Utilization of Genomics for Risk Assessment and Molecular Subtyping to Improve Treatment Strategy in Breast Cancer

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UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

UTILIZATION OF GENOMICS FOR RISK ASSESSMENT AND MOLECULAR SUBTYPING TO IMPROVE TREATMENT STRATEGY IN BREAST CANCER

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Death from breast cancer began a decline in the 1990’s.¹ One widely accepted reason for this reduction was the discovery that breast cancer can be uniquely defined at the molecular level by oncogenes and tumor suppressor genes including, but not limited to, EGFR, AKT, ErbB2, PI3K, TP53, BRCA1/2, and PTEN. These genes and potential mutations within, can drive constitutive activation of aberrant signaling that can induce and sustain tumorigenesis. More importantly, these genes represent new avenues for possible markers and therapies.

In the wake of this molecular biology discovery, there was an influx of new breast cancer treatments to the marketplace, most notably the launch of trastuzumab
(Herceptin) in 1996. Subsequently, the incidence of death continued to decrease peaking from 2002 to 2003 when it dropped by 7% in that one year alone. However, the current challenge in oncology is how to translate the wealth of information contained in the molecular biology of cancer and translate it into patient care. While progress has been made in developing treatments, less progress has been made identifying new markers for breast cancer to get that treatment to the right patients or selecting them for treatment trials.

Currently, the majority of providers and healthcare systems do not utilize molecular biology to determine risk of breast cancer recurrence and treatment decision making for breast cancer. Rather, they determine which patients require what adjuvant treatment based on some form of the breast cancer tumor-node-metastases (TNM) staging system and clinical-pathological criteria, such as lymphovascular invasion (LVI), nodal status, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2/neu) status and more. As a result, potential differences exist worldwide in the selection of patients who require adjuvant chemotherapy based on their risk of breast cancer recurrence.
All of these tests and criteria are based on the anatomical extent of the tumor, with little if any insight into the patient’s breast cancer biology. A significant percentage (30 to 50%) of all early stage breast cancer patients (50,000 to 75,000 patients annually in the US alone) have clinically ambiguous or confounding clinical-pathological characteristics, making it difficult for physicians to formulate clinical risk and supports the argument for development and utilization of molecular diagnostic testing.

By looking at the molecular biology of cancer, companies, such as Agendia, seek to create technologies beyond clinical screening, imaging or cell surface staining. Technologies like MammaPrint and BluePrint, reveal who is truly at risk by differentiating responders from non-responders based on genetics. By interrogating single genes or overall patterns of gene expression, it is possible to better understand the various sequences of biological events that give rise to breast cancer and in turn, better develop and guide treatments.
DEDICATION

I would like to dedicate this dissertation to Jill Swain, for your unconditional support, love and faith in me and in us.
ACKNOWLEDGMENTS

There are numerous people that I would like to acknowledge and thank for their support throughout this journey. First, my committee members Drs. Ralf Landgraf, Zafar Nawaz and Thomas K. Harris for their time and mentoring over the years. A special acknowledgment to Dr. Stefan Glück for introducing me the University of Miami and to Dr. William Audeh, for being a mentor, friend and great ambassador of our Agendia family. I would also like to acknowledge Dr. Stephen Lee, Annalise and especially Dr. Sylvia Daunert for her ongoing encouragement, which made this experience that much brighter.

Next thank you to all my lab mates, and colleagues both past and present, from USF, Moffitt, University of Miami and Agendia. I sincerely appreciate all I have learned and look forward to continuing to grow with all of you. I would especially like to express my thanks to all my friends, for the steady stream of smiles over the past several years.

Finally, I would like to acknowledge my family, My Uncle Roger and Aunts Charlotte, Karen and Mary. To my siblings, especially piglet and spouses and my nephews. Last, a big
hug and thank you to my parents and my grandmother. You taught me to “strive for excellence, but be prepared for the ups and downs because the journey is worth it”. Indeed, this has been worth it and I will carry these wonderful lessons with me forever.
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List of Abbreviations

AI: Aromatase Inhibitors
AJCC: American Joint Committee on Cancer
A!O Adjuvant! Online (A!O)
BC: Breast Cancer
CAP: College of American Pathologists
CLIA: Clinical Laboratory Improvement Amendments
DCIS: Ductal Carcinoma In Situ
DDAC+T: Doxorubicin Cyclophosphamide followed by paclitaxel or docetaxel chemotherapy regimen
DDAC+T+H: Dose dense Doxorubicin Cyclophosphamide followed by paclitaxel + trastuzumab chemotherapy regimen
DDAC+T+H+P: Dose dense Doxorubicin Cyclophosphamide followed by docetaxel + trastuzumab + Pertuzumab chemotherapy regimen
DMFI: Distant Metastasis Free Interval
DMFS: Distant Metastasis Free Survival
EBCTCG: Early Breast Cancer Trialists' Collaborative Group
EGF: Epidermal Growth Factor
EGFR: Epidermal Growth Factor Receptor
ER: Estrogen Receptor
FEC100+T: 5-Fluorouracil Epirubicin Cyclophosphamide followed by paclitaxel or docetaxel chemotherapy regimen
FDA:  Food and Drug Administration
FISH:  Fluorescence in situ hybridization
HER2: Human Epidermal Growth Factor Receptor 2
HRT:  Hormone Replacement Therapy
IDC: Invasive ductal carcinoma
ILC Invasive lobular carcinoma
IVDMIA In Vitro Diagnostic Multivariate Index Assays
LDT: Laboratory Developed Test
MAAA: Multianalyte Assays with Algorithmic Analyses
OS: Overall Survival
PR: Progesterone Receptor
PS: Progression free Survival

PTH+DDAC: Pertuzumab Docetaxel Trastuzumab followed by
dose dense Doxorubicin Cyclophosphamide chemotherapy
regimen

PTH+FEC: Pertuzumab Docetaxel Trastuzumab followed by 5-
Fluorouracil Epirubicin Cyclophosphamide chemotherapy
regimen

RT-PCR: Real Time Polymerase Chain Reaction
TAC: Docetaxel Doxorubicin Cyclophosphamide chemotherapy
regimen

TC: Docetaxel Cyclophosphamide chemotherapy regimen

TCH: Docetaxel Carboplatin Trastuzumab chemotherapy
regimen

T+HCEF+H: Paclitaxel + Trastuzumab 1 dose +
Cyclophosphamide Epirubicin 5-Fluorouracil + Trastuzumab
chemotherapy regimen
TNBC: Triple Negative Breast Cancer  
TNM: Tumor Nodal and Metastasis Staging
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Chapter 1: Introduction

In 2017 it is estimated that over 300,000 new cases of breast cancer will be diagnosed. The majority of these cases will be invasive breast cancer and over 40,000 are expected to die from the disease. Although about 5-10% of breast cancers can be linked to an inherited or germline gene mutation, the majority occur in patients with no family history of breast cancer. A woman’s lifetime risk of breast cancer is approximately 12% and a man’s is about 1 in 1,000. Despite treatment advances, better screening, an increase in awareness campaigns and earlier diagnosis, breast cancer continues to be the second leading cause of cancer related death behind lung cancer.\(^1\)\(^4,5\)

Breast Cancer Anatomy

The Breast

The human breast is primarily a collection of adipose tissue. Anatomically, the breast extends from the collarbone, including the tissue to the underarm and across to the middle of the ribcage. The breast can be sectioned into areas called lobes such as inner, outer and upper. The average female breast is comprised of up to 20 lobes.

\(^1\)\(^4,5\)
Each lobes is made up of many smaller glands. These glands are capable of producing milk in breastfeeding mothers. These glands are also referred to as lobules. As seen in Figure 1.1, the lobes and smaller lobules are intra-connected by milk ducts. This intra-mammary system’s primary purpose is to carry milk to the nipple. However, these ducts are traditionally where cancer begins to form, known as ductal carcinoma.

Figure 1.1. Anatomy of the female breast. Side view (left) of the interior anatomy including lobes, lobules, lymph nodes and ducts of the breast are also shown. Front view (right) of the exterior anatomy including the nipple and areola of the breast.
Lymph Nodes

Further within the adipose tissue of the breast is a network of lymph vessels and lymph nodes. The lymph system, including breast lymph nodes, is part of the immune system. These vessels and nodes are connected and part of a larger network that runs throughout the entire body. Lymph nodes under the arm, are called axillary nodes or nodes that connect above or below the collarbone, are supraclavicular or infraclavicular nodes and those that connect to lymph nodes in the chest are called internal mammary nodes. As an integral part of the immune system, the lymph system is tasked with a number of responsibilities, including immune surveillance, protection and disease fighting. Certain areas of the body, such as the breast, have large clusters of lymph nodes.

