Characterization of Male Breast Cancer

From Molecule to Clinical Outcome

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Abstract

The aim of this thesis was to investigate different aspects of male breast cancer (MBC), and to compare these with findings in female breast cancer (FBC). In paper I, a population-based study was performed to investigate possible differences in treatment and outcome between MBC and FBC patients. MBC and FBC presented with a similar distribution of stage. Although no differences in primary treatment strategy were demonstrated, MBC patients had significantly poorer overall and relative survival, indicating a more aggressive disease. Paper II aimed to assess the value of clinicopathological factors and molecular subtypes in MBC. One hundred and ninety-seven MBC tumors were characterized using immunohistochemistry (IHC) and the findings were correlated to outcome. Lymph node positivity, larger tumor size and ER-negativity were independent risk factors for breast cancer death. Tumor grade, HER2, Ki 67 or IHC classification into molecular subtypes did not demonstrate any prognostic information. In paper III, the same patient material as in paper II was used for evaluation of proliferation markers. High levels of cyclin A and cyclin B expression and an elevated mitotic count were predictive of breast cancer death. Ki-67 was re-evaluated using different cut-offs, but no prognostic value could be demonstrated. Contrarily, overexpression of cyclin D1 was associated with a lower risk of breast cancer death. In papers IV-V, the molecular background of MBC tumors was investigated. Global GEX analyses were performed and two novel subgroups of MBC tumors were identified; luminal M1 and luminal M2. When comparing the degree of similarity with the “intrinsic” subtypes in FBC tumors, more than half of the MBC tumors remained unclassified. Comparative genomic hybridization was used to investigate DNA aberrations. Two MBC subgroups were identified, of which one did not resemble any of the female subgroups. In both studies on the molecular level, a majority of patients were classified into the subgroup with a more aggressive tumor behavior. In conclusion, MBC seems to be a unique tumor entity. The established molecular subtypes in FBC are not applicable in MBC. Other prognostic profiles, specific for MBC, need to be identified.

Keywords: male breast cancer, immunohistochemistry, prognostic, cyclins, gene expression, genomic profiling

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To Eric, Victor, Lovisa and Gustav.
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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### Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aCGH</td>
<td>Array CGH</td>
</tr>
<tr>
<td>AI</td>
<td>Aromatase inhibitor</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostate hyperplasia</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CGH</td>
<td>Comparative genomic hybridization</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratin kinase</td>
</tr>
<tr>
<td>CNA</td>
<td>Copy number alterations</td>
</tr>
<tr>
<td>DAVID</td>
<td>Database for annotation, visualization, and integrated discovery</td>
</tr>
<tr>
<td>DMFS</td>
<td>Distant metastasis free survival</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FBC</td>
<td>Female breast cancer</td>
</tr>
<tr>
<td>FGA</td>
<td>Fraction of genome altered</td>
</tr>
<tr>
<td>GEX</td>
<td>Gene expression</td>
</tr>
<tr>
<td>GISTIC</td>
<td>Genomic identification of significant targets of cancer</td>
</tr>
<tr>
<td>HCL</td>
<td>Hierarchical clustering</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>MBC</td>
<td>Male breast cancer</td>
</tr>
<tr>
<td>NAT1</td>
<td>N-acetyltransferase-1</td>
</tr>
<tr>
<td>NHG</td>
<td>Nottingham histological grade</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SAM</td>
<td>Significance analysis of microarrays</td>
</tr>
<tr>
<td>SISH</td>
<td>Silver in situ hybridization</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor size, node, metastasis</td>
</tr>
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</table>
Background

Introduction

Male breast cancer (MBC) is a rare disease accounting for <1% of all breast cancer. Current knowledge about MBC is based mainly on population-based studies or descriptive studies that assess a limited number of patients. The optimal treatment for MBC is presently not known and the general recommendation is to use current guidelines for female breast cancer (FBC). Some differences regarding clinicopathological characteristics have been identified but it has not yet been established whether the prognostic and predictive factors used in FBC have the same impact in MBC. Our aim with this research project was to further examine possible differences in clinicopathological characteristics, treatment modalities and outcome in male and female breast cancer. In addition, we also wished to characterize MBC by using immunohistochemistry and molecular array techniques to identify factors with prognostic and/or predictive value which might be of importance for treatment decisions.

The male breast

The breast is morphologically similar in boys and girls until puberty and consists of small ductal structures in a fibroblastic stroma. The walls of the ducts consist of two layers of epithelial cells; basal (myo-epithelial cells) and luminal (epithelial) cells. In females the breast continues to develop during puberty with the proliferation of end-bud-like structures into terminal duct lobular units, which are the functional units of the breast. After puberty, the female breast continue to undergo developmental changes during the menstrual cycle and pregnancy and this constant remodeling under the influence of hormones and growth factors is thought to make the female breast particularly susceptible to carcinogenic events. In males no further development normally occurs after childhood, leaving the male breast in a rudimentary state. The most common abnormality in the male breast is gynecomastia, defined as an abnormal overgrowth of the breast tissue most often seen during adolescence or in middle-aged men. The condition is benign and often reversible. The histological appearance in gynecomastia is characterized by an increase in the number of ducts together with an increment in stromal cellularity and vascularity. Identified risk factors for gynecomastia
are different hormonal imbalances, which demonstrate that the male breast like the female breast can proliferate under the influence of hormonal alterations. The male breast does not normally develop lobular units.

Epidemiology

The incidence of MBC varies greatly worldwide, ranging from 5-15% of all breast cancer in certain African countries to less than 1% in North America and Europe. The higher incidence in certain African countries has been explained by infectious diseases that cause liver damage, which in turn leads to higher estrogen levels. In the Nordic countries, breast cancer accounts for 0.2% of all invasive cancer in men, with approximately 100 men diagnosed with the disease every year compared with 17,000 women. A slight increase in age-standardized incidence has been reported. In MBC, a unimodal age distribution is seen, with a peak incidence in the sixth decade of life. The mean age at diagnosis occurs approximately five years later than that for FBC. The mortality rate seems to be stable in MBC, which is in contrast to the decline observed in FBC and due to improved diagnostic procedures and treatment methods.

Risk factors and genetics

In several studies, obesity has been identified as a risk factor for MBC. Other factors which have been associated with an increased risk are: physical inactivity, bone fractures after the age of 45, gynecomastia, orchitis/epididymitis, liver cirrhosis and radiation exposure. Many of these risk factors are associated with an altered ratio of estrogen/androgen, resulting in a relative excess of estrogen. There are also case reports describing men that have developed breast cancer after treatment with exogenous estrogen, such as in prostate cancer and in transsexuals. An association between benign prostate hyperplasia (BPH) and MBC has also been discussed; however, it is unclear whether similar changes in sex hormone balance could predispose for both conditions or if treatment with finasteride in BPH could increase the risk of breast cancer. Genetic predisposition is considered to be of greater importance in MBC, compared with FBC. Approximately 20% of all MBC patients report a first-degree relative with breast cancer or ovarian cancer. BRCA2 mutations have been identified in 4-40% of MBC patients, whereas BRCA1 mutations are infrequent. The estimated risk for male carriers of BRCA 2 of developing breast cancer is 5-10%, whereas the risk for BRCA1 carriers is 1-5%. CHEK2, a cell cycle checkpoint kinase, has also been suggested to play a role in the development of MBC. The most well-known single condition...
predisposing for MBC is Klinefelter’s syndrome (carrier type XXY) with a 30-50 relative risk of developing breast cancer\textsuperscript{15,16}.

**Diagnosis**

The most common clinical symptom of MBC is a painless lump in the breast. The tumors are most often located in the retroareolar area and nipple involvement is a relatively early event, with retraction described in 7-9% and discharge in 2-6% of cases\textsuperscript{3,17}. The recommended diagnostic work-up is a combination of clinical examination, mammography and/or ultrasonography and histopathological verification with fine-needle aspiration or core biopsy\textsuperscript{3}. Mammography is a useful tool in the diagnostic procedure with an estimated sensitivity of 92% and a specificity of 90%\textsuperscript{18}.

At diagnosis all breast cancer tumors are described according to the TNM staging system. The system describes tumor size (T), lymph node status (N), and distant metastases (M), and has definitions for clinical and pathological staging. Clinical staging is based on information before surgery, encompassing the clinical examination and radiological imaging. Pathological TNM staging is denoted by the prefix p, for example pT1N0M0, and is based on the histopathologic examination which reveals additional information, especially in describing lymph node status (Table 1). Based on the three parameters in the TNM system, breast tumors are also grouped into different stages\textsuperscript{19} (Table 2).

**Table 1. TNM classification (7\textsuperscript{th} edition)**

<table>
<thead>
<tr>
<th>Tis</th>
<th>In situ</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>≤20 mm</td>
</tr>
<tr>
<td>T2</td>
<td>&gt;20-50 mm</td>
</tr>
<tr>
<td>T3</td>
<td>&gt;50mm</td>
</tr>
<tr>
<td>T4</td>
<td>Chest wall/skin ulceration, skin nodules, inflammatory</td>
</tr>
<tr>
<td>N1</td>
<td>Movable axillary</td>
</tr>
<tr>
<td>N2</td>
<td>Fixed axillary or internal mammary nodes clinically apparent</td>
</tr>
<tr>
<td>N3</td>
<td>Supra/infraclavicular nodes or internal mammary together with axillary nodes</td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>
### Table 2. Stage classification based on the TNM system

<table>
<thead>
<tr>
<th>Stage</th>
<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 I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T0-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-T2</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N1-N2</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>T4</td>
<td>N0-N2</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIC</td>
<td>Any</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any</td>
<td>Any</td>
<td>M1</td>
</tr>
</tbody>
</table>

MBC has often been reported to be diagnosed at a more advanced stage than FBC, with positive axillary lymph nodes reported in 40-50% and a high proportion of tumors classified as T4 because of skin or nipple involvement. The nipple involvement seen in MBC patients is a result of direct tumor extension to the epidermis and is not comparable to Paget’s disease of the nipple, which is most often associated with cancer in situ. Nipple involvement seems to be associated with a high risk of lymph node metastasis. It has been hypothesized that the association between tumor size and the presence of lymph node metastases is different in male breast cancer, where the smaller anatomical distances between breast parenchyma and skin and/or chest wall could possibly increase the risk of regional metastases. In line with this, studies in MBC have reported a high rate of axillary nodal involvement even in small tumors (pT1). The most common sites for metastases in advanced disease are the skeleton and lungs. Invasive breast tumors are classified according to histological appearance. In FBC, the most common histology is ductal carcinoma (75%) followed by lobular carcinoma (5-15%). The predominant histology in MBC tumors is invasive ductal carcinoma which is described in >90% of cases. Lobular carcinoma is infrequent, probably due to the fact that the male breast tissue only differentiates into lobules when excessive levels of endogenous or exogenous estrogen are present. Isolated in situ cancers account for approximately 5% of MBC tumors.
Outcome of MBC compared with FBC

MBC has in certain studies been described to resemble post-menopausal FBC, with a more indolent tumor behavior 3, 7. Other reports indicate that hormone-receptor-positive MBC tumors are often of higher grade and proliferation, suggesting a more aggressive behavior 8, 25. Comparisons of outcome in cases of male and female breast cancer are contradictory. Several studies report a poorer overall survival for MBC patients when compared with FBC 5, 26. However, this could be attributed to older age and the increased mortality of men in the general population. Disease-specific survival and relative survival have been evaluated in certain studies. In one register study, MBC patients had a statistically significant poorer breast cancer specific survival at longer follow up 22, whereas no difference in disease-specific survival could be seen in other smaller age- and stage matched series 27, 28.

