>P-value</th>
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<td>PR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>5 (17.9)</td>
<td>7 (25.0)</td>
<td>16 (57.1)</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>7 (9.2)</td>
<td>29 (38.2)</td>
<td>40 (52.6)</td>
<td>76</td>
<td>P=0.093</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>8 (10.8)</td>
<td>27 (36.5)</td>
<td>39 (52.7)</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>2 (13.3)</td>
<td>3 (20.0)</td>
<td>10 (66.7)</td>
<td>15</td>
<td>P=0.768</td>
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</table>

Values are listed as number of subjects, with percentage listed in brackets.

b P-value; Chi-square analysis (χ²) (Pearson’s test, two-tailed, 95% CI), statistical significance is assumed when P < 0.05

c Modified Scarff-Bloom-Richardson grading system

d FIGO staging system

*Her2/neu status was determined through IHC: 0=negative (no staining present), 1+=negative (Weak/barely perceptible membrane staining), 2+=Borderline results/case referred for FISH analysis (weak to moderate complete membrane staining), 3+=positive (strong complete membrane staining)
3.4 Number Cases used from the TMAs

Due to the inherent nature of TMAs, some cases could not be assessed. Cores missing from the TMA upon purchase and cores lifting off the slide during staining (more specifically during antigen retrieval) are reasons why some cases could not be evaluated. Cores which contained an insufficient amount of relevant tissue were also excluded from the assessment. Table 8 illustrates the categorical breakdown of the number cases used in this study.
Table 8  
Categorical breakdown of the number of cases used in this study: TMAs, WTS of breast tumor, and WTS of normal breast

<table>
<thead>
<tr>
<th>CATEGORY</th>
<th>TMAs</th>
<th>WTS:</th>
<th>WTS:</th>
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<tr>
<td></td>
<td>PURCHASED</td>
<td>ASSESSED</td>
<td>BREAST TUMOR CASES</td>
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<tr>
<td>NORMAL</td>
<td>204</td>
<td>180</td>
<td>85</td>
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<tr>
<td>HYPERPLASIA</td>
<td>91</td>
<td>81</td>
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<tr>
<td>DCIS</td>
<td>78</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>ILC</td>
<td>102</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>IDC</td>
<td>343</td>
<td>325</td>
<td>85</td>
</tr>
<tr>
<td>LN METASTASES (IDC TUMORS)</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

**Totals**

- NORMAL CASES ASSESSED = 311
- IDC CASES ASSESSED = 410

**Matched**

(TUMOR AND ADJACENT NORMAL) = 143 (58 TMA + 85 WTS)
3.5 MI-ER1α Expression Pattern in Normal Breast and Invasive Ductal Carcinoma Tissue

The initial part of this study involved assessing the expression pattern of MI-ER1α in normal and tumor breast tissue. In normal breast tissue, MI-ER1α was predominantly observed in the ductal epithelial cells (Figure 26). Staining within the flattened, discontinuous myoepithelial cell layer, which surrounds the ductal epithelial cells, was difficult to discern. However, those few which could be assessed were either negative or had weak, focal cytoplasmic staining (Figure 26D). Also notable, was the absence, or weak cytoplasmic expression, of MI-ER1α expression within the vascular endothelial cells (inset of Figure 26B) and the stromal cells. MI-ER1α expression pattern within the invasive ductal carcinomas was similar to that of normal breast tissue. In that, staining was observed within the tumor cells (derived from ductal epithelium), whereas expression in stromal cells was either absent or weak (Figure 26 G-L).

The intensity level of MI-ER1α expression within both the epithelial cells of the normal ducts and within the neoplastic cells of the breast tumor was variable. In that, the intensity level in the normal epithelial cells ranged from absent to weak, moderate or strong with no one prominent level; this observation was also noted for the breast tumor cells.

It was also recognized when comparing the staining intensity level of that in the normal ducts to that of the lobules, within a single specimen, that there was no observable difference between the two (Figure 27). The intensity of staining observed within the ducts (Figure 27A, C, E) is indistinguishable from that observed within the lobule (Figure 27B, D, F). The intensity level, in both the nuclei and the cytoplasm, was either negative
or a range between mild to strong. Indicating that microscopically, MI-ER1α appears to be equally expressed in the lobule and the ducts of normal breast tissue.
Figure 26  Expression pattern of MI-ER1α in normal breast tissue and invasive ductal carcinoma tissue

A-C, low magnification (4X):
Representative examples of MI-ER1α expression pattern within normal breast tissue; it is observed in the ductal epithelial cells with little or no expression in the stromal cells. The inset in B shows a higher magnification (40X) of the blood vessel, indicated by the long arrow, which demonstrates the weak staining observed within majority of the endothelial cells (arrowhead).

D-F, High Magnification (40X):
Examples of normal ducts illustrating both nuclear MI-ER1α staining (arrows) and negative nuclei (arrowheads). A myoepithelial cell which has positive cytoplasmic staining and negative nuclei is marked by an M. A stromal cell which is negative for MI-ER1α is indicated by an S.

G-I, low magnification (4X):
Representative examples of primary invasive ductal carcinoma.

J-L, High Magnification (40X):
Invasive ductal carcinoma samples exemplifying the exclusive cytoplasmic expression of MI-ER1α (brown staining) and negative nuclei (blue stain).