Breast Cancer Pathology

Type of Breast Cancer

Non-Invasive

Cancer can develop in any area of the breast. When it originates in the breast, this is considered the primary
site of the cancer, the most common of which is invasive type, as can be seen below in Figure 1.2.

![Figure 1.2. Subtypes of breast cancer.](image)

However, if the disease progresses to involve blood supply as well as evade the protection of the immune system, both lymph and blood infrastructure can carry the cancer to others areas of the body helping it to metastasize. Regardless of spread, the type of breast cancer is determined by the primary site of the cells. As previously discussed, the ducts are traditionally where cancer grows in the breast. This can be non-invasive, which is known as ductal carcinoma in situ (DCIS) as seen by a stained
patient sample in figure 1.3. Approximately 60,000 new cases of DCIS are estimated to be diagnosed this year.¹

Figure 1.3. Microscopy image of DCIS.⁸

Invasive

There are many types and forms of invasive breast cancer. The most common, invasive ductal carcinoma (IDC) or ductal breast cancers have several subtypes including but not limited to medullary, papillary, mucinous, and tubular. Less common, as seen in figure 1.4, are invasive lobular carcinomas (ILC), which make up about 10% or less of all breast cancers and originate in the lobes or lobules. Least common, occurring in less than 1-5% are inflammatory breast and Paget’s disease, a breast cancer in the ducts of the nipple.
Grade of Breast Cancer

All cancers, including breast are given a grade. The grade of the breast cancer is dependent on the cells of the tissue and how different from normal they look upon microscopic examination. Pathologist look at specific cellular features including the appearance of the nucleus, tubule formation or how many cells are in tubule formation and the mitotic rate or how fast the cells appear to be dividing.
Figure 1.5. Microscopy image of three grades of breast cancer. Grade one (Left). Grade two (center). Grade three (right). 

As demonstrated above in figure 1.5, the grading system ranges from one to three with a one grade indicating that cells are closer to normal. Low grade or well differentiated is a one, intermediate grade or moderately differentiated is a two and high grade or poorly differentiated is a three.

Size of Breast Cancer

The size of a breast cancer is the measurement taken of the tumor ex vivo. The tumor is measured at its widest point in metric (mm, cm). This primarily is used to define the stage of the breast cancer and is classified by the categories as described in table 1.1.
<table>
<thead>
<tr>
<th>Tumor size categories</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TX:</strong> Tumor size cannot be assessed</td>
</tr>
<tr>
<td><strong>T0:</strong> No tumor can be found</td>
</tr>
<tr>
<td><strong>Tis:</strong> Carcinoma in situ</td>
</tr>
<tr>
<td>Subcategories of Tis:</td>
</tr>
<tr>
<td>Tis (DCIS): Ductal carcinoma in situ</td>
</tr>
<tr>
<td>Tis (LCIS): Lobular carcinoma in situ</td>
</tr>
<tr>
<td>Tis (Paget): Paget disease of the breast (Paget disease of the nipple) with no DCIS, LCIS or invasive breast cancer</td>
</tr>
<tr>
<td><strong>T1:</strong> Tumor is 2 cm or smaller</td>
</tr>
<tr>
<td>Subcategories of T1:</td>
</tr>
<tr>
<td>T1mi: Very small tumor (0.1 cm or smaller)</td>
</tr>
<tr>
<td>T1a: Tumor is larger than 0.1 cm, but no larger than 0.5 cm</td>
</tr>
<tr>
<td>T1b: Tumor is larger than 0.5 cm, but no larger than 1 cm</td>
</tr>
<tr>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>T1c: Tumor is larger than 1 cm, but no larger than 2 cm</td>
</tr>
</tbody>
</table>

**T2:** Tumor is larger than 2 cm, but no larger than 5 cm

**T3:** Tumor is larger than 5 cm

**T4:** Tumor is any size, but has spread beyond the breast tissue to the chest wall and/or skin

<table>
<thead>
<tr>
<th>Subcategories of T4:</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4a: Tumor has spread to the chest wall</td>
</tr>
<tr>
<td>T4b: Tumor has spread to the skin, not inflammatory BC</td>
</tr>
<tr>
<td>T4c: Tumor has spread to both the chest wall and skin</td>
</tr>
<tr>
<td>T4d: Inflammatory breast cancer</td>
</tr>
</tbody>
</table>

Table 1.1. Breast cancer primary tumor size chart
TNM Staging System

The American Joint Committee on Cancer (AJCC) created a common classification system that is based on the basic elements involved in the development and spread of the disease. This includes the tumor, as discussed above, also the nodes and determining the extent of nodal involvement, and finally indicating if the cancer has metastasized. Together these three elements, tumor, node and metastasis (TNM) are the basis of the classification to define and stage breast cancer.

Breast Cancer Staging

Staging is essential to effectively and efficiently identify available treatments for breast cancer patients. Additionally, TNM staging (table 1.2) can also help to identify patients who may meet inclusion criteria for clinical trials for pipeline therapies. Staging patients can occur four different ways. They can be staged in the clinic upon initial physical examination, by pathologist via microscopy, or secondary staging that can occur after treatment such as surgery or neoadjuvant treatment or restaging if a recurrence should occur.
<table>
<thead>
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<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IA</td>
<td>T1*</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IB</td>
<td>T0</td>
<td>N1mi</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1*</td>
<td>N1mi</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T0</td>
<td>N1**</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1*</td>
<td>N1**</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIB</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>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>T4</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIC</td>
<td>Any T</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any T</td>
<td>Any N</td>
<td>M1</td>
</tr>
</tbody>
</table>

Table 1.2. AJCC Cancer Staging\textsuperscript{10}
Breast Cancer Genes and Receptors

Hormone Receptor

Estrogen Receptor

The estrogen receptor (ER) is well understood and binds to estrogen. Once bound and activated by estrogen, ER translocates to the nucleus where it then can bind to DNA, thereby regulating a number of gene functions. (Figure 1.6) ER is a well understood prognostic marker in breast cancer. The majority of breast cancer patients over express ER leading to hyper proliferation. However, ER is highly targetable and a number of treatment options are available for hormone receptor (HR) positive cancers.
Figure 1.6. Estrogen Receptor pathway. The estrogen receptor pathway, crosstalk pathways and co-regulatory proteins.  

Unfortunately, as can be seen above, the ER pathway is highly interactive and cross-talk with other pathways does make resistance and response to therapy an ongoing challenge for treatment providers.  

_Progesterone Receptor_

While the role the estrogen receptor and ER targeted therapies is clearly understood, the progesterone receptor
(PR) remains more confounding. The normal activity and use of progesterone is well documented. It can be useful in the premenopausal setting, when progestin is prescribed for contraception or postmenopausal setting for hormone replacement therapy (HRT). However, there is a paucity of information and studies on PR and its precise role in breast cancer. What is clear is the prognostic value of HR status. There is also a correlation to HR status and breast cancer specific survival with positive patients having a greater percent survival than HR negative patients. \(^{14,15}\)

Genes

**EGFR Family**

The epidermal growth factor family of receptor tyrosine kinases (RTKs) are also known as the ErbB or HER family. This family includes EGFR/ERBB1, HER2/ERBB2/NEU, HER3/ERBB3, and HER4/ERBB4. (Figure 1.7)

Each family member is unique. EGFR and ErbB4 can bind ligands and autophosphorylate. ErbB2 is highly promiscuous. It has no known ligand but dimerization preferentially with other family members. Finally, ErbB3
has no intrinsic tyrosine kinase activity but by interacting with active receptors, can transduce signals. When ligands, such as epidermal growth factor (EGF), bind a receptor with kinase activity, they induce a conformational change that facilitates subsequent receptor homo- or heterodimer formation. The result is activation of tyrosine kinase activity. This activated HER family member subsequently phosphorylates downstream targets that drive growth, survival and metastasis.

Figure 1.7. The EGFR Family of receptors.
Growth factor binding to HER family members results in activation of the EGFR pathway and potential cross activation to the MAPK signaling pathway (RAS-RAF-MEK-ERK) and the PI3K pathway (PI3K-AKT-mTOR) among others. The proper regulation of tyrosine kinases is essential for the control of cell growth, however, constitutive activation of the HER family has been connected with essential hallmarks of cancer such as tumor cell proliferation, evading apoptosis, neo-angiogenesis, invasive behavior and chemotherapy resistance. Tumors caused by constitutively active tyrosine kinases, mutations or over expression, have provided ideal targets for drug development. \textsuperscript{2,17-20}

HER2

While all ErbB family members and their ligands have been implicated in cancers, amplification of ErbB2 leads to receptor overexpression in up to a third of breast cancers. This overexpression is illustrated in Figure 1.8.
Figure 1.8. HER2 in breast cancer. Normal HER2 receptor expression (left), overexpression (center). HER2 overexpression leads to excessive cellular division.  