A recent population-based multi-institutional study, comparing men and women diagnosed with breast cancer over the last 40 years, concluded that MBC patients had a more advanced disease and higher age at diagnosis. The male patients underwent surgery and/or radiotherapy less often, but there were no differences in the administration of chemotherapy or endocrine treatment between the genders. MBC patients had a poorer overall and relative survival in an overall comparison. After adjusting for multiple parameters including stage, age and treatment, however, the male group had a better relative survival 29.

Two other population-based studies could not prove any difference in relative survival between male and female breast cancer patients, although one of them demonstrated a trend towards poorer survival in men 5, 30.

Treatment

Surgery

Surgery is the most important treatment modality in the management of localized breast cancer. In MBC, modified radical mastectomy is considered most feasible in the majority of cases because of the paucity of breast tissue together with the predominantly central location of tumors 13, 14, 22. However, breast conservative surgery followed by radiotherapy is a feasible option 31. Axillary lymph node involvement is a strong prognostic factor and should always be evaluated in invasive breast cancer. Two different procedures are used for determining the axillary nodal status; axillary dissection or sentinel lymph node biopsy. The sentinel node is by definition the first lymph node to receive lymphatic drainage from the tumor, and in FBC, several studies have shown that sentinel node biopsy is reliable in predicting axillary lymph
node status\textsuperscript{32, 33}. Sentinel node biopsy decreases the comorbidity related to axillary surgery and is currently the standard procedure in a majority of FBC patients with a clinically normal axilla. Sentinel node biopsy has been evaluated in small series in MBC and appears to be a reliable method.\textsuperscript{34, 35} In one of the largest series, 78 men with a clinically negative axilla underwent sentinel node biopsy. Biopsy was successful in 76 of 78 men. In 3 patients (8\%) with a negative SN, a positive non-SN was found. No axillary recurrences were seen after a median follow-up of 28 months. Males are considered to have the same risk of comorbidity after full axillary dissection as females and SN is, therefore, the recommended method\textsuperscript{13, 35}.

Radiotherapy
Postoperative radiotherapy after breast-conserving surgery or mastectomy reduces the risk of recurrence and improves overall survival in FBC\textsuperscript{36}. Radiotherapy is recommended after breast-conserving surgery and in all patients with >3 positive lymph nodes, regardless of the method of surgery\textsuperscript{36}. Post-mastectomy radiotherapy is recommended in the following situations; tumors >5 cm, uncertain surgical margins and skin involvement. Some studies also demonstrate a benefit from postoperative radiotherapy in patients with 1-3 positive lymph nodes\textsuperscript{37}. The use of postoperative radiotherapy in MBC has been described in a few retrospective studies. In one of the largest series, 85\% of patients received postoperative radiotherapy. A rate of 1.8\% chest recurrence and 5.3\% nodal recurrence was observed, which is considered low and suggests a benefit from radiotherapy\textsuperscript{17}. In some earlier studies, a higher risk for local recurrence was reported and it was hypothesized that this could be explained by the smaller anatomical distances between breast tissue and skin/chest wall\textsuperscript{38}. Consequently, some authors have suggested post-mastectomy radiotherapy on broader indications in MBC (tumors >1 cm)\textsuperscript{39}. However, in a study evaluating outcome in MBC patients who had been treated with mastectomy without radiotherapy, the risk of loco-regional recurrence was low and the recommendation was to follow the same indications as in FBC\textsuperscript{40}. This is supported by a study comparing male and female breast cancer patients treated with mastectomy, where no differences in the loco-regional relapse-free survival rates after radiotherapy was observed\textsuperscript{41}.

Endocrine treatment
A majority of MBC tumors express estrogen receptors (ER) and/or progesterone receptors (PR), and endocrine treatment is considered an important part of the treatment. Historically, different endocrine ablative treatments such as orchidectomy were used. Today, tamoxifen is considered
to be the first choice in the adjuvant and metastatic setting. There are no prospective studies evaluating endocrine treatment in MBC. Some retrospective reports have demonstrated a significantly improved outcome for patients administered adjuvant tamoxifen. In metastatic disease, response rates of 32-75% with different endocrine treatments have been described.

On the other hand, other studies have described an inferior survival in hormone receptor positive MBC cases when compared with FBC patients, despite similar endocrine treatment practices. Furthermore, it has been suggested that ER do not have the same function in MBC as in FBC and that the benefit achieved with tamoxifen is uncertain. Knowledge about potential side-effects produced by endocrine treatment in MBC patients is scarce. The role of aromatase inhibitors (AIs) in MBC is unclear. In men, approximately 80% of circulating estrogens are produced from peripheral aromatization of testicular and adrenal androgens with the remaining 20% being derived from direct testicular production. AIs do not affect the estrogen production in the testes and it is, therefore, recommended to combine this treatment with surgical or medical orchiectomy. However, some case reports have described objective responses after AI single treatment in the metastatic setting. In line with this, a small study (n=15) examining serum levels of estradiol in men on treatment with AI, reported that some patients with a total and partial response displayed corresponding low levels of estradiol in their serum samples.

Chemotherapy

To date, only one prospective evaluation of adjuvant chemotherapy in MBC is available. Thirty-one MBC patients with node positive disease were enrolled in a study in which adjuvant CMF (cyclophosphamide, methotrexate, and fluorouracil) was administered. Compared with historical controls, a benefit from the treatment was suggested. However, disease-specific survival could not be evaluated since the cause of death was not known in all cases. Furthermore, the chemotherapy regimen administered is not considered to be standard treatment today. Some additional small retrospective series suggest a benefit from adjuvant chemotherapy, although no statistically significant results have been demonstrated, which may be explained by the small patient samples. The general recommendation today is that male patients should be offered chemotherapy according to the guidelines recommended for FBC patients.
Prognostic and predictive factors

In FBC, significant improvements in disease-free and overall survival have been achieved by continuous improvements in diagnostic and treatment strategies. A majority of patients who undergo surgery for localized breast cancer are offered adjuvant radiation therapy and/or systemic treatment with the aim of decreasing the risk of breast cancer recurrence. Prognostic and predictive factors are used in order to identify patients with a higher risk for recurrence and to select the most appropriate adjuvant treatment strategy for each patient. Prognostic factors are factors that predict outcome independently of treatment (or with standard treatment), whereas predictive factors predict response to a specific treatment. Established prognostic factors in FBC are age, lymph node status, tumor size, histologic grade, PR and human epidermal growth factor 2 (HER2) status 48-53. According to some guidelines, Ki-67 is considered to have prognostic value and can also have a role in indicating the potential benefit of chemotherapy 54. The use of molecular gene expression analysis as a prognostic tool in clinical practice is still under debate but is considered to provide additional information in FBC patients with hormone-sensitive, lymph node negative tumors 53. ER and HER2 are also predictive factors, used to identify patients who might benefit from endocrine treatment (tamoxifen and AIs) and agents directed against the HER2 receptor, respectively 52.

Due to the rarity of MBC there are only a few previous studies that aim at evaluating these prognostic and predictive factors in MBC, and today it is unclear whether adjuvant treatment strategies recommended for FBC patients are applicable in MBC patients.

Nodal status and tumor size

In a register study based on 2,537 MBC patients, tumor size >20 and positive lymph node status were independently associated with poorer survival 5. In another analysis on >400 cases of MBC, nodal status but not tumor size were independently associated with increased risk for metastatic disease 17. The prognostic impact of positive nodal status seems to be at least equally negative in male as in FBC 7, 55.

Histological grade

In a study comparing male and female breast cancer, MBC was more often than FBC classified as low grade, thus indicating a more indolent growth pattern 7. However, in other studies, the contrary has been reported 8, 51. In a recent study, high tumor grade and lymph node status were the only risk factors for metastatic disease in a multivariate analysis 17. Other studies indicate that grade is not an independent prognostic factor 5.
Estrogen and progesterone receptor

Most studies report that MBC tumors express ER and PR to a greater extent than FBC (80-90% versus (vs.) 75% and 73-81% vs. 65%, respectively) \(^{13, 14, 17}\). Some studies indicate that ER positivity is associated with a better overall survival \(^{24}\), whereas other series could not find any prognostic value with ER and/or PR status \(^{5}\). The predictive and/or prognostic values of these parameters have not been confirmed in prospective studies.