The scale bar for A-C and G-I is 250μm and 25μm for D-F, J-L and inset in H.
Low to high magnifications of a representative sample of normal breast tissue stained with the anti-MIER1-α antibody. Staining within breast lobules is illustrated panels B, D and F whereas panels A, C and E represent staining of breast ducts from the same sample. Note the presence of both positively stained nuclei (arrows) and negatively stained nuclei (arrowheads) within the lobule and the duct. The strong staining intensity within the nucleus is evident and the cytoplasm is moderately stained. The scale bar is 100μm for A and B (25X), 50μm for C and D (50X) and 25μm for E and F (100X).
3.6 Shift in Subcellular Location of MI-ER1α from the Nucleus to the Cytoplasm

3.6.1. Subcellular Localization of MI-ER1α is Predominantly Nuclear in Normal Breast Tissue, but Cytoplasmic within IDC

Samples of both WTSs and TMAs were analyzed for the subcellular localization of MI-ER1α in matched cases. Matched cases are samples of breast tumor that also contain adjacent normal breast epithelium. Eighty-five WTSs of matched IDC and adjacent normal breast tissue were used in the Allred Scoring assessment. Fifty-eight cases of matched tumor and adjacent normal breast from the TMAs were also used in this assessment.

In this analysis, the result of each case was assigned to one of three following categories; those having nuclear staining only, cytoplasmic only or those which have both nuclear and cytoplasmic staining. It was noted upon review of these results that all of the positively stained samples had some cytoplasmic staining and as such the categories were reduced to 2; those which had nuclear staining and those that did not.

When comparing the results from cases of normal breast to that of IDC, the results were conspicuous. In that, a marked differential existed for the percent of nuclear staining observed within the normal compared to that of IDC; 72.3% versus 4.4%, respectively ($P<0.0001$; Figure 28A). The majority of the normal samples had both nuclear and cytoplasmic staining (Figure 29A, 29C and 29E and Figure 26D-F), whereas majority of the IDC cases had mainly cytoplasmic positivity (Figure 29B, 29D, 29F and Figure 26J-L). There was also a difference in the proportion of positive nuclei within a sample of normal breast tissue compared to that of IDC; greater than 50% of the nuclei
within normal breast cells were positive, however, IDC cells had less than 10% of positively stained nuclei.

Correlations with clinicopathological parameters could not be carried out as there were not enough cases within each category for statistical analysis. For example, for tumor grade the majority of cases fell into the moderately differentiated category, and again a 2cm - 5cm tumor size (T2) was most prevalent among all cases.
Panel A provides evidence that the percent of cases having positive nuclear staining decreases in the progression from normal to non-infiltrating breast abnormalities (hyperplasia and DCIS) to an invasive phenotype such as IDC. The percentage of DCIS samples having nuclear staining lies intermediate to that of the normal and IDC cases.

Panels B and C are cases of matched IDC and adjacent normal tissue from TMAs and WTSs, respectively.

Data gathered from assessment of WTSs of breast carcinoma (containing adjacent normal breast tissue), WTSs of normal breast (no history of breast carcinoma) and TMAs. The percentage of each sample expressing nuclear staining is shown along with the 95% confidence intervals. The number of samples for each category is shown above each bar. A ** indicates that the $P \leq 0.0001$. 

Figure 28  A shift in the localization of MI-ER1α from the nucleus to the cytoplasm in the progression from normal breast and pre-invasive breast sub-types to breast carcinoma
Figure 29 Localization of MI-ER1α is predominantly nuclear in normal breast and cytoplasmic in breast carcinoma

An example of the staining pattern observed within matched cases of IDC and adjacent normal breast tissue. Panels A, C and E illustrate the positive nuclear staining in adjacent normal breast from a case of IDC (panels B, D and F) where the expression of MI-ER1α is exclusively cytoplasmic. Negative and positive nuclei are indicated by arrows and arrowheads, respectively. The scale bar for A and B (25X) is 100μm, 50μm for C and D (50X), and 25μm for E and F (100X).
Infiltrating lobular carcinoma also had a significantly marked reduction in the percent of cases with nuclear staining, 25.3%, when compared to that of normal breast tissue (72.3%, \( P < 0.0001 \), Figure 28A). More specifically, staining in ILC cells was primarily cytoplasmic (Figure 30A-C) and the staining intensity within these samples was similarly to that for IDC, either negative or within the range of weak to strong.

When comparing nuclear positivity within the normal to that of IDC cases having LN metastases, the results were similar to that observed when normal breast was compared to IDC cases without metastases. Only 6.3% of IDC cases (with LN metastases) had nuclear staining; which was statistically significant from that of the 72.3% of normal breast cases which had nuclear staining (\( P < 0.0001 \), Figure 28A, Figure 30D-F). The percentage of IDC cases, with LN metastases, having nuclear staining was similar to that of IDC without LN metastases (\( P = 0.5009 \), Figure 26A).
Figure 30  Expression of MI-ER1α in cases of ILC and ILC with LN metastases

Illustrated in panels A-C are cases of ILC, in which the majority of the cases have cytoplasmic MI-ER1α expression. Panels D-F are cases of IDC that have LN metastases; localization of MI-ER1α is predominantly cytoplasmic which is similar to that of IDC without LN metastases. The single-filing of cells, a classic pathological identifier for ILC, is marked by arrows in panels B and C. Negative nuclei are indicated by arrowheads. Scale bar is 100µm for panels A and B (25X), 50µm for B and E (50X) and 25µm for C and F (100X).
3.6.3 Percentage of DCIS Cases having Nuclear Staining falls between that of the Normal Breast and that for IDC

Ductal Carcinoma In Situ, a non-infiltrating breast lesion, has the potential to develop into or recur as an invasive breast carcinoma; a phenomenon which heavily depends on the extent of the lesion and the treatment used to resolve the DCIS (Yen et al., 2005).