Against this background HER2 was identified as an appropriate target for the development of anti-cancer targeted agents that treat specific unregulated tyrosine kinase activities.  

Breast Cancer Subtypes  

Classification of breast cancer beyond staging has important prognostic value for breast cancer patients. Understanding the status of the three receptors, ER, PR and HER2 helps to identify appropriate candidates for adjuvant therapies. The subtypes are broken out by positive or negative receptor status. (Figure 1.9)
Figure 1.9. Breast cancer Subtypes. US incidence of breast cancer subtypes defined by HR and HER2 status.30

A triple positive, ER/PR+, Her2+, a triple negative, ER/PR–, Her2–, HER2– PR driven, ER–/PR+, Her2–, and HER2– ER driven ER+/PR−, Her2−. IHC studies have shown that the triple negative subtype has the lowest overall survival, while the triple positive has the highest compared to the other subtypes.
Chapter 2: Background

Treatment Strategies for Breast Cancer

Hormonal Treatment

Statistics vary, however approximately 70% of breast cancer patients are ER+ and therefore eligible for hormonal therapy. ER+ patients have about a 10 percent increase in five-year survival versus ER- patients, in part due to available therapies.\textsuperscript{31-33}

Tamoxifen

Tamoxifen was the first hormonal drug therapy available for ER+ breast cancer. As can be seen in figure 2.1, it binds to the estrogen receptor resulting in reduction of transcription. While there are some observed benefits to receiving tamoxifen, these can still be outweighed by the toxicities including gynecological side effects, ocular and others. As a result, compliance is an understood challenge.
Figure 2.1. Hormone treatment. Treatment in estrogen receptor positive and negative breast cancer.  

SERMs

Another class of drug, Selective Estrogen Receptor Modulators (SERMs) include the drug Fulvestrant, a steroidal antiestrogen. These drugs inhibit ER dimerization and reduce shuttling of the ER from the cytoplasm to the nucleus, as seen in figure 2.2.

AIs

A final major class for ER+ patients are Aromatase inhibitors (AIs). AIs work by inhibiting the action of the
enzyme aromatase. Like tamoxifen, there are a number of side effects described in table 2.2 that include vaginal bleeding, osteoporosis and hot flashes that can make patient compliance an issue.

Figure 2.2. Treatment effect. Mechanism of action of tamoxifen, aromatase inhibitors and fulvestrant.\textsuperscript{33}
Targeted Therapies

Trastuzumab

The targeted therapy Trastuzumab is a monoclonal antibody designed for HER2+ cancers and believed to block downstream signaling pathways as can be seen in figure 2.3 below.\textsuperscript{34,35}

![Figure 2.3: Anti-EGFR inhibitors.\textsuperscript{36}]

Pertuzumab

Pertuzumab, another targeted therapy for HER2+ cancers has a different proposed mechanism of action than trastuzumab. It binds to a subdomain of HER2 and blocks ligand-dependent heterodimerization with HER1, HER3, and HER4. These
targeted therapies have demonstrated the ability to stabilize tumor development, growth and progression in many HER2+ tumors. 34,35

Chemotherapy

To understand chemotherapy, it is critical to first understand tumor growth. Over the decades, several models for tumor growth and treatment have developed. One model assumes exponential cellular growth that is constant. In turn, when these tumors are treated with chemotherapy, the percentage of cells killed is assumed to be equally constant. (Figure 2.4) Just as cell growth is on a logarithmic scale, subsequent chemotherapy would combat this with exponential cell kill.
Figure 2.4. Log Kill Model. Tumor growth is exponential on a logarithmic scale (left). Chemotherapy kills tumor cells in a constant (right).\textsuperscript{37}

However, this earlier log-kill model was based on mathematical assumptions versus clinical patients. This model did not allow for enough variation, for example, solid tumors, like breast cancer have a different growth rate than hematologic tumors and other factors, such as tumor size or stage may be different from patient to patient. These tumor characteristics and more may affect the choice and result of a chemotherapy treatment. As a result, secondary models have been developed that take these other aspects into account, including tumor size and the stage of the cancer.

Most well-known is the Gompertzian growth curve which assumes the standard exponential cellular growth, but assumes that a tumor’s growth pattern may increase or slow prior to the constant. Regardless of which model is used, the goal is to help the oncologist to determine if chemotherapy is an option and when to give the therapy. Chemotherapy can be given either before surgery, called neoadjuvant chemo or after surgery called adjuvant chemo.\textsuperscript{38,39}
Chemotherapeutic Agents

During the Second World War, servicemen were exposed to mustard gas and were observed to develop hematological cancers. Subsequently, this agent was studied and eventually used to treat Hodgkin’s and non-Hodgkin’s lymphomas. Chemotherapy has progressed since the forties, most notably with the understanding that there is no one “silver bullet” chemotherapy drug. The majority of all modern chemo regimens are given in combination as can be seen in table 2.1.

Alkylating agents such as cyclophosphamide are DNA crosslinking agents. This means that these drugs cause intrastrand crosslinks within the DNA, which results in inhibiting tumor growth. Antimetabolites are also DNA interfering agents but are cell-cycle specific to the S-phase. Agents like 5-Fluorouracil either compete or inhibit the tumor’s DNA or RNA synthesis. Antineoplastic antibiotics interfere with DNA, leading to disruption of DNA synthesis, replication and ultimately function. Doxorubicin is an example of this class of drug. Platinum-based antineoplastic. As with all antineoplastic agents, platins are also DNA crosslinking agents, inhibiting DNA
repair, such as carboplatin or cisplatin. Taxanes are a class of drugs that is responsible for the disruption of microtubule function. Examples would be paclitaxel and docetaxel\textsuperscript{40-42}

<table>
<thead>
<tr>
<th>HER2-</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAC: Docetaxel Doxorubicin</td>
</tr>
<tr>
<td>Cyclophosphamide chemotherapy regimen</td>
</tr>
<tr>
<td>TC: Docetaxel Cyclophosphamide chemotherapy regimen</td>
</tr>
<tr>
<td>DDAC+T: Doxorubicin</td>
</tr>
<tr>
<td>Cyclophosphamide followed by paclitaxel or docetaxel chemotherapy regimen</td>
</tr>
<tr>
<td>FEC100+T: 5-Fluorouracil Epirubicin Cyclophosphamide followed by paclitaxel or docetaxel chemotherapy regimen</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HER2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCH: Docetaxel Carboplatin Trastuzumab chemotherapy regimen</td>
</tr>
<tr>
<td>Regimen</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>T+HCEF+H</td>
</tr>
<tr>
<td>DDAC+T+H</td>
</tr>
<tr>
<td>DDAC+T+H+P</td>
</tr>
<tr>
<td>PTH+DDAC</td>
</tr>
<tr>
<td>PTH+FEC</td>
</tr>
</tbody>
</table>
Table 2.1. Common chemotherapy regimens. Chemo regimens for breast cancer broken out by HER2 status.43-46

Treatment Toxicities

While the response to chemo has increased with combination chemotherapy regimens, so too have the toxicities. Chemotherapy has numerous side effects including alopecia, anemia cardiotoxicity and neutropenia. (Table 2.3) The risk of these toxicities must be carefully weighed against the benefit of giving the cytotoxic therapy to the patient.41

<table>
<thead>
<tr>
<th>Vaginal Complications</th>
<th>Tamoxifen poorer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrial Cancer</td>
<td></td>
</tr>
<tr>
<td>Thromboembolic events</td>
<td>AI poorer</td>
</tr>
<tr>
<td>Cardiac complications</td>
<td></td>
</tr>
<tr>
<td>Arthalgia /Myalgia</td>
<td></td>
</tr>
<tr>
<td>Osteoporotic fractures</td>
<td></td>
</tr>
<tr>
<td>Hot flushes</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. Hormone therapy toxicities. Toxicity of aromatase inhibitors versus tamoxifen 47-49
Table 2.3. Chemotherapy Toxicities. List of common chemotherapy toxicities and their related drugs.\textsuperscript{45,50-52}