HER2

HER2 is a member of the transmembrane epidermal growth factor family (EGFR) with the corresponding gene on chromosome 17. Overexpression and amplification are seen in 11-30% in FBC and correlate with a poorer prognosis \(^{23, 56}\). HER2 status can be determined with two different methods; immunohistochemical staining for evaluation of protein expression or in situ hybridization for evaluation of gene copy numbers. Different methods and arrays are used for HER2 testing, but in general, IHC staining 3+ (a uniform and intense membranous positivity in more than 30% of the tumor cells) or amplification with \(\geq 6\) copies of the HER2 gene or a ratio of HER2 gene signals/chromosome 17 signals exceeding 2.2 are all considered as HER2 positive. Different HER2 directed therapies have become a cornerstone in the treatment of HER2 positive patients. Trastuzumab is a monoclonal antibody that binds to the extracellular domain of HER2 and has been demonstrated to improve response rate, time to progression and overall survival when used in combination with chemotherapy or endocrine treatment and is today routinely used in the adjuvant and metastatic setting in HER2 positive patients \(^{57-59}\). Additional HER2 targeted therapies have demonstrated improved time to progression in HER2 positive patients with metastatic disease \(^{60, 61}\).

In MBC, the prognostic or predictive value of HER2 is unclear. Studies examining the prevalence of HER2 overexpression in MBC have shown highly varying results, with HER2 expression present in 0-37% and amplification in 0-11% of patients \(^{62, 63}\). Two of the largest series, with 129 and 99 evaluable patients, demonstrated an overexpression in 4 and 15% of patients, respectively \(^{63, 64}\). The prognostic and/or the predictive values of HER2 in MBC have not been examined in the referred studies.

Array-based analysis

The genome, which is equivalent to the DNA, encompasses the genetic information of the cell. The DNA molecule is built up of nucleotides or bases in two complementary strands, and is densely packed into chromosomes (Figure 1). All cancer arises as a result of accumulated changes, i.e.
mutations, in the DNA sequence of the genomes in the cancer cells. These mutations range from single nucleotide substitutions to quantitative and structural changes of larger chromosome regions. The genome contains segments of DNA which encode different genes. Genes are transferred to messenger RNA (mRNA) in a process called transcription. The mRNA molecules are thereafter translated to form proteins, the functional molecules and main building blocks of the cells (Figure 2). The difference between various cell types is mainly due to the different subsets of genes expressed in each cell type.

Figure 1. DNA structure. https://creationwiki.org/DNA

Figure 2. Protein synthesis. https://creationwiki.org/Protein synthesis
Gene expression (GEX) profiling is a method of determining which genes are active in a specific cell population. The activity of the genes can be detected by measuring mRNA levels. In several cancer entities, tumor subgroups with distinct gene expression patterns have been identified, probably reflecting basic differences in the cell biology of tumors 66, 67.

Normal cells contain two copies of each chromosome. By studying alterations of the chromosomal copy number (Copy Number Alterations (CNA)) in cancer cells and comparing these genomic profiles to normal cells, information about the genes involved in cancer development and progression can be achieved. Additionally, the different patterns of DNA alterations can give information about the underlying mechanisms of genomic instability 68. Comparative Genomic Hybridization (CGH) is a method used for analyzing CNA. GEX or CGH can be examined with microarray based technologies, by which thousands of genes or CNAs can be analyzed at the same time. There are different types of microarrays, but typically a microarray is built up of thousands of fragments of DNA molecules or oligonucleotides (spots) printed on a solid substrate, for example a glass slide. One common application is to compare the gene expression levels in two different samples, for example reference sample and tumor sample. From each sample, RNA is extracted and labeled with fluorescent dyes (green for reference sample and red for tumor sample). Samples are thereafter hybridized onto the microarray and, by analyzing the fluorescence intensities and colors for each spot, the relative expression level of the genes in the tumor sample can be estimated. For CGH, overlapping (tiling) bacterial artificial chromosomes (BAC) are often used for the construction of whole genome arrays with a high degree of resolution. Reference and tumor sample are co-hybridized onto the array and by analyzing the fluorescence intensities, the chromosomes in the tumor are compared with normal chromosomes, and gains and losses can be detected. A microarray experiment produces a very large amount of complex raw data that need to be processed by different computational and statistical methods in order to obtain a comprehensive picture of the gene expression patterns.

In FBC, it has been found that the classification of tumors based on gene expression patterns is of prognostic importance. In the primary studies, a set of genes was identified which consisted of genes which varied significantly between different tumors but with less variation in paired samples from the same patients, the so-called “intrinsic gene set”. Based on this gene set, FBC can be classified into 5 different subtypes, viz. luminal A and B, basal-like, HER2-like and normal-like. The diversity between different subtypes is largely driven by the expression of ER-related, proliferation-related and HER2-related genes 69, 70. The subtypes can broadly be characterized as follows:

- **Luminal A type**: high expression of ER and ER-related genes
• **Luminal type B**: expression of ER-related genes together with proliferation-related genes
• **Basal-like**: low expression of ER, PR and HER2, together with expression of basal cytokeratins
• **HER2-type**: expression of HER2 and related genes
• **Normal-like**: expression of genes seen in adipose and other non-epithelial tissue.

The most important finding is that ER positive and ER negative breast cancers are fundamentally different diseases on the molecular level, with the greatest separation between luminal and basal-like tumors. Luminal tumors, which are clinically ER positive, are characterized by a high expression of genes expressed by luminal (epithelial) cells, whereas basal-like tumors demonstrate gene patterns seen in basal (myo-epitelial) cells \(^70\). Comparisons between the subtypes have demonstrated significant differences in recurrence free survival and overall survival, with the basal-like and HER2-like groups having the poorest outcome. Significant differences were also seen between the hormone-receptor positive groups, luminal A and B.

One of the main challenges in clinical oncology is to distinguish between tumors with high or low risks of recurrence, in order to more specifically identify those patients in need of more intensive treatment and concurrently identify patients in whom it is safe to omit adjuvant systemic treatment with potential harmful side-effects. Gene arrays mirror the heterogeneity of breast cancer tumors and may more accurately describe the tumors clinical behavior compared with the traditional clinicopathological prognostic factors. Gene array profiles have been developed for the prediction of outcome in specified subsets of FBC patients. A majority of these profiles are applicable in patients with hormone receptor positive and lymph node negative disease and have, in this context, demonstrated additional prognostic information when compared with clinicopathological parameters. A unifying characteristic of these profiles is a high expression of proliferation-related genes \(^71\). Two of the most validated, commercially available, gene profiles are the 70 gene profile (Mammaprint) and the 21-gene recurrence score (Oncotype DX). The 70-gene profile was identified in a group of young (<55 years) and primarily node-negative women. The genes included are involved in the cell cycle, invasion and metastasis, angiogenesis and signal transduction \(^72\). The 21-gene recurrence score was developed for prognostication in hormone receptor positive, node- negative patients. Of the 21 genes, 16 are tumor related with the main emphasis on proliferation \(^73\).

FBC tumors have also been classified by array CGH (aCGH), and different genomic sub-groups have been identified, viz. luminal simple, luminal complex, basal complex, amplifier, 17q12 and mixed. The groups demonstrate different tumor biological features and clinical behavior and four of the subgroups (luminal simple, luminal complex, basal complex, 17q12) demonstrate clear similarities with the gene expression subgroups \(^74\).
Today, large-scale subtyping using array-based techniques on paraffin-embedded tumor tissue is not feasible due to its high costs. As an alternative, combinations of immunohistochemical (IHC) markers have been identified, broadly matching the different GEX subtypes. One of the most commonly used definition for IHC subtypes are: luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), core basal-like (ER-, PR-, HER2-, CK5/6 positive and/or EGFR+), HER2-like (ER-, PR-, HER2+) and unclassified (negative for all 5 markers) \(^{75, 76}\). Other definitions use grade or proliferation markers in addition to hormone status and HER2 status to better differentiate between the luminal groups \(^{77, 78}\).

MBC tumors have previously not been classified into subtypes by GEX arrays or high resolution aCGH. In one study, gene expression analysis was used to compare male and female breast tumors with similar clinicopathological features. Differences in gene expression were found, possibly indicating different pathways for tumor progression and treatment response in MBC compared with FBC \(^{79}\).

On the contrary, a small study using chromosomal CGH in MBC revealed the same patterns of chromosomal imbalances as in FBC, suggesting similar genetic events behind tumor progression in male and female breast cancer \(^{80}\). However, chromosomal CGH can only detect larger chromosomal aberrations.

Immunohistochemical characterization of MBC tumors indicates that luminal A is the most common subtype (seen in 75-98% of cases) followed by luminal B (0-21%). A very small subset of tumors in performed studies have been classified as basal-like, and the HER2-like subtype seems to be even more infrequent \(^{81-83}\). The prognostic relevance of the different subtypes has not been evaluated, due to a lack of outcome data.

**Proliferation and the cell cycle**

A key step in tumor growth is the cells capacity to proliferate, i.e. to duplicate and divide. Proliferation is driven by the cell cycle, which consists of a precisely defined set of phases; gap1 (G1); synthesis (S); gap2 (G2); mitosis (M) and the quiescent phase (G0) (Figure 3). In the G1 phase, the cells are prepared for DNA synthesis, which occurs during the S-phase. In G2, cells grow before entering the M-phase, in which mitosis takes place. Non-dividing cells remain in G0. The different steps of the cell cycle are executed by cyclin-dependent kinases (CDK) which, in turn are, controlled by changes in the availability and levels of the different cyclins (Figure 4). This complex system is also regulated by CDK inhibitors.

In tumor cells, different steps of the cell cycle can be affected leading to a dysregulated cell cycle process and uncontrolled proliferation.
The prognostic and predictive value of cyclins and other proliferation markers have been investigated by looking at gene alterations and/or the corresponding protein product.

**Figure 3.** The cell cycle. The figure illustrates how each type of cyclin pairs with a specific cyclin-dependent kinase and in which phase the complexes are active. Adapted from *The Biology of Cancer, Robert A. Weinberg, 2007.*

**Figure 4.** Cyclic changes in the availability and levels of the different cyclins

**Proliferation markers in FBC**

In recent years, there has arisen a renewed interest in proliferation markers, partly explained by the increasing interest in molecular subtyping. As previously mentioned, proliferation genes are important in the differentiation
between the molecular subtypes. In the commercially available 21-gene recurrence score, Ki-67 and cyclin B are two of 16 tumor-related genes. However, the utility of gene arrays is limited by high costs and several studies suggest that proliferation factors evaluated by IHC may provide similar prognostic information.

Protein overexpression of cyclins and the proliferation marker Ki-67 can be evaluated by IHC and several studies have shown that the overexpression of Ki-67, cyclin A, cyclin B and cyclin E is prognostic of a poorer outcome in FBC patients.