Seventy-one cases of DCIS were evaluated for subcellular localization of MI-ER1α. Thirty-six of these cases (50.7%) had nuclear staining; a value which lies intermediate to that of the normal breast (72.3%) and that of the IDC (4.4%, $P = 0.0001$, Figure 28A). The staining intensity of DCIS cases ranged from weak to strong, with majority of cases displaying a strong expression of MI-ER1α. As illustrated in Figure 31, MI-ER1α localization scores for all DCIS cases comprised of an equal distribution of both categories: approximately 50% had exclusive cytoplasmic staining (Figure 31A-C) with the remaining cases having both nuclear and cytoplasmic staining (Figure 31D-F).

To determine if clinicopathological parameters, namely lesion size or nuclear grade, correlates with DCIS cases which have nuclear staining and those that lack nuclear staining, a correlation analysis was attempted. Unfortunately, this could not be carried out as the clinical data was either unavailable (lesion size) or there was not enough cases in each category within a parameter such as grade. For the TMA DCIS cases, 21 cases were nuclear grade II, 9 cases were nuclear grade I, 9 cases were nuclear grade III; for the remaining cases of DCIS the grade was not available.

Four cases of comedo DCIS, a specific subtype of DCIS, were also stained with the anti-MI-ER1α antibody. While the case number is too low to infer any
significant conclusions, it was noteworthy that all four cases presented with only cytoplasmic expression of MI-ER1α. A representative case of this result is illustrated in Figure 31 G-I.

Four cases of LCIS were also included in the TMAs and stained with the anti-MIER1α antibody, however no one category of localization pre-dominated. In that, 2 cases had cytoplasmic only staining and 2 cases had nuclear and cytoplasmic staining.
Figure 31 Localization of MI-ER1α in DCIS and comedo-DCIS

The percent of cases having nuclear MI-ER1α in DCIS falls in between that for normal and IDC; approximately half the cases have nuclear positivity. Panels A-C shows a representative case lacking nuclear MI-ER1α and panels D-F shows a representative case where MI-ER1α is expressed within the nucleus. Exclusive cytoplasmic localization of MI-ER1α in a case of comedo-DCIS is illustrated in panels G-I. Positive and negative nuclei are indicated by arrows and arrowheads, respectively. In panel G, “Necrosis” indicates an area of central necrosis, a typical feature of comedo-type DCIS. In addition, microcalcifications are also observed microscopically in comedo-DCIS and such areas are indicated in panel H with an “M”. Scale bar is 100μm for panels A, D and G (25X), 50μm for panels B, E and H (50X) and 25μm for panels C, F and I (100X).
Subcellular Localization of MI-ER1α in Breast Hyperplasia Cases is Strikingly Similar to that of Normal Breast Samples

Eighty-one cases of non-atypical hyperplasia (atypical having a more abnormal pattern of growth) were stained with the anti-MI-ER1α antibody and 62 (76.5%) of these cases had nuclear expression, a result similar to that of normal breast tissue ($P = 0.8831$,
Figure 28A and Figure 32A-D).

Staining intensity, as in normal breast tissue, was negative, weak, moderate or strong, with majority of the cases having a moderate expression level (Figure 32).

Also noteworthy was that 8 of the 10 atypical hyperplasia cases from one of the TMAs had cytoplasmic staining; the other two cases could not be assessed.
Figure 32 Localization of MI-ER1α in cases of breast hyperplasia is predominantly nuclear

Two representative examples of breast hyperplasia demonstrating nuclear and cytoplasmic MI-ER1α expression. Positive and negative nuclei are marked by arrows and arrowheads, respectively. Asterisks in panels A and D indicate areas which have an increase in the number of abnormal cells; a trait which is used to identify breast hyperplasia. The scale bar is 50μm for panels A and B (50X) and 25μm for D and F (100X).
3.6.5 A Differential in Subcellular Localization exists between Matched Cases of IDC and Adjacent Normal Breast Tissue

To establish that the difference in the subcellular localization of MI-ER1α between normal breast tissue and IDC was not due to individual patient differences, nuclear staining in IDC cases was compared with that of the adjacent normal breast tissue (matched cases). Eighty-five WTSs of IDC cases (Figure 28C), having adjacent normal breast tissue, as well as 58 TMA cases of IDC (Figure 28B) with matched cores containing adjacent normal breast tissue were examined. Matched cases of WTSs and TMAs give results similar to that for the unmatched cases described in Section 3.5.1. In that, the majority of the breast tumors lacked nuclear staining (WTSs: 5.9%, TMAs: 12.1%), whereas the adjacent normal tissue displayed nuclear staining (WTSs: 85.9%, TMAs: 74.1%. Figure 28B and 28C, Figure 27).
4. **DISCUSSION**

This study was done to assess the pattern of expression, the level of expression and the subcellular localization of MIER1α, a novel ER co-regulator, in both normal breast tissue and breast cancer tissue.