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>Drug(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal</td>
<td>cisplatin</td>
</tr>
<tr>
<td>Hepatic</td>
<td>cyclophosphamide</td>
</tr>
<tr>
<td>Cardiac</td>
<td>doxorubicin</td>
</tr>
<tr>
<td>Neurologic</td>
<td>cisplatin,</td>
</tr>
<tr>
<td></td>
<td>paclitaxel</td>
</tr>
<tr>
<td>Immunosuppressive</td>
<td>cyclophosphamide</td>
</tr>
</tbody>
</table>
As can be seen above in figure 2.5, a breast cancer sample is processed and examined via IHC. These steps will include primary and secondary antibodies, substrates, fluorescent detection, stains and mounting on a slide. Then a pathologist will review the slide under a microscope and document the interactions with target antigens on a pathology report. \textsuperscript{70-85}

ER/PR/HER2 Expression

Depending on the stains a pathologist uses, which can highlight different cellular functions and features, IHC can help determine a number of elements of the breast cancer. This can be seen in figure 2.6 below.
IHC can provide additional information, beyond hormone receptors or HER2 status. Specific stains can also inform about proliferation status via Ki67, cell origin and other proteins of interest including p53.\textsuperscript{87,88}

Over the years however, the College of American Pathologists (CAP) has come to understand there is a great deal of discordance among IHC test results. In breast cancer, the largest culprit is HER2 tests with CAP
reporting that nearly a third of all HER2 test results may be wrong. The challenge is in the HER2 scoring system and a lack of consistency. Dependent on the amount of HER2 receptor protein on the surface of the cells, IHC gives a score to a patient’s breast cancer from 0 to 3+. A 0–1+, negative, 2+ is borderline and 3+ positive. Different institutions and pathologists have different criteria for determining positive or negative as well as what to do if the results are borderline. In many cases, when the result is borderline, the tumor sample is sent for further testing by fluorescence in situ hybridization (FISH). 

**FISH**

![Figure 2.7: FISH and HER2. Fluorescence in situ hybridization (FISH) assessment for Her2 status in breast cancer. Her2 negative (left) and Her2 positive (right).](image)
HER2 Amplification

FISH is different from IHC. Protein over-expression is detected by IHC vs HER2 gene amplification status when analyzed by FISH. (Figure 2.7) Regardless of which standard clinical pathological test is used for assessing breast cancers, inaccurate test results are an ongoing topic of study and concern. These results, in absence of any genomic testing, might be the only information driving treatment decisions on a patient. As a result, they may not receive appropriate therapy.

Standardized Clinical Factors

Adjuvant! Online

With the cracking of the genome and the rise of the internet, came a number of online algorithm tools for breast cancer treatment providers. These tools were created with the intent to somehow creating an umbrella under which common clinical factors could be collected, considered and calculated for risk.

The hypothesis was that an individual’s treatment decisions might be improved upon using these online calculators that are based on large national data sets vs an individual
physician or intuition. A physician using an online tool would input the standardized clinical factors and a determination whether or not to give chemotherapy, toxicities and survival could then be generated by the tool.

The most successful and widely used of these online algorithm tools was Adjuvant! Online (A!O). This tool assesses a breast cancer patient’s risk of recurrence and death at 10 years. This risk assessment is based on the surveillance, epidemiology, and end-results (SEER) database. It estimates adjuvant therapy data from the Early Breast Cancer Trialists' Collaborative Group (EBCTCG). To use, the physician would enter various tumor characteristics such as size, grade and nodal status as can be seen in figure 2.8. A 10-year mortality rate and benefit of treatment determination would be reported.
Figure 2.8: Adjuvant! Online sample. A web-based breast cancer predictive algorithm for adjuvantchemotherapeutic decision making.$^{89}$

However, much like traditional clinical pathology, A!O has its limitations. For example, as previously discussed, HER2 status in breast cancer patients can be challenging and discordant. SEER did not include HER2 and therefore it was not included in the A!O algorithm. For this reason, as well as other challenges, A!O is now currently offline.$^{90-92}$
Chapter 3: Innovation

Genomic Tests for Assessment of Breast Cancer

In the early 2000’s Gene Expression Profile (GEP) tests for breast cancer became commercially available. The hypothesis was that these Multianalyte Assays with Algorithmic Analyses (MAAA) would be able to further inform the decision of whether to treat with adjuvant chemotherapy. A number of factors contributed this age of personalised medicine in cancer and the rise of MAAAs. However, the main catalyst for these genomic tests was a lack of reproducibility among pathologist grading the breast tumors and a desire to identify tumor biology as predictors of therapeutic response.\textsuperscript{93}

As discussed previously, inaccurate HER2 testing is well documented. Traditional IHC pathology may initially be borderline and FISH may be negative. These patients would likely be denied, trastuzumab, when in fact, molecular testing may show that the tumor is HER2 positive and could have benefited from treatment.\textsuperscript{94} As is the case with HER2, there is equal controversy over Ki67 and its cutoff values.
In this case, women may be classified as having a rapid proliferation rate and a high grade tumor. This high proliferation rate and subsequent grade would traditionally indicate that these tumors would be good responders to chemotherapy. The result would be that these patients may receive chemotherapy when genomic testing may reveal they are not high risk. For these patients the risk of receiving chemotherapy outweighs the benefits, if they are correctly classified as lower risk by genomic testing.93,95-97

**Commercialization of MAAAs**

FDA and CLIA certification

Once a company has developed an MAAA or a laboratory-developed test (LDT) and they want to make their genomic signature commercially available, a choice should be made between Food and Drug Administration (FDA) and Clinical Laboratory Improvement Amendments (CLIA) approval. The FDA has not been the choice for the vast majority of companies in this space. The main reasons are because of its strict regulatory standards as the gatekeepers of patient safety. This leads to high standards of quality control as well as clinical data. The result is a much higher cost and time
for route to market than CLIA. However, while avoiding the more heavily regulated FDA path may lower cost upfront, it may increase a company’s challenges selling the test to certain institutions. One of the most critical steps to success for any commercial assay is adoption. Payers and providers alike want to know that a test has met and exceeds both clinical and regulatory validation.\textsuperscript{98}

Clinical validation

Phases of Trials

In order to bring a new signature to market, the company must test and validate its product. This is done via a clinical trial. Trials escalate in their levels of availability and testing on consented human subjects in three phases. (Figure 3.1)
Figure 3.1. Clinical trials. Human clinical trial with increasing validation from left to right.\textsuperscript{99}

Levels of evidence

Level of evidence is a designation that is given to the data put forward by industry. It is a standardized system that indicates the level of strength of the evidence supporting the use of a specific tool, intervention or approach. (Figure 3.2) Levels of evidence are taken into consideration in various aspects of cancer care from guideline inclusion to reimbursement to everyday clinical practice.
Table 3.2. Levels of Evidence. Levels of evidence with decreasing validation from top (1A) to bottom. \(^{100}\)

<table>
<thead>
<tr>
<th>Level of Evidence</th>
<th>Type of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Systematic reviews of randomized controlled trials (RCTs)</td>
</tr>
<tr>
<td>1b</td>
<td>Individual RCTs with narrow confidence interval</td>
</tr>
<tr>
<td>2a</td>
<td>Systematic reviews of cohort studies</td>
</tr>
<tr>
<td>2b</td>
<td>Individual cohort studies and low-quality RCTs</td>
</tr>
<tr>
<td>3a</td>
<td>Systematic reviews of case-control studies</td>
</tr>
<tr>
<td>3b</td>
<td>Case-control studies</td>
</tr>
<tr>
<td>4</td>
<td>Case series and poor quality cohort and case-control studies</td>
</tr>
<tr>
<td>5</td>
<td>Expert opinion</td>
</tr>
</tbody>
</table>

Multianalyte Assays with Algorithmic Analyses (MAAAs)

The 21 gene Oncotype DX (Odx) and the 70 gene MammaPrint (MP) are the two most dominant commercially available MAAAs on the market. Both have been available for more than ten years and both tests have accrued outcomes in clinical studies attesting to their usefulness in clinical decision making for treatment purposes. Although both of these assays are believed to provide equivalent information, the platforms and development are quite different, resulting in
discordant results and observed differences in risk assessment. This in turn can cause a downstream effect of differences in treatment decision recommendations.

Reverse transcription polymerase chain reaction (RT-PCR)

The Odx assay was developed using a reverse transcription polymerase chain reaction (RT-PCR) platform. RT-PCR, is a highly proven method for allowing scientists to copy DNA. However, it is not a high throughput platform. For that reason, the developers needed to batch samples as opposed to being capable of running a patient’s entire genome.