The Ki-67 antibody binds to a nuclear antigen present in all phases of the cell cycle except the quiescent phase (G0) and is, as such, a marker of proliferating cells. Initially, the Ki-67 antibody could only be used on fresh or frozen tissue, which reduced its clinical usefulness. MIB1 is an equivalent antibody developed for the evaluation on paraffin embedded tissue. Studies in FBC have demonstrated an association between high levels of Ki-67 expression and a poorer outcome. The expression level defining high proliferating tumor have varied from 5-34%, with 10 and 20% as the most frequently used cut-offs. In the St Gallen consensus conference 2011, the recommended cut-off was specified as 14%.

Cyclin A is a protein which increases in early S-phase and decreases during mitosis. Several studies in FBC have demonstrated that the overexpression of cyclin A can predict recurrence and breast cancer death. In a study evaluating Ki-67 and cyclin A in the same patient cohort, it was suggested that cyclin A was a stronger prognostic factor than Ki-67. Furthermore, it has been indicated that cyclin A expression is a stronger prognostic factor in ER positive patients.

Cyclin B increases at the beginning of the S-phase and reaches its maximum during mitosis. Overexpression of Cyclin B in early FBC is predictive of poorer outcome. It has been shown that Cyclin B is associated with tumor grade and HER2, but does not seem to correlate with tumor size. Cyclin B overexpression has been demonstrated more often in luminal B and basal-like FBC.

Cyclin E regulates the progression of the G1 phase and the entry into the S-phase. Overexpression of cyclin E1 protein in FBC has been associated with a poorer outcome in several retrospective studies. There seems to be a correlation between cyclin E and high tumor grade or ER-negativity. High cyclin E expression has also been reported to be predictive of resistance to tamoxifen.

The D-type cyclins are mainly controlled by extracellular signals and have a role in the G1/S transition. There are at least three different cyclin D proteins, of which cyclin D1 has been most extensively studied but has produced contradictory results. Cyclin D1 is encoded by the gene CCND1 but amplification of the gene does not always seem to correspond with an overexpression of the protein. Overexpression of cyclin D1 appears to promote cell proliferation by binding to specific cyclin-dependent kinases. It also
seems to act independent of Cdks by acting as a co-factor for the ER-α. Cyclin D1 protein overexpression has demonstrated variable results in predicting breast cancer outcome and does not seem to be a useful prognostic factor in FBC. Tamoxifen exerts its effect by blocking ER-α and certain studies suggest that cyclin D1 can have a predictive role, with overexpression linked to tamoxifen resistance. However, many of the referred studies are retrospective and with heterogeneous study populations, thus making the interpretation of data more difficult. In addition, there has been a lack of consensus regarding IHC staining methodology, scoring and cut-off levels. As a consequence, only KI-67 is today recommended for use in routine clinical practice. The other proliferation markers have yet to be validated in further studies.

**Proliferation markers in MBC**

Ki-67 is the most studied proliferation marker in MBC but results are inconclusive. Some studies indicate an association between high levels of Ki67 expression and a poorer outcome, whereas other studies fail to demonstrate any prognostic importance. A few small series have examined the prognostic value of Cyclin D1 in MBC, but results are contradictory. The prevalence of CCND1 amplification and cyclin D1 expression was examined by Bärlund et al, and revealed CCND1 amplification in 12% and protein overexpression in 63% of cases. However, due to a lack of outcome data, the prognostic significance of cyclin D1 could not be evaluated. To date and to our knowledge, other cyclins have not been investigated in MBC.

As previously described, MBC tumors express hormone receptors to a great extent and tamoxifen is often the recommended adjuvant treatment. In line with data presented in FBC, it is of interest to examine whether cyclin D1 could be predictive of tamoxifen resistance in MBC.

In summary, information about the molecular biology in MBC comes from small series, with only a few studies having more than 100 cases. Different methodologies have been used as well as different cut-off points, in order to achieve what has been considered a positive result. All in all, this leads to a great inconsistency between studies. Furthermore, in a majority of studies, the prognostic value of different markers has been difficult to assess because of small patient samples and a lack of outcome data.
Aims of the thesis

The aim of this study was to investigate whether MBC differs from FBC. If this is the case, it could have potential implications for the clinical management of male breast cancer. In the first project, male and female breast cancers were compared with regard to treatment and outcome. In the following studies MBC tumors were characterized using IHC and molecular array techniques to evaluate the prognostic impact of clinicopathological factors and to learn more about the tumor biology background.

The specific aims were:

Paper I: To investigate whether there were any differences in adjuvant treatment and outcome in MBC and FBC patients.

Paper II: To evaluate the prevalence and prognostic impact of routinely used immunohistochemical markers in MBC. Furthermore, to classify into molecular subtypes using IHC markers according to alternative definitions and to evaluate their prognostic impact.

Paper III: To evaluate the prevalence and prognostic value of proliferation markers in MBC.

Paper IV: To characterize MBC tumors on the transcriptional level, to classify into comprehensive subgroups and to compare identified subgroups with established subgroups in FBC.

Paper V: To characterize MBC on the genomic level using high resolution aCGH, compare with FBC and, furthermore, to investigate whether the genomic subtypes in MBC have prognostic value.
Materials

Paper I
All men diagnosed with invasive breast cancer (n=99) in the Uppsala-Örebro region between the years 1993-2007 were identified and data retrieved from the Regional Breast Cancer Register. The same register was used for the random sampling of four FBC patients for each MBC patient. The Regional Breast Cancer Register has a high level of completeness and a high level of agreement as regards data on primary treatment retrieved from patients files\textsuperscript{106}.

Paper II-III
The National Cancer Register was used to identify male patients diagnosed with breast cancer between the years 1990 and 2007 in two regions of Sweden, encompassing a population of 1.70 (Lund region) and 1.96 million people (Uppsala-Örebro region), respectively. In the Lund region, 131 patients and in Uppsala-Örebro, 131 patients were identified. All patient charts were reviewed and accessible paraffin-embedded tumor blocks collected. Patients with in situ cancer (Uppsala-Örebro n=4, Lund n=2) and cases with missing patients charts (Uppsala-Örebro n=0, Lund n=2) were excluded. In Lund, patients from the years 2006-2007 were excluded since a higher proportion of tumor blocks were missing when data from the National Cancer Register was utilized. Finally, a cohort of 109 patients from Uppsala-Örebro from 1990 to 2007 and 88 patients from Lund from 1990 to 2005 were identified. Updated information about the patients’ vital status and cause of death were retrieved from the National Population Register.

Paper IV-V
MBC patients from Skåne University Hospital, Uppsala University Hospital and Örebro University Hospital with available fresh frozen and paraffin-embedded tissue were identified. In Paper IV, a total of 66 and in paper V, a total of 56 MBC tumors with sufficient tumor material were identified and could be included in the studies. Medical charts were reviewed for the collection of data on clinicopathological characteristics, treatment and outcome. All tumors were graded according to current pathological standards by a board-certified breast pathologist (ST). ER, PR and HER2 were re-evaluated as described in paper II.
Methods

Paper I

In a retrospective setting, cohorts of men and women diagnosed with invasive breast cancer were analyzed. A sampling procedure was conducted in order to achieve comparable groups. To increase the power of the study, each male (n=99) was randomly sampled with four females (n=396). The sampling procedure was performed using age at diagnosis (<49, 50-59, 60-69, 70-79, ≥ 80 years of age) and time of diagnosis (1993-1997, 1998-2002, 2003-2007). Data on tumor stage, hormone receptor status, tumor grade and primary treatment were retrieved from the register. In all patients, updated information about vital status was obtained from the National Population Register.

Paper II-III

The Nottingham histological grade (NHG) was evaluated on hematoxylin and eosin (HE) stained conventional slides while immunohistochemical (IHC) stainings were evaluated on tissue microarrays (TMA).

Construction of TMA block

Hematoxylin and eosin sections from the original paraffin-embedded tumor blocks were used to determine the most representative areas of invasive tumor. From the tumor blocks, two 1 mm biopsies were punched and brought to recipient paraffin blocks to construct tissue microarrays (TMA). The TMAs were then cut into 4µm thick sections and transferred to glass slides (Figure 3).
Immunohistochemical staining

All immunostainings were performed on sections from the TMA blocks. For the preparation of IHC markers used in paper II, a fully automated IHC staining machine (BenchMark Ultra, Ventana) was used for deparaffinization, cell-conditioning and staining. EGFR was stained using DAKOs EGFRpharmDX. HER2 SISH, a fully automated silver in situ hybridization (SISH) from Ventana, was used for evaluation of HER2 amplification. For the staining of cyclins in paper III another process was implemented. Slides were deparaffinized and rehydrated through a ladder of graded ethanols (absolute ethanol, 95%, 80% and distilled water). For cyclin A and cyclin D1, antigen retrieval was performed in a microwave oven for 10 minutes (750W) + 15 minutes (350W) with the use of a TE-buffer. For Cyclin B, antigen retrieval was carried out in a pressure cooker for four minutes with the use of TRS-buffer. After antigen retrieval, all TMA slides were processed in an automatic immunohistochemistry staining machine according to standard procedures (Autostainer, Dako, Sweden). See Table 3 for antibodies and dilutions.