Previous studies have demonstrated that MI-ER1α has the capacity to act both as a co-repressor and a co-activator (Ding et al., 2003; Paterno et al., 1997; Paterno et al., 1998). Recently, we demonstrated, that MI-ER1α interacts with ERα *in vivo* (McCarthy et al., 2008). It was also shown that induced expression of MI-ER1α, in a T47D breast carcinoma cell line, leads to a reduction of anchorage independent growth (McCarthy et al., 2008). Together, these results infer that MI-ER1α plays a role in ER-dependent cell proliferation. The latter 2 sets of results are illustrated in Appendix B.

4.1 **Allred Assessment Revealed that there is No Significant Correlation Between MI-ER1α Expression and Mammary Clinicopathological Parameters**

The Allred Scoring System was used to determine if a relationship existed between the level of MI-ER1α and clinicopathological parameters. No one category, tumor score less than normal, greater than normal or equal to normal, dominated and neither of which correlated with clinicopathological parameters. A study reviewing the same parameters but with a larger sample size, with the hope of obtaining an even distribution of stage I-IV cases as well as more Her2/neu positive cases, is warranted since the probability associated with each of these parameters approached significance at $P = 0.082$ and $P = 0.086$, respectively.
4.2 Proteins that have a Shift in its Subcellular Localization may Contribute to the Progression of Breast Carcinoma and Impact the Therapeutic Response

A decrease in the number of cases that have nuclear MI-ER1α expression, as one compares the pre-invasive types to the invasive carcinomas, demonstrates its potential role in the progression of an invasive breast carcinoma.

While the literature demonstrates opposing views, some researchers still believe that normal cells can advance to an invasive carcinoma sequentially; simplistically, they can become hyperplastic, then develop into a DCIS/LCIS lesion and finally an invasive carcinoma. A model proposed from the results of this study, in conjunction with work carried out by M. Savicky and C. Mercer (Appendix B), is that during the progression from a normal phenotype to DCIS and then to IDC, MI-ER1α is shuttled from the nucleus to the cytoplasm. This shift in localization is what prevents MI-ER1α from exerting its genomic effects through its interaction with the ERα. However, it is also possible that this shift allows for the cytoplasmic interaction between MI-ER1α and the ERα where it acts as a co-activator to enhance ERα non-genomic activities, such as cell growth stimulation through signal transduction pathways, as seen with MTA1 (Kumar, Wang, Mazumdar, Talukder, Mandal, Yang, et al., 2002)

There are many examples whereby a shift in the localization of a protein can be associated with the development of a carcinoma, as well as the tumor's clinical response to therapy. For example, an increase in nuclear YB-1 is associated with up-regulation of the P-glycoprotein; a protein known to increase the likelihood of chemotherapy resistance in many cases of breast carcinoma (Fujita et al., 2005). From this, one would infer that
nuclear YB-1 connotes a poor prognosis for women diagnosed with breast carcinoma and for whom chemotherapy is a part of their treatment regime. Again, in another study using mammalian cancer cell lines to investigate the role of Apoptin (produced by the VP3 gene of the chicken anemia virus and known to induce cell death) in cancer development found that it was held in the cytoplasm and therefore could not exert its apoptotic effects. Casteel, et al. (2008) found that PKGs (Type I cGMPs-dependent protein kinases) which translocates to the nucleus to regulate gene expression are found to be anchored by the binding of a specific protein (inositol 1,4,5-triphosphate (IP(3)) receptor-associated cGMP kinase substrate (IRAG)) and hence retained within the cytoplasm; an event that prevents PKGs ability of exerting its transactivational influences. Metastatic tumor antigen 1 (MTA1) is a protein whose expression is associated with an increased risk for development of invasive carcinomas and metastases (Nawa, Nishimori, Lin, Maki, Moue, Sawada, Toh, Fumitaka, & Nicolson, 2000). Kumar et al. (2002) found that metastatic tumor antigen 1 (MTA1), which localizes to the cytoplasm, anchors ERα in the cytoplasm where it can enhance the non-genomic effects of ERα. In this study it was shown that when a specific portion of the MTA1 gene was deleted, there was loss of its co-repressor activity, its interaction with ERα and is associated with restoration of nuclear ERα expression (Kumar et al., 2002).

From this, one might suggest that MI-ERα becomes exported from the nucleus or anchored in the cytoplasm, where it can no longer act as a co-repressor or ER-dependent proliferation, as it progresses from normal an invasive breast phenotype. However, it is also possible that MI-ER1α is sequestered in the cytoplasm during the
progression to an invasive carcinoma, where it acts to promote the proliferative activities of ERα.

4.3 Absence of a Differential in the Expression Levels of MI-ER1α Between Normal and Tumor

There was no differential in the level of expression of MI-ER1α between normal and tumor. Some cases had an equal expression of MI-ER1α in the tumor and adjacent normal, while others either had higher or lower expression in the tumor versus normal. In a previous study by Paterno et al. (1998) using PCR mRNA expression analysis, it was shown that *mi-er1* was expressed at higher levels in breast tumor tissue than that of normal breast tissue. This inconsistency can be explained through the fact that its expression in normal breast tissue if limited to ductal epithelial cells, but in a tumor it is expressed in every cell. Therefore, the number of cells expressing *mi-er1* mRNA in the normal sample would be expected to be lower when compared to the same volume of tumor.