OncotypeDX/21-Gene Assay

Odx, or the 21-gene assay has been offered commercially since 2004 for testing ER+, Her2−, T1-2, N0, M0, patients. To create Odx, the developers looked at a selected candidate gene set from treated breast cancer patients and launched the Odx assay from these validated results. The Oncotype DX Recurrence Score (RS) provides a risk assessment of high, low high, but gives an uncategorized intermediate recurrence score (RS 18-30 clinical range, 11-25 trial range) for 38-67% of tested patients. The RS is
derived from 21 genes total, 16 genes and 5 reference genes. Included in these 16 selected candidate genes are ER, PR, HER2 and Ki-67. The RS is calculated on a scale from 0 to 100 and is derived from the reference-normalized expression measurements. Therefore an equation is used to derive a reference normalized expression measurement. The RS is dependent on these traditional clinico-pathological CPF factors.102-112

Microarray

A key difference between MammaPrint and Oncotype is platform. MP was developed utilizing microarray. Microarray provides a number of platform difference and potential advantages for genomic testing. There is a very small sample size required to run the microarray which means better cost as well as conservation of extremely valuable breast tissue for future studies. (Figure 3.3) IHC may be able to slice and fix up to 100 sections off a block of breast cancer specimen. Comparably, microarray may be able to retrieve up to 300 times as many samples from the same block. Additionally, the high throughput
nature of microarray means that compared to RT-PCR, rapid, multiple sample assays can be performed.

Figure 3.3. A microarray. Diagram of a microarray.
Unlike RT-PCR, microarray is also capable of interrogating a patient’s entire genome, critical to the understanding of a tumor’s biology and generating an appropriate treatment strategy. High throughput capability also improves uniformity over a larger number of tissue samples. This makes microarray a robust and highly reproducible platform. A key factor in the breast cancer space which has a history of challenges with both pathology, online tests and discordance.\textsuperscript{113-119}

MammaPrint/70-Gene Assay

MP or the 70-gene assay was developed from untreated patient samples. These sample were processed for whole genome sequencing via microarray. (Figure 3.4) The MammaPrint test is a laboratory developed test (LDT) which falls into the class of \textit{In Vitro Diagnostic Multivariate Index Assays (IVDMIA)}. MammaPrint was the first (2007) IVDMIA to be cleared by the FDA in a \textit{De Novo Classification Process (Evaluation of Automatic Class III Designation) and}
is the only molecular diagnostic test with level 1A evidence validating clinical utility

Figure 3.4: MammaPrint development.\textsuperscript{120-140}

The test uses RNA isolated from tumor samples and run on custom glass microarray slides in order to determine the expression of a 70-gene signature. The expression profile is then used in a proprietary algorithm to categorically classify the patient as being at binary high or low risk of breast cancer recurrence.\textsuperscript{141-145}

BluePrint/80-Gene Assay

BluePrint (BP) or the 80-gene assay is a molecular subtyping assay that determines the mRNA levels of 80 genes
that best discriminate between Luminal-type or ER driven tumors, HER2-type, and Basal-type tumors or triple negative tumors. (Figure 3.5)

![Figure 3.5](image.png)

Figure 3.5. BluePrint and estrogen receptor activity. Protein expression does not necessarily indicate the pathway is functioning$^{13,42,94}$

BP was developed in a supervised training method, using samples with concordant ER, PR, and HER2 status by IHC and single-gene readout, ensuring the capture of ER/PR/HER2-regulated processes. Further stratification of the Luminal
group into types A and B based on MP high and low risk stratification determines the need for chemotherapy in these ER driven tumors.

BluePrint allows for a more truly functional assessment of the tumor biology. For example, by looking at luminal tumors driven by the estrogen pathway, the differences can be seen between the tools and surface versus functional measurement (Figure 3.5). As previously discussed, traditional subtyping, by IHC or FISH can have a high level inaccuracy. With microarray, proper estrogen expression and function is confirmed versus merely the presence of the estrogen receptor through IHC testing.

While it is important that the estrogen receptor is present, if the genes are not activated, the entire pathway won't function. This can result in incorrect pathology reports and subsequent treatment recommendations. Herein lies one of the many challenges with traditional clinical pathological tests or even genomic signatures like Odx, which give results dependent on these same traditional clinical factors.
Validation data from both MP and BP supports that breast cancer patients may benefit from treatment developed utilizing the results of genomic assays developed independent of traditional clinical risk factors.13,94,146-153
Chapter 4: Methods

All Aims

For molecular and clinical risk assessment, MP and BP was performed according to standard protocols and have been previously described\textsuperscript{154} This test is based on microarray gene expression analysis of RNA extracted from FFPE breast tumor tissue, and uses custom-designed array chips manufactured by Agilent Technologies (CA, USA). The 70-GS provides a numerical index between 1.000 and -1.000, as well as a dichotomous categorization of Low Risk or High Risk depending on whether the index is > 0 (Low Risk) or ≤ 0 (High Risk).

Specific Aim One Methods

Multi Institutional Neo Adjuvant Therapy MammaPrint Project (MINT) patient samples were utilized for this aim. Array data from pre-treatment samples were obtained and classified as MammaPrint High Risk, subtyped by IHC and treated with neo-adjuvant chemotherapy according to protocol.
A frozen sample of the resected tumor were shipped on dry ice to Agendia (fresh frozen specimen on dry ice, RNA-retain fixed material in a sealed tube or unstained FFPE slides). H&E stained slides were prepared and a qualified pathologist assessed tumor cell percentage. A minimum of 30% invasive tumor cells were required to qualify for analysis. Total RNA were isolated, amplified and labeled with fluorescent dye. The amplified labeled cRNA were hybridized on a DNA microarray. A scanner scanned the DNA microarray slide and quantified each probe on the array. Proprietary software extracted the information from the scan and using the 70-genes printed in 9 fold and 465 normalization genes, calculated the MammaPrint Index.

Responses were measured by centrally assessed residual cancer burden pursuant to guidelines. Patients were then further stratified based on this MammaPrint Index per their classification threshold between MP1/MP2.

GraphPad prism 7 was used to assess significance of association with risk groups as well as perform all biostatistics and create all graphs.
The primary list of patients were kept in miami.box.app which was password protected for compliance with any matters concerning health information privacy and security for patient records exchanged or stored with the University of Miami and Miller School of Medicine. Files within miami.box.app could only be accessed by the Principle Investigator and the research team that had been certified to participate in reviewing patient records and therefore received passwords. A list of patient genomic reports came from Agendia as a .csv file and were converted to excel. Patient clinical pathology reports came from hospitals in report form. Any research findings or reports were free of PHI and constructed in a Microsoft Word file. Statistical analysis performed in GraphPad were also free of PHI. No paper records were kept for this study and no PHI were disclosed to anyone, nor will be except as required by law for compliance review.

Specific Aim Two Methods

As described above, MINT patient samples were again utilized. Similarly, array data from pre-treatment samples were obtained and classified as MammaPrint High Risk. This
was again performed as follows: A frozen sample of the resected tumor were shipped on dry ice to Agendia (fresh frozen specimen on dry ice, RNA-retain fixed material in a sealed tube or unstained FFPE slides). H&E stained slides were prepared and a qualified pathologist assessed tumor cell percentage. A minimum of 30% invasive tumor cells were required to qualify for analysis. Total RNA were isolated, amplified and labeled with fluorescent dye. The amplified labeled cRNA were hybridized on a DNA microarray. A scanner scanned the DNA microarray slide and quantified each probe on the array. Proprietary software extracted the information from the scan and using the 70-genes printed in 9 fold and 465 normalization genes, calculated the MammaPrint Index. Responses were again measured by centrally assessed residual cancer burden pursuant to guidelines. Patients were then further stratified based on this MammaPrint Index per their classification threshold between MP1/MP2, but then further subtyped.

For aim two specifically, this were conducted by both IHC and BluePrint to compare a multi-gene classifier to conventional local IHC/FISH subtyping and its ability to predict chemosensitivity as defined by pCR.
GraphPad prism 7 was also used in this aim to assess significance of association with pCR overall and within hormone receptor (HR) and HER2 subtypes and produce graphs.