For evaluation of HER2 status, all cases were analyzed by IHC and by SISH. Using IHC, cases were classified as 0, 1+, 2+ or 3+ according to standard procedures. With SISH, cases were scored as no amplification if 5 or fewer copies of the HER2 gene were present per nucleus in more than 50% of the tumor cells. Tumors were classified as HER2 positive when amplified or when HER2 IHC 3+ if amplification was not evaluable.
Table 3. Summary of antibodies, clones, dilutions and preparations used in paper II-III

<table>
<thead>
<tr>
<th>Marker</th>
<th>Clone</th>
<th>Vendor</th>
<th>Dilution</th>
<th>Preparation</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>SP1</td>
<td>Ventana</td>
<td>Ventanas Ready to Use</td>
<td>CC1 Standard</td>
<td>II</td>
</tr>
<tr>
<td>PR</td>
<td>16</td>
<td>Leica</td>
<td>1:25</td>
<td>CC1 Standard</td>
<td>II</td>
</tr>
<tr>
<td>Ki-67</td>
<td>MIB-1</td>
<td>DAKO</td>
<td>1:50</td>
<td>CC1 Mild</td>
<td>II</td>
</tr>
<tr>
<td>CK5/6</td>
<td>D5/16B4</td>
<td>DAKO</td>
<td>1:25</td>
<td>CC1 Standard</td>
<td>II</td>
</tr>
<tr>
<td>EGFR</td>
<td>1494</td>
<td>DAKO</td>
<td></td>
<td>EGFR pharmDx</td>
<td>II</td>
</tr>
<tr>
<td>HER2</td>
<td>4B5</td>
<td>Ventana</td>
<td>Ventanas Ready to Use</td>
<td>CC1 Mild</td>
<td>II</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>NCL-cyclin A</td>
<td>Novo Castra Laboratories</td>
<td>1:100</td>
<td>Microwave oven and TE-buffer</td>
<td>III</td>
</tr>
<tr>
<td>Cyclin B</td>
<td>1495-1</td>
<td>Epitomics Inc</td>
<td>1:200</td>
<td>Pressure cooker and TRS-buffer</td>
<td>III</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>RM-9104-S</td>
<td>NeoMarkers</td>
<td>1:75</td>
<td>Microwave oven and TE-buffer</td>
<td>III</td>
</tr>
</tbody>
</table>

Evaluation of immunoreactivity scores

Estrogen receptor status and Ki67 were analyzed by one investigator (CA), Progesterone receptor (PR) status by a second investigator (CN) and EGFR, CK5/6 by a third investigator (IJ) The percentage of positively stained cells was assessed by choosing the high-power field with the largest number of positively stained cells out of the two biopsies, also called hot-spot, and dividing positively stained cells by the entire number of cells from the same high-power field. A minimum of 200 cells per tumor were counted. Cells were counted manually in high-power fields using a light microscope. The method used for scoring has been validated in previous studies 90, 107, 108. ER and PR were considered positive when more than 10% of tumor cells were stained. For Ki-67 a cut-off point of 14% was used 93 and, in the assessment on EGFR and CK5/6, any staining was considered as positive result.

Immunohistochemical markers were used to classify tumors into subgroups derived from molecular classification. Three different combinations of IHC markers defining molecular subtypes were used; hereafter called the five biomarker classification, classification/NHG and classification/Ki-67. See Table 4 for IHC markers used in the different classifications.
Table 4 Summary of immunohistochemical criteria for defining breast cancer intrinsic subtypes according to three different classifications

<table>
<thead>
<tr>
<th>Classification</th>
<th>Subtype</th>
<th>ER and PR</th>
<th>HER2</th>
<th>NHG</th>
<th>Ki-67</th>
<th>CK5/6 and EGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Five biomarker</strong></td>
<td><strong>classification</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>ER and/or PR pos.</td>
<td>neg.</td>
<td>NA</td>
<td>NA</td>
<td>any</td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER and/or PR pos.</td>
<td>pos.</td>
<td>NA</td>
<td>NA</td>
<td>any</td>
<td></td>
</tr>
<tr>
<td>HER2-like</td>
<td>neg.</td>
<td>pos.</td>
<td>NA</td>
<td>NA</td>
<td>any</td>
<td></td>
</tr>
<tr>
<td>Core-basal</td>
<td>neg.</td>
<td>neg.</td>
<td>NA</td>
<td>NA</td>
<td>CK5/6 and/or EGFR pos.</td>
<td></td>
</tr>
<tr>
<td>SNP</td>
<td>neg.</td>
<td>neg.</td>
<td>NA</td>
<td>NA</td>
<td>neg.</td>
<td></td>
</tr>
<tr>
<td><strong>Classification</strong></td>
<td>/NHG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>ER and/or PR pos.</td>
<td>neg.</td>
<td>I-II</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
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<td>HER2 pos. and/or NHG III</td>
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<td>NA</td>
<td>NA</td>
<td></td>
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<tr>
<td><strong>Classification</strong></td>
<td>/Ki-67</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
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<td>neg.</td>
<td>NA</td>
<td>low</td>
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<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER and/or PR pos.</td>
<td>neg.</td>
<td>NA</td>
<td>high</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Luminal HER2</td>
<td>ER and/or PR pos.</td>
<td>pos.</td>
<td>NA</td>
<td>any</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

* Only receptor-positive subtypes are described. The classification also includes HER2-like subtype (ER and PR negative, HER2 positive) and basal-like (ER, PR negative and HER2 negative).

In paper III, cyclin A and D1 were analyzed by one investigator (CN) and cyclin B by a second investigator (AK). The percentage of positive cells was determined using the same criteria as described in paper II. For cyclin A and D1, cells with unequivocal nuclear staining and for cyclin B, nuclear and cytoplasmic stainings were considered positive.

**Paper IV**

Only tumors with >70% tumor cell content were included. RNA was extracted from fresh frozen tumor tissue and RNA integrity was assessed. Samples with sufficient RNA quality were finally hybridized to gene arrays (Human HT-12 v3.0 Expression BeadChips) in three batches. BioArray Software Environment (BASE) was used for data normalization and management. Normalization is performed with the aim to remove technical variation without removing the biological variation between samples. To be able to adjust for platform related biases, four samples from batch 1 and 2 were re-hybridized in the third batch. A batch effect was detected and adjusted for.

Unsupervised hierarchical clustering (HCL) was performed in order to identify GEX subgroups in the MBC cases. HCL, based on Pearson correlation
distance and complete linkage, identified MBC subgroups which were further analyzed using significance analysis of microarrays (SAM) to identify differentially expressed genes. Up- and down regulated genes were analyzed in the Database for Annotation, Visualization and Integrated Discovery (DAVID), to investigate whether genes with specific biological themes differed between subgroups.

The male subgroups were compared with an external FBC GEX data set. Seven GEX modules associated with key biological processes in FBC were used to detect biologically meaningful differences between MBC subgroups and additionally, as a comparison with FBC. The external female data set was also used for analyzing the degree of similarity between the MBC GEX subgroups with FBC GEX subgroups. Vectors of mean values (centroids) representing the MBC and FBC subgroups, respectively, were constructed. Samples were thereafter classified according to the centroid with which they displayed the highest correlation. With the same method, identified subgroups in MBC were validated to an external GEX data set from 37 MBC cases.

The protein expression of N-acetyltransferase-1 (NAT1) and class 1 human leukocyte antigen (HLA), two of the genes which differed significantly between the male subgroups, were evaluated using immunohistochemistry on TMAs in an extended MBC cohort.

Paper V

Only samples with sufficient tumor cell content were used. DNA was extracted using a modification of a back-extraction protocol from the organic phase of Qiagen Lipid miniRNA kit. BioAnalyzer was used for the assessment of DNA quality. Tumors of sufficient DNA quality were analyzed by high-resolution tiling BAC aCGH. BAC arrays with ≈32,000 BAC clones mapped to the UCSC Human Genom build 17 were produced at the SCIBLU Genomics Resource Centre, Lund University. The PopLowess method was used for the normalization of aCGH data. The Circular Binary Segmentation algorithm was used as a method of dividing the genome into regions of equal copy number and segments containing at least four consecutive probes were used in the following analyses. Gains and losses within the genome were detected and the fraction of genome altered (FGA) was calculated. To identify genomic aberrations in common between cases, a Genomic Identification of Significant Targets in Cancer (GISTIC) analysis was run. GISTIC is an evaluated statistical approach aimed at distinguishing regions of aberration that are more likely to be of importance in cancer pathogenesis. The method identifies regions that are aberrant more often than would be expected by chance. An in-house set of 359 FBC samples run on the same aCGH platform, and analyzed in a similar way was used for comparison between male and female cancer. GISTIC regions
derived from the FBC samples were used for hierarchical clustering of the MBC tumors. Using the female tumors as a reference set, the male tumors could be classified into molecular subgroups. In addition, mean values for all GISTIC regions were calculated for all samples in each of the FBC reference CGH groups, and a centroid characterizing each subgroup was constructed. Thereafter, the correlation of each MBC sample to each of the FBC subgroups was calculated using the Pearson correlation.
Statistics

Paper I
The differences in proportions between the male and female cohorts were analyzed using the Chi-square test and Fisher’s exact test where appropriate. Analyzes were done without regard to the initial sampling procedure. The differences in overall survival between genders were analyzed using Kaplan-Meier with log-rank statistics. Relative survival was used to analyze gender specific differences in survival. Relative survival is a model which estimates the ratio of observed survival to the expected survival of comparable groups in the standard population. The standard population used was Sweden 2000. Differences in survival between genders were analyzed using The Poisson model 113.

Paper II-III
All statistical analyses were performed in SPSS version 17.0-19.0. The study end-point was breast cancer death, other cases were censored. A Cox proportional regression model was used to assess the hazard ratio for the different clinicopathological variables. Correlation between clinicopathological variables were analyzed using Spearman’s correlation test. To define the optimal cut-off of the different proliferation markers in MBC, a previously described method was used 114. Briefly, the material was divided into 10 equal groups, so-called deciles, and the cut-off values corresponding to each decile limit were used to separate the material into a higher and a lower proliferating group. For each cut-off, the hazard ratio for breast cancer death was calculated, using Cox proportional hazard model. For comparison of categorical data chi-square statistics were used.