4.4 MI-ER1α Localization in DCIS Cases may be a Determinant of *in situ* Recurrence and or Progression to an Invasive Carcinoma

In this study, the results of the DCIS subcellular localization analysis revealed that only half of the cases lack nuclear MI-ER1α expression. However, majority of the IDC cases had loss of nuclear MI-ER1α. Not all cases of DCIS, following treatment, have an *in situ* recurrence or progress to an invasive carcinoma. However, those that have DCIS are at an increased risk for such. Taken together, these raise important questions
regarding the potential role of MI-ER1α in the recurrence of DCIS and/or if it promotes the progression to IDC.

One of the things that a pathologist takes into account when determining the possible risk of recurrence for a patient having been diagnosed with DCIS is the margin of resection. This margin is defined as the distance between the perimeter of the removed lesion and the normal area that was removed. Vargas, et al. (2005) (2008) found that individuals whose lesion’s margin of resection were less than or equal to 2mm (which are considered “close” or “positive” margins), or if the lesion was of high grade (poorly differentiated), there is a higher risk for breast cancer. Rashtian, et al. (2008) also found that small tumor margins and high grade lesions were associated with higher risk for local recurrence. In addition, they found that comedo DCIS lesions which have a central necrotic area increase the risk of local recurrence. Another recent study by Korourian, et al. (2008), determined the tumor-associated carbohydrate antigen (TACA) expression and correlated it with things such as tumor size, nuclear grade and ER and PR status. They found a significant correlation between TACA expression and high nuclear grade. From this, they predicted that TACA might contribute to the development of a higher-grade DCIS lesion and disease recurrence. One other study, using immunohistochemical analysis, found that expression of COX-2 and PPARγ in DCIS lesions have opposite associations with recurrence. COX-2 is an enzyme involved in prostaglandin synthesis; prostaglandins are fatty-acid derivatives that contribute to inflammation and immune response effects. PPARγs play a role in cell differentiation, inflammation and lipid metabolism. High expression of COX-2 was found to be associated with recurrence, whereas nuclear PPARγ expression in DCIS lesions appeared to have a protective effect.
This, together with the data from this study, one might predict that a specific pattern of MI-ER1α subcellular localization within a DCIS lesion might contribute to and/or be prognostic for recurrence of an in situ carcinoma or an invasive phenotype.

Although there are no clear tests that distinguish which cases of DCIS are at an increased risk for developing a recurrence, there is a specific DCIS subtype that imposes a greater malignant potential than others, namely, comedo DCIS. This type of DCIS has a specific histological feature that distinguishes it from others, and that is the presence of a central necrotic area. One study proposes that this necrotic area arises as a result of apoptosis due to the influence of both environmental and internal genetic insults. These apoptotic events permits elimination of both myoepithelial cells and luminal cells and allows for expansion of the apoptotic-resistant cancer cells (Shekhar, et al., 2008).

This study had four cases of comedo-DCIS; all of which had loss of nuclear MI-ER1α. In relating this result to the above information, it is possible that loss of nuclear MI-ER1α might be one such internal alteration which influences a comedo DCIS cell to undergo apoptosis.

4.5 IHC and TMA limitations

Immunohistochemistry is an effective tool for evaluating the location and level of expression of proteins and one which has long been employed in both laboratory research and in clinical settings. However, this technique brings with it some limitations that need to be considered. One artifact that is common after performing IHC is the uneven staining of samples which is often due to poor fixation and hence non-uniform
antigen retrieval. In this study, attempts were made to control for this through exclusion of samples that were not appropriately fixed.

The other issue is that maximum quantitative evaluation achieved through IHC is semi-quantitative and hence to quantitatively assess the protein, other assays such as those which combine digital imaging with fluorescence microscopy would need to be used. Fluorescent immunohistochemistry can be utilized to quantify light emitted from each cell within a paraffin tissue section.

With regard to staining of TMAs, it is essential to use a large sample size and/or compare the results to a few cases of whole tissue sections. Tumors are heterogeneous in nature and for this reason when taking a small core from a larger sample, any results from such may not be representative of what is going on in the whole tissue. In this study, a large number of whole tissue sections were utilized and analysis was compared to that of the TMAs.

4.6 Future Directions

A complete understanding of the mechanism ER-stimulated cancer cell growth is essential to the development of better treatment regimes for breast cancer, prognostic indicators and ideally the development of early detection/screening markers.

This study has identified that MI-ER1α does indeed play a role in this pathway. It has also established that one of the functional implications of an interaction between MI-ER1α and the ERα is a reduction in ERα-mediated cancer growth. However, the mechanistic details of how this occurs should be investigated. Further to this, it would be
particularly important to determine how it is that MI-ER1α is either held in the cytoplasm or shuttled from the nucleus.

Initially, it would be advantageous to use a protein microarray, which contain hundreds or thousands of human proteins fixed to an array. To this array one would apply the antigen of interest (MI-ER1α) followed by a detection method such as fluorescence. Positive staining or fluorescence will indicate a protein-protein interaction involving the antigen of interest. Following this, one would review the properties of the other protein, to which the antigen of interest binds, and determine where it might be expressed (its localization) and what its function is; all of which could provide evidence as to the role MI-ER1α plays in ERα-mediated breast cell growth as well as its mechanism of localization. If it is determined that MI-ER1α interacts with a cytoplasmic protein, then RNA interference can be utilized to see the functional effects of blocking this interaction.