The primary list of patients for this aim were also kept in miami.box.app which was password protected for compliance with any matters concerning health information privacy and security for patient records exchanged or stored with the University of Miami and Miller School of Medicine. Files within miami.box.app could only be accessed by the Principle Investigator and the research team that had been certified to participate in reviewing patient records and therefore received passwords. A list of patient genomic reports came from Agendia as a .csv file and were converted to excel. Patient clinical pathology reports came from hospitals in report form. Any research findings or reports were free of PHI and constructed in a Microsoft Word file. Statistical analysis performed in GraphPad were also free of PHI. No paper records were kept for this study and no PHI were disclosed to anyone, nor will be except as required by law for compliance review.
Specific Aim Three Methods

ER, progesterone receptor (PR), HER2, and Ki67 status were determined by the local institution on pre-treatment samples according to ASCO/CAP testing guidelines \(^{155-157}\). All patients were also categorized as either Clinically-low or Clinically-high risk using clinicopathological factors. A modified version of Adjuvant! Online 8.0 \(^{158}\) (A!O) including HER2 was used to standardize the clinical risk assessment. While the algorithm is not currently available, a guideline defining risk outcome based on ER status, HER2 status, nodal status, tumor grade and tumor size was adapted from the MINDACT trial (Appendix A) \(^{159}\).

Study Endpoint assessment of breast cancer mortality is directly associated with distant metastasis, therefore the distant metastasis free interval (DMFI) was evaluated in addition to distant metastasis free survival (DMFS) \(^{160}\). Only a distant metastatic event was considered the endpoint for DMFI, and only death due to a metastatic event was considered the endpoint for DMFS.

Data for IRB approved patients (Appendix B & C) who did not have an event were censored at the time of last follow-up
for DMFI and DMFS. Patients who had a non-breast cancer related death were censored at time of death for DMFI and DMFS. Survival analysis was performed using the Kaplan-Meier estimator and log-rank test, stratifying patients by the MammaPrint, Clinical Risk, and combined clinical-genomic risk categories. All calculations were performed with R version 3.2.2. 154-160
Chapter 5: Results

Given the previous detailed overview of breast cancer, and background on the past and current testing methods, it was hypothesized that utilization of tumor biology specifically, genomic signatures for risk assessment and molecular subtyping, could improve treatment development and strategies in breast cancer. New genomic signatures seek to understand a tumor beyond clinical screening, imaging or cell surface staining. By looking at the molecular biology of cancer, interrogating single genes or overall patterns of gene expression, it is possible to better understand the various sequences of biological events that give rise to breast cancer and in turn, it is possible to better develop and guide treatments.161

Specific Aim One

The purpose of this aim was to determine if there is a significant difference in MammaPrint risk stratification signatures (MP1/MP2) between pre-neoadjuvant chemotherapy treatment (NCT) breast cancer specimens and post-NCT treated breast tumors. NCT has been shown to clinically down-stage many large or locally advanced breast cancers.
For patients who do not achieve a pathologic complete response (pCR), there is limited research that has been conducted to evaluate how NCT affects the gene signature profile on residual tumors.\textsuperscript{162,163}

Specific Aim Two

The purpose of this aim was to determine if there is a significant association with BluePrint molecular signature and breast cancer receptor subtype for MP1/MP2 risk classes. Beyond risk assessment, classification of breast tumors into correct molecular subtypes is critical for the selection of therapy for patients with breast cancer.

The neo-adjuvant I-SPY 1&2 TRIALs demonstrated that further stratification of patients into MammaPrint High 1 (MP1) and MammaPrint High 2 (MP2) risk groups may help predict chemosensitivity. There were significant differences in pathological complete response (pCR) rates for early stage, locally advanced breast cancer patients who were not HR+HER2- MammaPrint Low Risk. Specifically, the PARP inhibitor veliparib in combination with carboplatin recently graduated the I-SPY 2 phase 2 screening trial, having met the 85% predictive probability criterion with a triple-negative breast cancer signature, which was the
subset recommended for this regimen's subsequent development.

Given these data, we wanted to determine whether the Multi Institutional Neo Adjuvant Therapy MammaPrint Project (MINT) patient population confirmed the MP1/MP2 risk stratification, clarify if there is an associated receptor subtype for MP1/MP2 risk classes, and conclude if the stratification correlates to a significant MP1 vs MP2 risk classes yielded subsets with significant (p=0.007) differences in pCR. (Figure 5.1) 44% (40/92) of MP2 patients achieved a pCR, compared to 24% (21/88) of MP1 patients.
Figure 5.1. MINT patient population and pCR. Data confirms the MP1/MP2 risk stratification with significant differences in pCR Student’s t-test on MP1/2 data. \( t = 5.4767, \text{df} = 171.45, \text{p-value} = 1.521\times10^{-07} \) (mean pCR - 0.6809037, mean No pCR - 0.4213295) (A). MammaPrint Low Risk (0% pCR) 31 Luminal A, 1 HER2 and MP1 (27% pCR), MP2 (40% pCR) (B).

Next, we investigated whether the MP1 and MP2 risk classes were associated with receptor subtype. MP1 demonstrated a significant association. (Table 5.1) 32% (21/66) of triple-negative patients were classified as MP2 vs only 3% (2/66) MP1. (Figure 5.2) Similarly, in the overall population,
28% (51/180) HR+HER2- are classified as MP1 vs 4% (8/180) MP2.

Table 5.1. MP1/2 by IHC receptor subtype.

<table>
<thead>
<tr>
<th>IHC Hormone Receptor Status</th>
<th>pCR</th>
<th>No pCR</th>
<th>Grand Total</th>
<th>% pCR by line</th>
<th>% of total pCRs</th>
<th>% of HR group</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRpos_HER2pos</td>
<td>18</td>
<td>20</td>
<td>38</td>
<td>47.4%</td>
<td>29.0%</td>
<td></td>
</tr>
<tr>
<td>MP1</td>
<td>12</td>
<td>13</td>
<td>25</td>
<td>48.0%</td>
<td>19.4%</td>
<td>65.8%</td>
</tr>
<tr>
<td>MP2</td>
<td>6</td>
<td>7</td>
<td>13</td>
<td>46.2%</td>
<td>9.7%</td>
<td>34.2%</td>
</tr>
<tr>
<td>HRpos_HER2neg</td>
<td>11</td>
<td>54</td>
<td>65</td>
<td>16.9%</td>
<td>17.7%</td>
<td></td>
</tr>
<tr>
<td>MP1</td>
<td>4</td>
<td>44</td>
<td>48</td>
<td>8.3%</td>
<td>6.5%</td>
<td>73.8%</td>
</tr>
<tr>
<td>MP2</td>
<td>7</td>
<td>10</td>
<td>17</td>
<td>41.2%</td>
<td>11.3%</td>
<td>26.2%</td>
</tr>
<tr>
<td>HRneg_HER2pos</td>
<td>19</td>
<td>10</td>
<td>29</td>
<td>65.5%</td>
<td>30.6%</td>
<td></td>
</tr>
<tr>
<td>MP1</td>
<td>5</td>
<td>3</td>
<td>8</td>
<td>62.5%</td>
<td>8.1%</td>
<td>27.6%</td>
</tr>
<tr>
<td>MP2</td>
<td>14</td>
<td>7</td>
<td>21</td>
<td>66.7%</td>
<td>22.6%</td>
<td>72.4%</td>
</tr>
<tr>
<td>HRneg_HER2neg</td>
<td>14</td>
<td>37</td>
<td>51</td>
<td>27.5%</td>
<td>22.6%</td>
<td></td>
</tr>
<tr>
<td>MP1</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>50.0%</td>
<td>3.2%</td>
<td>7.8%</td>
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<tr>
<td>MP2</td>
<td>12</td>
<td>35</td>
<td>47</td>
<td>25.5%</td>
<td>19.4%</td>
<td>92.2%</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>121</td>
<td>183</td>
<td>33.9%</td>
<td>100.0%</td>
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</tr>
</tbody>
</table>
Figure 5.2. MP1/2 and subtype. MP1 and MP2 risk classes are associated with receptor subtype. By IHC classification (A). By BP molecular classification (B).

Results in the pCR population were reflective of these subtype trends. (Figure 5.3) 63% (58/92) of MP2 patients were classified triple-negative, of which nearly one quarter (21/92) had a measured pCR, whereas 58% (51/88) MP1 patients were HR+HER2- with 3% (3/88) achieving pCR.
Figure 5.3. BluePrint molecular subtyping reclassification vs IHC. IHC subtyping (A) and via BluePrint (B).

This analysis in the MINT patient population supports previously published data and suggests that the MammaPrint High 1/2 risk classification may help predict chemotherapy sensitivity. Given the statistical significance of these data, we are currently investigating the biological
mechanisms distinguishing the MP1/MP2 subgroups that may account for its use as a specific biomarker of response to chemotherapy treatment.