Paper IV-V
All statistical analyses were performed in R 115. Distant metastasis free survival (DMFS) was used as end-point in all survival analyses. The Wilcoxon test or Students t-test were used for calculation of p-values.
Results

Paper I

Current guidelines generally recommend that MBC patients should be managed similarly to FBC patients, but some reports have indicated that MBC patients receive less treatment \(^{30, 116}\). Many studies have described a poorer overall survival in MBC patients which may partly be explained by reported differences in stage at diagnosis and more advanced age \(^{26, 30}\). Other explanations could be differences in tumor characteristics or treatment intensity. In the present study, we compared MBC and FBC cohorts which were sampled according to age and time of diagnosis in order to obtain comparable groups. No differences in the distribution of stage at diagnosis were seen between the male and female patients. MBC patients underwent mastectomy more often, and breast conserving surgery less frequently compared with FBC patients. Radiotherapy given after partial (80% vs. 75%, \(p=1.00\)) or full mastectomy (44% vs. 39%, \(p=0.47\)) did not differ between the groups. No differences in adjuvant chemotherapy (16% vs. 21%; \(p=0.31\)) or adjuvant endocrine treatment (59% vs. 52%, \(p=0.24\)) could be demonstrated. Not even when analyzing those patients most likely to receive adjuvant chemotherapy (<70 years and node-positive) could any difference in treatment intensity be demonstrated between genders. Overall survival (41% vs. 55%, \(p=0.001\)) and relative survival (74% vs. 88%, \(p=0.015\)) at five years were significantly inferior in MBC (Fig 6-7). Although no differences in the distribution of stage or treatment strategy was demonstrated, the male group demonstrated a significantly poorer overall survival. However, the observed difference in overall survival may be explained by the fact that men in general have a shorter life expectancy and higher comorbidity burden. With the aim of adjusting for this potential bias, we compared the relative survival between genders and could observe that the MBC patients had significantly poorer relative survival. In conclusion, no differences in stage at diagnosis or primary treatment intensity were seen, but the male group had a significantly poorer survival which might indicate differences in tumor biology.
Paper II

The IHC markers routinely used in clinical practice have not been sufficiently validated in MBC. Previous reports in MBC are contradictory, and in many studies survival data are lacking, thus precluding prognostic evaluation. IHC marker combinations broadly matching the molecular subtypes are recommended for clinical use in FBC. Previous reports in MBC have demonstrated that there are distinct differences in the prevalence of molecular subtypes compared with FBC, and it is unknown whether
the subtypes have similar prognostic information. In our study, ER- positivity was found in 93% of patients and PR-positivity in 78%. High tumor grade (NHG III) was demonstrated in 41% and HER2 positivity in 11%. Tumor size >20 mm, positive nodal status, ER negativity and PR negativity were significantly associated with an increased risk of breast cancer death in the univariate analysis, whereas no associations could be demonstrated for grade, PR, HER2 status, Ki-67 or age. In a multivariate model; node positivity, tumor size > 20 mm and ER negativity remained as independent prognostic factors (Table 5). The three definitions for molecular subtypes; “the five biomarker classification”, “classification/NHG” and “classification/Ki-67” identified luminal A and luminal B in 81% vs 11%; 48% vs. 44% and 41% vs. 42% of cases, respectively. Two cases of core-basal-like were revealed but no cases of HER2-like. One interesting finding was that luminal A and luminal B were near equally common according to “classification/NHG” and “classification/Ki-67”, thus differing from data reported in FBC. However, no significant difference between Luminal A and B regarding risk for breast cancer death could be demonstrated by any definition (Table 6). The latter finding also differs from FBC, where a majority of studies show a significantly poorer outcome in luminal B cases.
Table 5. Tumor markers in univariate and multivariate models by Cox Regression. Only variables with significant values in the univariate model were included in the multivariate model.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HRa</th>
<th>95% CI</th>
<th>p-value</th>
<th>HRb</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size; &gt;20 vs. ≤20 mm</td>
<td>2.9</td>
<td>1.5 - 5.7</td>
<td>&lt;0.01</td>
<td>3.3</td>
<td>1.4 - 7.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Nodal status; pN1 vs. pN0</td>
<td>5.4</td>
<td>2.2 - 13.0</td>
<td>&lt;0.01</td>
<td>4.5</td>
<td>1.8 - 11.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tumor grade; III vs. I-II</td>
<td>1.5</td>
<td>0.8 - 2.8</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER; ≤10 vs. &gt;10%</td>
<td>6.2</td>
<td>2.7 - 14.2</td>
<td>&lt;0.01</td>
<td>10.9</td>
<td>3.2 - 37.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PR; ≤10 vs. &gt;10%</td>
<td>2.4</td>
<td>1.2 - 4.7</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-67; ≥14 vs. &lt;14%</td>
<td>1.3</td>
<td>0.7 - 2.4</td>
<td>0.43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2; pos vs. neg</td>
<td>1.4</td>
<td>0.5 - 3.6</td>
<td>0.48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age; ≤60 vs. &gt;60</td>
<td>1.1</td>
<td>0.6 - 2.2</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HR Hazard Ratio; CI, confidence interval
a Univariate analysis
b Multivariate model including tumor size, nodal status and ER

Table 6. Univariate analysis of different classifications of molecular subtypes. Cox proportional regression was used with breast cancer death as an end-point.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Subtype</th>
<th>Prevalence n</th>
<th>%</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Five biomarker classificationa</td>
<td>luminal A (Ref)</td>
<td>160</td>
<td>81.2</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>luminal B</td>
<td>21</td>
<td>10.7</td>
<td>1.5</td>
<td>0.6 - 4.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Classification/NHG³</td>
<td>luminal A (Ref)</td>
<td>94</td>
<td>47.7</td>
<td>1.2</td>
<td>0.6 - 2.3</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>luminal B</td>
<td>86</td>
<td>43.7</td>
<td>1.2</td>
<td>0.6 - 2.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Classification/Ki-67³</td>
<td>luminal A (Ref)</td>
<td>80</td>
<td>40.6</td>
<td>1.0</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>luminal B</td>
<td>82</td>
<td>41.6</td>
<td>1.4</td>
<td>0.7 - 2.9</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>luminalHER2</td>
<td>19</td>
<td>9.6</td>
<td>1.1</td>
<td>0.3 - 3.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Paper III

The clinical utility of proliferation markers in MBC has not yet been fully elucidated. In paper II, we evaluated Ki-67 and NHG in MBC but no prognostic impact could be demonstrated. In this study, we evaluated other proliferation associated variables; cyclin A, B, D1 and mitotic count. Furthermore, Ki-67 was re-evaluated using different cut-off levels.

Cyclin A, B, mitotic count and Ki-67 demonstrated strong positive correlation with each other (Spearman correlation coefficient > 0.4) showing that these variables are linked in tumor biology. Cyclin D1 did not demonstrate any correlation with the other cyclins, mitotic count or Ki-67. The 7th decile cut-off for cyclin A (12%) corresponded well to the previously defined cut-off in FBC (11%). When using these cut-offs, overexpression of cyclin A was associated with a trend to poorer breast cancer survival (p= 0.11 and p= 0.07, respectively). The prognostic impact of cyclin A was enhanced when using cut-offs corresponding to the 8th (14%) and 9th decile (17%) with HR 2.1; p=0.05 and HR 3.7; p=0.001, respectively. Overexpression of cyclin B was prognostic of poorer breast cancer survival when using the cut-off corresponding to the 9th decile (15%), demonstrating HR 2.7; p=0.02. Additionally, high mitotic count (>10 per field of view) was associated with a poorer survival, HR 2.5; p=0.01 (Table 7). Ki-67 did not demonstrate any prognostic value by any cut-off values used in this material. Hence, our results indicate that cyclin A and B may be more reliable markers than Ki-67 for assessment of proliferation. Contrary to the other cyclins, overexpression of cyclin D1 was predictive of better survival (HR 0.3; p=0.001). The prognostic value of cyclin D1 remained in a multivariate model including tumor size and nodal status (HR 0.48; p=0.05). Hormone receptor positive patients were analyzed separately to investigate whether cyclin D1 could have an influence on the response to endocrine treatment. We found that patients undergoing adjuvant endocrine treatment had a significantly poorer breast cancer survival, which may be explained by the fact that endocrine treatment was given more often in higher stage disease. However, adjusting for cyclin D1 did not change the prognostic value of endocrine treatment, which contradicts the hypothesis that cyclin D1 is associated with tamoxifen resistance.
Table 7. Prognostic impact of proliferation markers compared with other clinicopathological parameters. Univariate analyses using the Cox proportional hazard regression model.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate models</th>
<th>HR</th>
<th>95 % CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodal status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN0 (ref.)</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN1</td>
<td></td>
<td>5.4</td>
<td>2.2 – 13.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20 mm (ref.)</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;20 mm</td>
<td></td>
<td>2.9</td>
<td>1.5 – 5.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ER</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10% (ref.)</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤10%</td>
<td></td>
<td>6.2</td>
<td>2.7 – 14.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10% (ref.)</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤10%</td>
<td></td>
<td>2.4</td>
<td>1.2 – 4.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Cyclin A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤17% (ref.)</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;17%</td>
<td></td>
<td>3.7</td>
<td>1.6 – 8.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cyclin B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤15% (ref.)</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;15%</td>
<td></td>
<td>2.7</td>
<td>1.2 – 6.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤62% (ref.)</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;62%</td>
<td></td>
<td>0.3</td>
<td>0.2 – 0.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mitotic count</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤10 (ref.)</td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 10</td>
<td></td>
<td>2.5</td>
<td>1.3 – 5.0</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Paper IV

FBC has been extensively investigated on the transcriptional level, and distinct subgroups providing prognostic information have been identified; luminal A, luminal B, HER2-like, basal-like and normal-like. Whether MBC tumors can be classified into similar subgroups associated with differences in clinicopathological characteristics and survival has previously not been evaluated. In the present study, 66 MBC tumors were characterized using gene expression arrays. An unsupervised hierarchical clustering analysis revealed two stable subgroups of MBC; luminal M1 encompassing 70% of tumors and luminal M2 30% of tumors. There was a tendency for luminal M1 to be associated with a younger age at diagnosis and a poorer survival. No differences in NHG or tumor size between the subgroups could be demonstrated. To further specify differences between the MBC subgroups, the expression of seven GEX modules associated with key biological processes were investigated. In this analysis, five of the modules - ER-signaling, tumor invasion and metastasis, HER2, proliferation and immune response - displayed significant or near significant differences between the subgroups. Luminal M1 was associated with higher scores of invasion and metastasis, proliferation and HER2, indicating a more aggressive tumor behavior. Luminal M2, on the other hand, displayed expression of genes involved in ER-signaling and immune response. Interestingly, ER-signaling
differed significantly between the subgroups even though the majority of tumors were ER positive at IHC assessment. Similarly low ER-signaling scores have only been seen in ER negative cases in FBC. None of the MBC subgroups displayed GEX module-patterns resembling any of the intrinsic subgroups of FBC. When using centroids based on a FBC GEX dataset, more than half of the MBC tumors remained unclassified. In comparison with the intrinsic subtypes in FBC, luminal M1 displayed higher correlations to HER2-like and basal-like tumors whereas luminal M2 displayed higher correlations to luminal A and luminal B subgroups. To further investigate the clinical relevance of the identified differences between the MBC subgroups, protein expression of NAT1 and HLA which are involved in ER signaling and the immune system, respectively, were investigated. NAT1 positivity was more common in luminal M2 tumors, and was found to be predictive of better DMFS. The prognostic impact of NAT1 was confirmed in a multivariate model including node status, NHG and tumor size (HR 2.8; 95% CI 1.0-7.2). High expression of HLA was associated with a better DMFS when compared with a moderate level of expression.