In this study there were 4 cases of LCIS, 2 cases had nuclear and cytoplasmic staining and the other two had only cytoplasmic staining. While this number is low, it warrants further investigating through staining of a larger sample size to see if this pattern continues and is therefore similar to that of DCIS whereby approximately 50% of the samples lacked positive nuclear staining.

To reiterate what was stated in the discussion, while 50% of the DCIS cases lacked nuclear staining, almost all of the IDC cases displayed loss of nuclear MI-ER1α (95.6%). This is of particular importance since having DCIS is not a predetermining factor for development of IDC and currently there are no tests that can predict the outcome for patients with DCIS (Wiechmann & Kuerer, 2008). Therefore, it would be
important to conduct a study using a larger sample size of DCIS cases and determine if MI-ER1α is a prognostic indicator. For this, one would obtain cases that had been diagnosed with DCIS approximately 15-20 years prior. These cases can be stained with anti-MI-ERα1 and localization results can be cross referenced with the categories of those who did develop IDC and those who did not to see if there is a correlation. Another possibility is to do a prospective study using a sample of patients who have had surgical removal of DCIS lesions, then staining these samples with anti-MI-ER1α, following them for a period of time to see if they develop IDC to determine if there is a correlation. The goal is to determine if there is a specific MI-ER1α staining pattern within DCIS lesions that can accurately predict one's risk for development of an invasive breast carcinoma. Using the same set of cases it would be important to determine if there is a correlation between the loss of nuclear MI-ER1α and 5 and 10-year survival time points.

The advantage of using a larger sample size of invasive lobular carcinomas would be to see if there is a correlation between the staining pattern of MI-ER1α in this carcinoma and clinicopathological parameters. In particular, it would be interesting to compare the expression pattern of MI-ER1α in both ER positive and ER negative breast tumors. It would also be useful to conduct a study, looking at the expression pattern of MIER1α in special types of breast carcinoma such as Paget's disease and inflammatory breast cancer. In the current study there were not enough cases for each category of DCIS grade and hence a statistical analysis could not be carried out. Therefore, future studies should include DCIS cases for which the grade of the lesion is available.

Ki-67 is often included in the pathological workup following removal of a breast lesion. It is a protein whose expression correlates with the mitotic index. A higher
mitotic index is associated with a higher neoplastic proliferation rate and hence a higher possibility of developing an invasive or metastatic carcinoma. Ki-67 is one of the 21 genes included in the Oncotype DX test that is utilized throughout the US and in some areas of Canada. The Oncotype DX test enables physicians and the patient to learn more about the biological activity of their breast tumor. More specifically, it is a test that assesses the expression of 16 genes known to be associated with breast cancer, as well as 5 control genes. The results of the test are reported as a quantitative recurrence score; a number between 0 and 100. The recurrence score allows physicians to predict both the risk of recurrence in node-negative, ER positive, early stage (I or II) invasive breast cancer patients, as well as their response to chemotherapy.

IGFBP-rP1 is a protein whose expression has been previously shown to be associated with progression from an in situ lesion to IDC (Wiechmann & Kuerer, 2008). Correlating the loss of nuclear MI-ER1α with either Ki-67 or IGFBP-rP1 may provide insight into the role of MI-ER1α in the development of IDC.

To date, the molecular mechanisms involved in the progression of comedo DCIS cells to that which is invasive, are not yet known. Comedo DCIS is a more aggressive form of DCIS (Maxhimer et al., 2005). In this study there were 4 such cases and all of which had loss of nuclear MI-ER1α. Therefore staining a larger sample size of various DCIS subtypes to see if the loss of MI-ER1α is associated with more aggressive DCIS subtypes.
4.7 Conclusions

Evidence that MI-ER1α functions as an ERα co-repressor was provided through experiments demonstrating the ability of MI-ER1α to physically interact with the ERα and that its overexpression results in a decrease of E₂-stimulated breast cancer cell growth (McCarthy et al., 2008).

Estrogen receptor-stimulated breast cell growth requires interaction between the ERα and a number of co-regulatory molecules; such molecules have been the target of many therapeutic drugs designed to control the tumor growth as well as those aimed at preventing tumor recurrences. Several ERα co-regulatory molecules have been identified, some of which have been confirmed to act as co-repressors of ERα activity (Baniahmad, 2005; Hall & McDonnell, 2005). These include NCoR and SMRT, both of which are ligand-independent co-repressors, as well as MTA1-3 (J. D. Chen & Evans, 1995; Horlein et al., 1995; Manavathi & Kumar, 2007).

The results of this study confirm that a shift of MI-ER1α from the nucleus to the cytoplasm is associated with the progression to an invasive lobular or ductal breast carcinoma. Hence, a loss of nuclear MI-ER1α may be a contributing factor in the development of an invasive breast carcinoma. In this regard, MI-ER1α represents a promising breast cancer prognostic marker and possibly a therapeutic target.
APPENDIX A:

Description of Non-proliferative Breast Changes and Proliferative Diseases with Atypia
**Non-proliferative Breast Changes**

These group of breast changes are not associated with an increased risk for the development of breast carcinoma. Complex fibroadenomas, which is associated with an increased risk of future breast cancer development, was included under this section so that it could be simultaneously compared to the non-complex fibroadenomas. Also, included in this section are moderate and florid hyperplasia which are associated with an increased risk for developing breast cancer. Again, these are included in this section to allow for a simultaneous comparison to that of mild hyperplasia.