Specific Aim Three

The purpose of this aim was to determine if the combination of MammaPrint and BluePrint for breast cancer genomic risk assessment and molecular subtyping can safely stratify patients and improve treatment decision making in a community clinical population.

The MINDACT trial which reported in 2016 was a European prospective, randomized study comparing the 70-gene signature MammaPrint with commonly used clinical-pathological criteria for selecting node-negative or 1-3 node positive breast cancer patients for adjuvant chemotherapy. The trial was intended to address whether tumor biology (genes) could improve on existing methods of risk assessment by tumor anatomy (clin-path) and treatment decision-making by assisting oncologists to select between patients who need adjuvant treatment and those who do not.

The primary objective of the trial was to confirm whether the number of patients safely spared adjuvant chemotherapy
was significantly increased when the decision was based on
genetics (the 70-gene signature) rather than on clinical-
pathologic factors alone. The trial enrolled 6,693
patients from 2007 to 2011 and reported its primary
analysis in The New England Journal of Medicine in 2016 when the patient population reached a 5-year average
follow-up. This prospective randomized trial reported
positive and its primary endpoint was met.

The full analysis of the COPPER data included 517 patients
from BayCare Healthcare Network (FL, USA) from 2009-2016.
642 patients total analyzed, 608 adjuvant patients, 527
adjuvant had MammaPrint and/or Clinical-pathology result
and 525 of these adjuvant patients had both MP and
clinical-pathology results making them eligible for
COPPER. Of these, 517 results were used for the final
analysis. Of all patients, 15 had duplicate samples,
therefore 8 duplicates were removed. The 8 duplicates were
either re-submissions or bilateral/multifocal. If
bilateral, the sample used was the reported result with a
higher risk result given treatment is systemic.
The objectives of COPPER were to one, determine if MammaPrint could safely stratify patients into Low Risk and High Risk categories based on prognosis of a recurrence of the disease in a community clinical population. (Figure 5.4) Two, COPPER was designed to determine if the combination of MammaPrint and BluePrint for breast cancer genomic risk assessment and molecular subtyping improved treatment decision making in a community clinical population when DMFI was used as a study endpoint (figure 5.7). \(^{164-166}\)

Of 103 CHR/GLR 59 (57%) omitted CT. This was in alignment with their LR MP result. (Table 5.2) 2 had events (deaths due to metastasis).

<table>
<thead>
<tr>
<th>Treatment (n=517)</th>
<th>70-GS HR</th>
<th>70-GS LR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count All</td>
<td>Event %s</td>
</tr>
<tr>
<td>Chemo (with or without Endo)</td>
<td>216</td>
<td>8</td>
</tr>
</tbody>
</table>
No Chemo (Endo Only or Untreated) | 48 | 2 | 4.2% | 168 | 2 | 1.2%
Unknown/Incomplete | 19 | | 20 |
All | 283 | 10 | 3.5% | 234 | 3 | 1.3%

Table 5.2. COPPER MP patient risk stratification.

Of 78 CLR/GHR, 47 (60%) received CT. This was in agreement with their MP result.
Figure 5.4. Survival curves of MammaPrint vs A!O. MP risk classification was superior as demonstrated by probability of DMFS MP (A) vs A!O (B).

There were 4 events recorded in this HR MP group. The median follow-up of the group overall was 44 months, 13 deaths, 25 metastases with 1 censored patient at death because of non-BC related complication.
Figure 5.5. COPEER subtyping by IHC BluePrint molecular subtyping (A) and IHC (B). Survival was statistically significant when determined by BP vs IHC.

BluePrint MS classified 14% TN as Luminal B. (Table 5.3) MS classified 38% as Basal-type among the HER2+ by PS. MS classified 47% as Luminal (A and B) and Basal-type among the TP by PS.
<table>
<thead>
<tr>
<th>IHC/PS classification</th>
<th>Concordant subtyping</th>
<th>Reclassified subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 TN</td>
<td>18 Basal-type</td>
<td>3 LuminalB</td>
</tr>
<tr>
<td>8 ER-/HER2+</td>
<td>5 HER2-type</td>
<td>3 Basal-type</td>
</tr>
<tr>
<td>19 ER+/HER2+</td>
<td>10 HER2-type</td>
<td>1 Basal, 7 LuminalB, 1 LuminalA</td>
</tr>
<tr>
<td>2 ER+/HER2equiv</td>
<td></td>
<td>2 1 LuminalB, 1 LuminalA</td>
</tr>
<tr>
<td>204 ER+/HER2-</td>
<td>196 Luminal-</td>
<td>8 7 Basal, 1 HER2</td>
</tr>
<tr>
<td>254</td>
<td>229</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 5.3. BluePrint molecular subtyping reclassification vs IHC

Overall BluePrint Molecular Subtyping reclassified 10% of all COPPER patients who received MS.
Of note, several interesting subgroups are currently under analysis from the overall COPPER analysis group (n=517). First among these sub-analyses is an interesting finding of patients who received both MP and Odx (n=78). As can be seen in the above survival data in figure 5.6, both low and high patients had statistically poor survival with the uncategorized intermediate recurrence score group doing best.

Overall, COPPER confirmed MammaPrint safely stratified patients into Low Risk and High Risk of recurrence
categories (p=0.0232) (figure 5.4) and BluePrint was also significant at molecular subtyping (p=0.0212) (Figure 5.5). Additionally, the second objective was met as the probability of distant metastasis free interval (DMFI) was clearly increased (p=0.058) (figure 5.7) when the decision was based on MammaPrint/BluePrint versus clinicopathological testing alone.

Figure 5.7. COPPER patient groups.

These data highlight the critical clinical importance of considering tumor biology in treatment decisions
Chapter 6: Discussion

In biology, an underlining principle is that, successful growth requires well-orchestrated control of gene expression. Gene expression is regulated by the interaction between proteins and DNA. One key to this regulation is messenger RNA (mRNA). The result of these interactions in turn drives transcription, replication, repair and a number of other regulatory DNA sequences.

Regulating transcription is fundamental to the control of gene expression. Conversely, transcriptional deregulation is equally fundamental in the development of cancer. For example, any number of mutations might activate transcription factors. These transcription factors could be oncogenes or tumor suppressor genes and depending on the specific mutation, could lead to increased transcriptional activation of the gene. This in turn could result in increased transcription, gene expression and cell growth or a cancer.

The majority of work in the area of MAAAs, including Agendia’s work, has focused almost exclusively on gene
expression and tools, such as microarray that measure this
expression via mRNA. The catalyst for the development of
MammaPrint and BluePrint breast cancer mRNA assays was to
improve upon previously used techniques, chiefly IHC/FISH.
As previously discussed, molecular classification of breast
tumors by IHC/FISH has been subject to broad ranges of
discordance both nationally and within institutions.

Other tools or platforms that came before MammaPrint and
BluePrint had also looked to determine a patient’s risk,
however these tools had looked at gene activity and merely
measured mRNA levels of single genes. These methods all
had their limitations in that they were dependent on the
literal presence of protein or mRNA. However, nothing on
the market had demonstrated that the protein, or mRNA was
truly functional. Agendia, and others subsequently
developed gene signatures that have genes or targets of
interest and correlate the measurement of the expression of
that signature of genes.

The focus of Agendia’s studies and subsequent development
of its signatures has examined the differences in gene
expression between high and low risk of metastasizing
breast cancer patients. However, it should be noted, that
this presents certain limitations. There are other biological mechanisms that can lead to gene modification and subsequent function alteration but do not change the nucleotide sequence, also known as epigenetics. For example, methylation of DNA is an enzyme-induced modification to the DNA structure and can affect how genes are expressed without a modification with a direct effect on the base sequence. While this process is present in normal biology, its role in cancer is becoming increasingly understood.

Critical to our understanding and future development of signatures and treatments is that epigenetic changes do not alter the sequence of DNA. Specifically, DNA methylation is a rearrangement of chromatin or chromatin remodeling. This remodeling takes the chromatin from a condensed state, where it is more challenging for transcription factors and proteins to access the DNA to an accessible state, where the chromatin is more loosely packed, and more easily accessible. Transcription activation and turning a gene on occurs by adding an acetyl group to histones by histone acetylase. Repressing transcription or conversely turning the gene off occurs by methylation of cytosine residues by DNA methyltransferase. The key difference is that whether
tuning on or off, this process of DNA methylation, chromatin remodeling and histone modifications, regulates gene expression without disrupting the base sequence.