Paper V

In line with the previously discussed intrinsic subgroups based on gene expression, FBC can also be classified by genomic aberrations, and distinct subgroups with differences in clinical outcome have been demonstrated. High resolution aCGH in this MBC cohort revealed that MBC is a heterogeneous tumor type. The most common genomic aberrations were similar in MBC and FBC. Although no significant difference in total FGA could be observed between genders, genomic gains were significantly more common whereas genomic losses seem to be less common in MBC tumors. Whole chromosome arm gains were more frequent in MBC. MBC could be classified into two genomic subgroups with distinct genomic patterns; male-simple and male-complex. Twenty percent of cases were classified as male-simple and 77% as male-complex. In the male-simple group, no high-level amplifications or deletions were observed. The male-simple group correlated with significantly smaller tumor size and lower proliferation. Additionally, a higher percentage of male-simple samples were diploid, when compared with the male-complex group. The male-complex group encompassed all PR negative (n=8), all HER2 positive tumors (n=2) and all patients with a known BRCA2 mutation (n=3). When stratifying patients by age, 14 of 15 patients younger than 60 years of age at diagnosis clustered in the male-complex group. Furthermore, all but one patient with a generalized recurrence clustered into the male-complex subgroup. The observed differences indicate that male-complex is a more aggressive tumor entity. However, no differences in DMFS could be observed, probably due to the small number of patients. The male-complex group displayed evident
similarities with the female luminal-complex group, whereas the male-simple subgroup did not seem to be comparable with any of the female subgroups. A Kaplan-Meier analysis comparing the male-complex with female-complex cases revealed equally poor DMFS.
General discussion

In this thesis, we present five papers aimed at increasing our understanding of MBC. It is known that MBC patients have a higher median age at diagnosis compared with FBC. Furthermore, it has been described that advanced stage at diagnosis is more common, particular in regions where women are offered regular mammography. In contrast, MBC are more often hormone receptor positive, often leading to the interpretation that MBC is a more indolent disease. Due to the rarity of MBC, there is a lack of prospective studies evaluating different treatment strategies and the optimal treatment is not known. Today, the general recommendation is to treat male patients according to guidelines in FBC. Breast cancer mortality in women has declined during the last decades due to improvements in diagnostic procedures and treatment but the corresponding observation has not been made in MBC. Several reports have indicated that MBC patients do not receive as intensive treatment as FBC patients. The older age and higher comorbidity in the male group is one explanation for less treatment, but the lack of evidence for different treatment modalities in MBC may also contribute.

In all, the results of our studies indicate that MBC is a unique breast cancer entity, different from FBC. In paper I we demonstrated that MBC is an aggressive disease with poorer disease-specific outcome in comparison with FBC. These findings are in line with some previous reports showing a poorer overall survival or breast cancer specific survival in the male group. On the other hand, a recent population-based study collecting data from registers in six countries concluded that MBC had a better relative survival in comparison with FBC, after adjusting for multiple variables including age, stage and treatment. The latter study had larger patient cohorts than our study but the registers from the different countries varied in accessible data and used different definitions for stage and treatment modalities. The access to high-quality, detailed registry data strengthens our results.

In papers II-III our aim was to examine MBC tumors as regards the prevalence and prognostic impact of different clinicopathological parameters. Additionally, we wished to classify MBC into molecular subtypes using IHC and evaluate the prognostic values. Previous studies have revealed that lymph node status is an important prognostic factor in MBC, but the knowledge regarding the prognostic value of other clinicopathological
parameters is scarce and contradictory \textsuperscript{5, 17, 103-105}. In recent years, the classification into “intrinsic” subtypes by gene expression has become more important in FBC. These molecular signatures are considered to mirror the heterogeneity of tumors in a better way and have proven to make certain conventional clinicopathological parameters redundant by identifying a higher proportion of low-risk patients not necessarily in need of adjuvant systemic treatment \textsuperscript{69, 70}. One important finding in FBC is that proliferation is the most important parameter in the classification of hormone receptor positive tumors into luminal A and B, where the latter subgroup is associated with a poorer prognosis \textsuperscript{70}. Based on these findings, commercial gene arrays have been developed most importantly to improve the risk-assessment of hormone receptor positive tumors \textsuperscript{72, 73}. However, gene arrays are expensive and not always accessible; therefore, combinations of immunohistochemical markers broadly matching the “intrinsic” gene expression subtypes have been identified and may be more feasible in clinical routine \textsuperscript{76-78}. Today, IHC based molecular subtyping is recommended by several guidelines for the prognostication and treatment decision-making in FBC \textsuperscript{93, 118}.

In paper II, we could conclude that node status, tumor size and ER status are independent prognostic factors in MBC. A large subset of cases was assessed as highly proliferating according to NHG or Ki-67, but we could not demonstrate that this was of any prognostic value. HER2 did not reveal any prognostic information but interestingly, the interpretation of HER2 assessment by IHC seem to differ from that in FBC. Of all HER2 amplified cases, only a few had a corresponding strong (3+) IHC expression. Three commonly used models for classification into subtypes were evaluated and, in all models, the vast majority of patients were classified into the hormone receptor positive luminal subgroups. Interestingly, luminal A and B were almost equally common in the two models adding tumor grade or Ki-67 for classification into luminal subtypes, which differs from reports in FBC \textsuperscript{77, 78, 119}. Furthermore, the molecular subtyping did not by any classification seem to give similar prognostic information to that reported in FBC.

Recently, two papers have been published describing molecular subtypes assessed by IHC in MBC. Shaaban et al used HER2 status to separate the luminal subtypes. The male cohort was compared with a matched female cohort and no survival difference between the male and female luminal A was seen; however, in the same paper, tumor biological differences were discussed indicating that luminal A in MBC is not comparable with luminal A in FBC. Furthermore, international praxis on evaluation of HER2 status was not adhered to: no FISH testing was performed and only IHC 3+ was considered HER2 positive. None of the tumors had IHC 3+ and consequently, no tumors were classified as luminal B \textsuperscript{83}. In the second paper, a classification including proliferation and HER2 was used but the prognostic
relevance of the different subtypes was not evaluated due to lack of outcome data. To date, our study is the first to correlate molecular subtypes with breast cancer survival. Non-significant results should be interpreted with caution due to the moderate level of power but the results imply that the molecular subtypes validated in FBC are not applicable in MBC, and molecular classifications specific for MBC tumors need to be identified.

In papers IV-V, the molecular background of MBC was investigated. In paper IV global GEX analyses in MBC tumors were performed and compared with a previous data set of FBC tumors. Two subgroups among MBC tumors were identified; luminal M1 and luminal M2. When evaluating the degree of similarity to the “intrinsic” subtypes in FBC tumors, more than half of the MBC tumors were left unclassified. To enlighten the differences between the MBC subtypes, tumors were analyzed for seven different GEX modules representing key biological processes; proliferation, tumor invasion, immune response, angiogenesis, apoptosis, ER-signaling and HER2. The larger subtype (luminal M1), encompassing 70% of patients, displayed higher scores in proliferation, tumor invasion and HER2 modules, whereas luminal M2 tumors were characterized by a higher expression of genes related to the immune system and ER-signaling. The observed differences in GEX modules indicate that luminal M1 displays a more aggressive tumor behavior, which was further supported by a survival analysis suggesting poorer DMFS in this group. The observed significant difference in ER-related gene signaling between the groups is different from FBC, where only tumors ER-negative by IHC display similar reduced ER-signaling. One of the genes correlating to ER-signaling, NAT1, was significantly higher expressed in luminal M2 tumors. The protein expression of NAT1 was investigated in an extended MBC cohort. Half of the patients were assessed as NAT 1 negative, which in FBC has been found to be associated with an inferior response of tamoxifen. In our material, NAT1 displayed independent prognostic information, but unfortunately, there were too few cases to evaluate the association of NAT1 to tamoxifen response.

In paper V, MBC tumors were evaluated on the DNA level by CGH, revealing differences compared with FBC. As in the GEX analyses, two subgroups were identified and a majority of patients were classified into the subgroup with more aggressive tumor behavior. One unique MBC subgroup, not resembling any of the FBC subgroups, was identified. Hence, both studies on the molecular level indicate that there are distinct differences in key biological processes between male and female breast cancer and that MBC has unique molecular subtypes with probable prognostic importance. Furthermore, the findings demonstrate that MBC is a heterogeneous tumor entity.
In both studies on the molecular level, proliferation was one important tumor biological feature that differed between the MBC subgroups. In paper II, however, we could not show that NHG or Ki-67, two parameters highly associated with proliferation and today used in clinical routine, had any prognostic value.