**A: Duct ectasia** is usually present in post-menopausal women and is characterized by a dilation of the larger ducts. The dilated ducts are marked by the presence of inspissated debris that is both acellular and contains evidence of an inflammatory reaction. Due to the presence of fibrosis around the ducts, a palpable mass is sometimes detected and mistaken for breast cancer.

**B: Cysts** can develop through the dilation of acini, which are lined by a flattened epithelium or cuboidal cell layer and vary in size, shape and number present. As with duct ectasia, it is not associated with an increased risk for breast cancer (Table 1).

**C: Apocrine change**, another non-proliferative condition refers to cysts that are lined with columnar epithelium that resembles that of normal apocrine sweat glands. These cells also contain an eosinophilic ("pink-staining") cytoplasm.

**D: Adenosis** is another non-proliferative breast condition that is not associated with an increased risk in breast cancer development. It is an enlargement of lobules as a result of an increase in the number of acini, however the structure of lobular unit persists.
E: Fibroadenomas are classified into two groups: Fibroadenomas without complex features and Fibroadenomas with complex features. Those that present without complex features, are non-proliferative and not associated with an increased risk of developing breast cancer. Secondly, are those that are in direct contrast to the latter—present with complex features, are classified as proliferative without atypia and are associated with an increased risk of breast carcinoma. Fibroadenomas are benign lesions that arise from both the stroma and the glandular epithelium. Histologically, the ducts appear as elongated and thinned which is surrounded by an expanded stroma and does not have any clinicopathological characteristics that increase the risk of developing breast cancer. However, those that are associated with complex features, such as cysts that are larger than 0.3cm, sclerosing adenosis (discussed in the next section), calcifications, papillary apocrine changes are associated with an increased the risk for developing breast carcinoma 1.5-2.0 fold (Lester, 2005).

Proliferative Disease with Atypia

A: Fibroadenoma with complex features—discussed in previous section

B: Moderate and florid hyperplasia—discussed in previous section

C: Sclerosing adenosis is an extension of adenosis mentioned in the previous section. In addition to the increase in the size of the lobular unit, through an increase in the number of acini, sclerosing adenosis arises when these acini are compressed and distorted in the central region of the lesion, but often dilated at the perimeter.

D: Complex sclerosing lesions, often referred to as radial scars if greater than 10mm, focal lesions that consists of a central region with small ducts embedded in a dense
fibrous stroma. Radiating from this central region are epithelial projections ("arms") which contain cysts and hyperplastic cells. These lesions may be mistaken for an invasive breast carcinoma on mammograms and histologic examination (Kennedy, Masterson, Kerin, & Flanagan, 2003; Lester, 2005).

**E:** **Papillomas** can exist as a solitary growth within a large dilated duct, usually within the lactiferous sinus, or as multiple small papillomas that usually arise in the posterior regions of the ductal system. Papillomas are branched papillae (small projections) structures with fibrovascular cores, that are lined by both epithelial and myoepithelial cells. They can exist alone or co-exist with atypical ductal/lobular hyperplasia (discussed in following section). If there are multiple papillomas, which may or may not co-exist with ADH or ALH, present there is a significantly higher risk for breast cancer development than when a single papilloma is associated with ADH or ALH (Lewis et al., 2006)
APPENDIX B:

Extra Figures from McCarthy et. al. (2008)
Figure 1 MI-ER1α interacts with ERα in vivo.

(A) HEK293 cells were transfected with pcDNA3-herα and either pCS3+MT-mier1α (lanes 3 and 4) or control empty vector (lanes 1 and 2) and treated with vehicle (lanes 1 and 3) or 10 nM E2 (lanes 2 and 4) for 3 h before extraction. Extracts were subjected to immunoprecipitation with anti-ERα (top panel) or loaded directly onto the gel (middle and bottom panels). Western blots were stained for MI-ER1α (top and middle panels) or ERα (bottom panel). (B) Extracts from MCF-7 cells treated with vehicle (lanes 1, 3 and 5) or 10 nM E2 (lanes 2, 4 and 6) were subjected to IP with anti-mier1α (lanes 1 and 2), pre-immune (lanes 3 and 4) or loaded directly onto the gel (lanes 5 and 6); Western blotting was performed with anti-ERα.

[Work carried out by M. Savicky and published in McCarthy, et. al. (2008)].
Figure 2  Overexpression of MI-ER1α reduces anchorage-independent growth of T47D breast carcinoma cells.

Control (TDc22)- or MI-ER1α (TDa5 and TDa7)-expressing Tet-On T47D clones were cultured in 0.35% agarose, in the presence or absence of 2 mg/ml dox and in the presence or absence of 10 nM E2. Colonies were stained with crystal violet, and colony size was measured using an ocular micrometre. A minimum of six fields from each plate was analysed; the number of colonies larger than 100 mm in size, expressed as a percentage of the total number of colonies, was recorded for each treatment. (A) Representative fields for each treatment combination for each clone are shown. (B) Histogram showing the average values and error bars for three independent experiments, performed in duplicate. (C) Western blot analysis to verify dox-specific induction of MI-ER1α expression. A representative blot of extracts from TDc22, TDa5 and TDa7 cells, cultured in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of 2 mg/ml dox, is shown. The position of MI-ER1α is indicated.