Microarray and mRNA have served Agendia's needs thus far and exhibited their superiority to traditional clinical methodologies including IHC/FISH. However, as our understanding of cancer biology grows, we must also consider new ways to examine tumors and develop signatures and treatment strategies. Certainly, continuing to grow our understanding of DNA methylation events and then applying that to observations from the clinic could catalyze advances in diagnostic tests. Developing methylation profile signatures by analyzing the patient samples and observing the differences might be a consideration, with an understanding between hypo or hypermethylation and how it can lead to gene activation and repression. Also, observing the differences in methylation patterns from circulating serum tumor DNA (ctDNA) would also be a reasonable consideration. Currently, several ctDNA trials utilizing samples from patients with breast cancer are tracking the response of tumors to certain therapies including histone deacetylase inhibitors (HDAC inhibitors) that inhibit histone deacetylase in hopes of
optimizing therapy development. Finally, moving to platform, next generation sequencing or NGS would be an option, where a more comprehensive and high-resolution examination of DNA and the human genome can be done. NGS would allow for molecular biology research beyond microarray, such as epigenetic studies including, but not limited to evaluation of histone modifications. Fully evaluating molecular functionality, such as transcription is essential to truly understanding a patient’s tumor biology and essential to developing the correct tumor treatment strategy.
Chapter 7: Future Directions and Conclusion

Future projects are already underway in the wake of this project.\textsuperscript{101,168,169} Several interesting subsets of data that warrant investigation have been discovered from the above specific aims and results. These are including but not limited to the impact of MP on quality of life, economic impact and better clinical trial design.\textsuperscript{44,170-184} Additional MP results are also being analyzed looking at multifocal breast cancer as well as heterogeneity, MP in subpopulations such as African Americans, Hispanics and its use in an elderly patient data set from COPPER. There are also collaborations underway as a result of this project including a meta-analysis of head to head data with Odx as well as the potential development of a new genomic biomarker for resistance to a cyclin-dependent kinase (CDK) 4/6 inhibitor, which is currently being evaluated in the adjuvant setting for patients with HR+, HER2_ breast cancer.\textsuperscript{174,175,177,178}

This project supports previously published validation data that patients were safely spared or assigned treatment
based on MammaPrint risk and BluePrint molecular subtyping results. These data highlight the importance of considering tumor biology in treatment decisions versus clinico-pathological methods alone. Furthermore, MammaPrint and BluePrint may help predict chemo-sensitivity and its use as a biomarker of response to specific breast cancer treatments, is a topic for current and future trials.
LIST OF REFERENCES


7. National Cancer Institute: the Surveillance Epidemiology and End Results (SEER) database of the National Cancer Institute (NCI). 2010


77. Mitch Dowsett1, Roger A’Hern3, Janine Salter1,2, Lila Zabaglo1,2 and Ian E Smith4: Who would have thought a single Ki67 measurement would predict long-term outcome? Breast Cancer Research


118. Lander ES: Array of hope. Nat Genet


APPENDICES

Appendix A

Classification of patients according to clinical risk assessment by the modified version of Adjuvant!Online.

<table>
<thead>
<tr>
<th>ER Status</th>
<th>HER2 Status</th>
<th>Grade</th>
<th>Nodal Status</th>
<th>Tumor Size</th>
<th>Clinical Risk in MIND ACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER positive</td>
<td>HER2 negative</td>
<td>well differentiated</td>
<td>N-</td>
<td>(\leq 3) cm</td>
<td>C-low</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>3.1-5 cm</td>
<td>C-high</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\leq 2) cm</td>
<td>C-low</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.1-5 cm</td>
<td>C-high</td>
</tr>
<tr>
<td></td>
<td></td>
<td>moderately differentiated</td>
<td>N-</td>
<td>(\leq 2) cm</td>
<td>C-low</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.1-5 cm</td>
<td>C-high</td>
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<tr>
<td></td>
<td></td>
<td>poorly differentiated or undifferentiated</td>
<td>N-</td>
<td>(\leq 1) cm</td>
<td>C-low</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1.1-5 cm</td>
<td>C-high</td>
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<td></td>
<td></td>
<td></td>
<td>(\leq 1) cm</td>
<td>C-low</td>
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<td>C-high</td>
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<tr>
<td></td>
<td></td>
<td>moderately differentiated</td>
<td>N-</td>
<td>any size</td>
<td>C-high</td>
</tr>
<tr>
<td></td>
<td>HER2 positive</td>
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<td>N-</td>
<td>(\leq 1) cm</td>
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<td>tiated or undiffer entiated</td>
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<tr>
<td></td>
<td>1-3 positive nodes</td>
<td>any size</td>
<td>C-high</td>
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<tr>
<td></td>
<td>1-3 positive nodes</td>
<td>≤ 2 cm</td>
<td>C-low</td>
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<tr>
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<td>any size</td>
<td>C-high</td>
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<tr>
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<td>any size</td>
<td>C-high</td>
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</tbody>
</table>
MEMORANDUM: EXPEDITED

TO: Peter Blumencranz, MD.
FROM: Kevy Albert, IRB Specialist
SUBJECT: IRB File #2016.068-BMP

PROTOCOL: "COPPER study: Community-based retrospective study comparing the 70-gene signature with the common clinical-pathological criteria in selecting patients for neo or adjuvant chemotherapy in breast cancer with 0-1 positive nodes" Category 65

The Co-Chairperson of the Institutional Review Board (IRB) has reviewed and approved the above protocol under expedited review for this submission. Dr. Ian Matheson, Co-Chairperson, has determined that this project is exempt from Continuing IRB review (45 CFR 46.110 and 21 CFR 56.110).

Your project should not be resubmitted for a yearly continuing review to the IRB. A Final Report [DB Progress Report] and/or Journal article(s) should be submitted at the completion of the project. This information will be maintained in the study folder.

This action will be reported at the July 26, 2016 IRB Meeting.
Appendix C

BayCare Health System IRB Waiver Form

Study #

Study Title:
COPPER study - Community based retrospective study comparing the 70 gene signature with the consensus clinical Pathological criteria in selecting patients for neo- or adjuvant chemotherapy in breast cancer with 0-3 positive nodes.

Phone #: 727-462-7119 or 727-461-8326
Email: kylen.barlowe@baycare.org

To be completed by the Principle Investigator or his/her representative.

Principal Investigator (Please Print): Peter W. Kliman, MD

Authorized Representative (If Applicable): Kylen Barlowe, BS, CCPRP;
Debra Ellis, RN, CCPRP

Facility Address (Hospital/Organization Entity): The Comprehensive Breast Care Center of Tampa Bay, 400 Pinellas Street, Suite 200 Clearwater, FL 33756

BayCare Health System Institutional Review Board Waiver

The investigators and/or authorized representative named here are authorized to review Health Information for the purpose of conducting research at the (Hospital/Organization) for the following purposes:

A. To be advised that BayCare entities may use or share information for purposes of research if the entities obtain from the researcher information that represents that:

1. The information is requested solely to review health information as necessary to prepare a research protocol or for similar purposes preparatory to research.
2. The information is not to be removed from the BayCare entity by the researcher in the course of reviewing and
3. The health information is necessary for the research purpose.

HIPAA Waiver Requirements:

1. If you plan to use or share protected health information (PHI) when conducting your research, you must obtain your study in accordance with the Privacy Rule of the Health Insurance Portability and Accountability Act (HIPAA). This means that when applying for

BCIRB Waiver Form 2014
Page 1 of 2
In a waiver of documentation of consent, you will also have to request a waiver or alteration of HIPAA Authorization. The IRB can grant the waiver of alteration if it deems the following criteria are met; The protocol must include, at a minimum, the following elements:

a. An adequate plan to destroy identifiers at the earliest opportunity absent (without) a health or research justification or legal requirement to retain them, and
b. An adequate plan to protect health information identifiers from improper use or disclosure,

c. Adequate written assurances that the PHI will not be used or disclosed to a third party except as required by law, for authorized oversight of the research study, or for other research uses and disclosures permitted by the Privacy Rule;

d. Research could not practically be conducted without the waiver or alteration; and

e. Research could not practically be conducted without access to and use of PHI.

I (Principle Investigator, MD.), certify that I have notified BayCare Health System Institutional Review Board as noted in the protocol of the activities to be conducted at the hospital/office in conjunction with the investigational research study identified above.

PI's Signature: [Signature]

Date: 6/25/16

For IRB Stamp of Approval:

BayCare Health System - IRB
IRB File #: 3016-068-14
Approved Date: 07/06/16
Expiration Date: 07/05/18
IRB Signature: [Signature]