[Work carried out by C. Mercer and published in McCarthy et. al., (2008)]
APPENDIX C:

Tissue and Fixation Processing Procedures: TMAs
Tissue and Fixation Processing Procedures

When fixation is inadequate it can adversely affect both a pathologists' diagnosis and presentation of requested stains. Adequate fixation of a sample is also critical for its subsequent use in future investigations through either a patient's request for a second opinion or consultation work involving other hospitals or laboratory research methods such as immunohistochemistry (Lester, 2005; Parnell, 2008; Rosai & Ackerman, 2004).

TMAs

A Biochain Insitutue Inc.

Normal tissues used in the construction of the TMAs were excised from death donors and tumors were removed during surgery. The tissue was fixed in formalin immediately after harvesting for varying times depending on the tissue, for a maximum of 48 hours. The tissues are then serially dehydrated overnight through various grades of alcohol (50%, 70%, 80%, 90%, 100%), cleared in xylene and then embedded into a paraffin wax.

B US Biomax Inc.

Tissues removed during surgery were immediately fixed in a 10% neutral PBS buffered formalin, while post-mortem tissues are placed in fixative within six hours after removal. Both surgically removed and post-mortem tissues were fixed for 24 hours before placement in a Leica tissue processor. The processing involved dehydration through an alcohol gradient, clearing in xylene and paraffin embedding.
Tissues used in the construction of the TMA were obtained from the University of Virginia Hospital. Tissues were removed during surgery or post-mortem and immediately fixed in zinc formalin (Z-fix, Anateach Ltd.) for 24 hours. The tissues are then processed in a Tissue Tek VIP 2000 (Sakura Finetek USA, Inc.), which involves the same steps as the previously described TMAs: dehydration followed by clearing in xylene and paraffin embedding.
APPENDIX D:

Labeled Streptavidin-Biotin (LSAB) Method
**Labeled Streptavidin-Biotin (LSAB) Method**

This kit relies on the strong binding affinity of Steptavidin for the vitamin biotin. Similar to Avidin, Streptavidin has four binding sites for biotin, but in contrast to avidin it has a more neutral isoelectric point and no carbohydrate moieties. The latter of which permits a reduction in non-specific binding (DakoCytomation, 2006).

Biotinylated secondary antibodies binds easily to primary antibodies and serves as the link between the primary antibody and the strepavidin-peroxidase conjugate. In the end, because of the previously mentioned 4 biotin-binding sites on strepavidin, a single antibody is linked to multiple peroxidase molecules (Appendix C).

Horseradish peroxidase (HRP) activity, isolated from the root of a horseradish plant, in the presence of an electron donor such as 3,3'-diaminobenzidinetetrahydrochloride (DAB), results in an enzyme – substrate complex then oxidation of the electron donor (DakoCytomation, 2006). When DAB becomes oxidized it produces an insoluble brown end product. When performing IHC with the anti-MI-ER1α antibody, it is this brown staining that is indicative for the presence of MI-ER1α.
Labeled Streptavidin-Biotin (LSAB) Method
The primary antibody is applied to the tissue section (Step 1) and is incubated overnight to allow binding to the tissue antigen. A biotinylated anti-mouse/rabbit secondary antibody is applied and attaches to the primary antibody. The biotinylated secondary antibody acts as a link between the primary antibody and the Streptavidin molecule; the latter of which has a high binding affinity for the biotinylated secondary antibody. (DakoCytomation, 2001)
APPENDIX E:

Protocols for making solutions used in IHC
1. **1% BSA/PBS, pH 7.4**
   A powdered form of PBS which contains BSA was ordered from Sigma – Aldrich. Dissolve one sachet in 1L of deionized water; which yields and 1% w/v of BSA and a solution with a pH of 7.4.

2. **Citrate Buffer 10mM**
   Dissolve 1.92g citric acid in 1L deionized water and adjust the pH to 6.0 using 1N NaOH.

3. **PBS, pH 7.4**
   A powdered form of PBS which when one sachet is dissolved in 1L of deionized water yields a 0.01M PBS solution at a pH of 7.4.

4. **Hematoxylin**
   Combine 180g of aluminium ammonium sulfate dissolved in 1200ml water with 12g hematoxylin dissolved in 75ml of 95% alcohol in a transparent glass bottle. Expose the mixture, unstoppered, to light and air for 1 month; filter into a second transparent bottle. Add 300ml glycerin and 300ml 95% alcohol. Allow solution to stand in light until colour is sufficiently dark before filtering into a dark glass bottle. Store for up to one month at room temperature in a dark glass bottle.

5. **Scott’s Tap Water**
   Add 3.5g sodium bicarbonate to 1L of tap water and 20g magnesium sulfate to a second litre of tap water and store separately. Immediately prior to use combine, in a 50:50 ratio, each of the above solutions.

6. **1% Acid Alcohol:**
   To 1L of 70% Alcohol add 10mL of 12N HCl
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