In paper III, other proliferation-related markers were assessed. We found that the overexpression of cyclin A and B were near significantly or significantly associated with a poorer outcome in the subset of patients with the highest expression. In addition, we could demonstrate that high mitotic count was associated with an increased risk of breast cancer death, which further strengthens the conclusion that proliferation is of prognostic importance in MBC. These findings are in line with some previous reports 17, 103, 122 but contrary to others 5, 17, 103, 104, 122. Ki-67 was re-evaluated using different cut-offs, but no prognostic value was demonstrated in this cohort. This is in accordance with studies in FBC indicating that Ki-67 may be a weaker prognostic factor than cyclin A and B 87, 90, and, consequently, a larger cohort may be needed to be able to confirm its prognostic impact. Another issue with potential influence on our results is that a subset of our patients had received adjuvant and/or palliative chemotherapy treatment which is known to diminish the prognostic impact of proliferation markers 114, 123. To summarize, our observations indicate that cyclins A and B and mitotic count may be more reliable markers than Ki-67 for assessment of proliferation, but additionally studies are warranted to establish the optimal marker and cut-off. In line with previous reports in MBC, overexpression of Cyclin D1 was independently predictive of better breast cancer survival 103, 105. The reason for cyclin D1 overexpression seeming to be associated with a better outcome despite its proliferation-activating potential is not fully understood. One hypothesis is that there might be an interaction between key biological processes in the process of metastasis. This is supported by in vitro studies where a lower expression of cyclin D1 was linked to an enhanced infiltrative potential in the tumor. The explanation for the latter finding was that cells in the quiescent phase had a greater migratory capacity 124. Previous studies in FBC have suggested that Cyclin D1 has dual roles, in addition to its cell cycle related actions, it has been suggested that it can stimulate the ER \( \alpha \) receptor in a ligand-independent manner and thereby have the ability to reverse the ER blocking effect of tamoxifen, leading to tamoxifen resistance 101. In our study, patients undergoing adjuvant endocrine treatment had a poorer breast cancer survival, possibly explained by the fact that treatment was given more often in higher stage disease. However, cyclin D1 did not seem to influence the response of endocrine treatment. One reason for this could be that ER-signaling is different in MBC, as postulated in paper IV. This notion is supported by other studies, suggesting a reduced ER functionality in MBC. Further studies regarding this issue are needed 43, 79.
A number of limitations concerning this thesis need to be discussed.

In Paper I, we used registry data for information on clinicopathological parameters, treatment data and vital status. An alternate method is to collect corresponding data from patient files which may give more accurate information. However, the Regional Breast Cancer Register has been validated and a very high level of agreement on data regarding primary treatment has been demonstrated. Furthermore, the Regional Breast Cancer Registry has shown a high level of completeness with >95% of diagnosed breast cancer cases registered. One limitation in this study was the absence of data on breast cancer deaths, preventing us from analyzing breast cancer specific survival. Instead we used relative survival, a method which estimates the ratio of the observed survival of patients in a cohort to the expected survival of a comparable group from the general population. In this model, commonly used in population-based studies, the cause of death is not required but the excess mortality related to the cancer diagnosis can be estimated.

In Papers II-III, MBC tumors were characterized by IHC markers on TMA. Hot-spots were chosen for evaluation and a minimum of 200 cells per tumor were counted. The TMA technique enables rapid screening of a large number of tumors on a single microscopic slide. One drawback with the technique is that only a small amount of tumor is analyzed which leads to the question of how representative the TMA core is, and how much tumor heterogeneity could affect the results. However, several studies have shown that even though the result from an individual TMA core in comparison with a conventional histological slide may vary, the correlation to histopathological variables and prognostic implications are in agreement when a large number of tumors are studied. It could also be questioned why we did not validate the scoring results with two or more investigators but this was not considered necessary as previous studies have demonstrated good reproducibility between different investigators with the scoring method used in our studies. One known disadvantage with the TMA technique is that tissue cores can be lost or damaged during sectioning, which was also a problem in our studies, leading to loss of tumor marker information in 2-6% of cases.

In papers IV–V, two distinct male subgroups were identified both on the transcriptional level (luminal M1 and luminal M2) and on the genomic level (male complex and male simple). In FBC, at least five subgroups with clinical relevance have been identified and it cannot be excluded that additional subgroups can exist in MBC. Although our material with fresh frozen tissue from MBC cases is the largest reported to date, the small number of patients included may prevent as accurate sub classification as that performed in FBC. However, the results were validated in an external GEX data
set from MBC tumors and comparable subgroups and a similar distribution of subgroups was observed. For the comparison between MBC and FBC GEX groups, an external FBC data set was used. Even though the FBC data set was derived from GEX analysis performed in a similar way, methodological differences between analyzes may influence the comparison between genders. Another concern which needs to be enlightened is the issue of patient selection. It is today not routine practice to save fresh frozen tissue from all patients diagnosed with breast cancer, which is reflected in the small subset of patients where frozen tissue was available. We collected all accessible fresh frozen tumor samples but there is a risk that this patient material may not be entirely applicable to the entire MBC population.

Our patient materials are comprehensive in comparison with previous studies in MBC. However, the problem of achieving sufficiently large patient materials is a matter that concerns all papers presented in this thesis. As a consequence, the power of the studies is moderate which makes interpretation of non-significant results difficult.

In conclusion, MBC is a unique breast cancer entity. Positive nodal status, larger tumor size, ER-negativity, low expression of cyclin D1 and low expression of NAT1 are independent predictors of poorer breast cancer survival. The established molecular subtypes used in FBC are not applicable in MBC. Two MBC subtypes were identified on both the transcriptional and genomic levels, but additional subtypes of clinical importance may exist. Molecular subtypes are today used in clinical routine, but we have yet to discover which IHC markers to use to define the MBC subtypes. Furthermore, MBC tumors ER positive by IHC do not seem to correspond to ER-signaling as highly as in FBC. This could imply that MBC tumors respond differently to endocrine treatment.
General conclusions

Paper I: When comparing cohorts of MBC and FBC patients, sampled according to age and year of diagnosis, no differences in primary treatment intensity could be demonstrated. At time of diagnosis, MBC and FBC patients presented with a similar distribution of disease stage. Although no differences in treatment intensity or stage was observed, the male group had significantly poorer overall and relative survival.

Paper II: In this study we demonstrated that ER-negativity, tumor size >20 mm and positive lymph nodes are independent risk factors for breast cancer death in MBC. Age, HER2, grade or Ki-67 did not reveal any prognostic impact, but the moderate power of this study must be taken into account. The classification using IHC markers into molecular subtypes does not seem to provide similar prognostic information as in FBC and prognostic profiles specific for MBC need to be identified.

Paper III: In this paper we showed that proliferation is important in MBC. Overexpression of cyclin A, cyclin B and high mitotic count is predictive of poorer survival in MBC and seems to provide more reliable prognostic information compared with Ki-67. Contrary to the other cyclins, overexpression of cyclin D1 is associated with better breast cancer survival. The prognostic information of cyclin D1 adds independent prognostic information to the other cyclins. However, further studies are warranted to be able to establish the optimal markers and what cut-offs to use.

Paper IV: In this MBC material two unique and stable gene expression subgroups were identified; luminal M1 and luminal M2. The subgroups displayed differences in tumor characteristics and outcome. The MBC subgroups differed from the acknowledged intrinsic subgroups in FBC. The findings may indicate that MBC patients may need specific treatment strategies, and further studies are warranted. NAT1 protein expression was identified as a prognostic factor, with overexpression associated with better outcome.

Paper V: With high-resolution genomic profiling, differences between male and female breast cancer could be demonstrated. MBC could be divided into two different sub-groups, male simple and male complex, which may have prognostic value. The male simple group seems to represent a new subgroup, unique for MBC patients.
Future perspectives

The present study emphasizes the need of further investigations into different fields of MBC. Regarding the molecular subtyping of MBC, several questions need to be answered. Our colleagues in Lund are planning to perform a GEX analysis with MBC tumors together with FBC tumors. In paper IV, the GEX patterns of MBC patients were compared with previous data from a FBC tumor data set analyzed in a similar way. The reason for the comparison with historical data was mainly due to the high costs of GEX arrays. A GEX analysis with male and female tumors analyzed together will hopefully validate the MBC subtypes but will also give a clearer picture of the differences in subtypes between genders. Another important issue is to identify IHC marker combinations defining the MBC molecular subtypes. It would in that case be logical to focus on the GEX modules that differed significantly between the MBC subtypes and identify additional corresponding protein products which could be used for IHC classification. Further GEX analyzes on larger MBC cohorts are warranted for the evaluation of the prognostic significance of the MBC subtypes.

The findings in papers II-III also need to be validated in, preferably, larger studies. Due to the rarity of the disease, multi-institutional collaboration is crucial in order to achieve larger patient materials. Different research collaborations have been discussed and we have been asked to participate in a research project involving a number of countries in Europe. In this project, two different studies are being considered. Firstly, a retrospective part assembling clinical data and tumor tissue from MBC patients diagnosed during the last 20 years is planned, with the goal of assembling more than 1000 cases. Paraffin-embedded tumor tissue will be collected for analyses of tumor biology. A central pathology review is planned with the aim of overcoming the problem of lack of harmonization in techniques and cut-off definitions. Clinical data and histopathological parameters will be correlated to outcome data for the evaluation of prognostic impact. Additionally, GEX analyses will be performed on fresh frozen tissue. The second part will be a prospective registration of all new cases diagnosed during two years. The registration will include information on risk factors, clinicopathological variables, treatment and follow-up data. If the collaboration succeeds, it will open up the possibility of launching randomized, controlled trials for the male group. Most importantly, the value of different endocrine treatments needs to be evaluated. It is also important to
allow the inclusion of MBC patients in all future larger clinical trials in the breast cancer field. Many clinical trials today collect blood samples and frozen tissue to identify molecular profiles predictive of treatment response, and this could prove valuable for male patients. Another way of learning more about factors behind tumor development and progression or the interaction between different treatments and molecular pathways, would be to use animal models. A method for the induction of mammary tumors in male transgenic mice has been developed\textsuperscript{128}.

In the future, we will have an excellent resource for research in MBC. Research centers in Sweden plan or have already started projects where fresh tissue and blood samples from all patients diagnosed with breast cancer are collected\textsuperscript{129, 130}. Molecular analyses will be conducted using high resolution genomic techniques and the molecular information will be linked to clinicopathological, treatment and outcome data from our comprehensive breast cancer registries mandatory for all patients diagnosed with breast cancer. This will probably give us the possibility to better answer the question regarding the optimal treatment and the optimal prognostic tools for MBC.
Manlig bröstcancer är en mycket ovanlig sjukdom som står för ca 0,6 % av all bröstcancer, vilket innebär att ca 30-50 män drabbas av sjukdomen i Sverige varje år 4. Vid kvinnlig bröstcancer har man de senaste decennierna sett en minskad dödlighet till följd av tidigare diagnos och effektivare behandlingar, vilket dock inte har kunnat visas vid manlig bröstcancer. Idag finns mycket lite kunskap om hur vi på bästa sätt ska behandla män med bröstcancer. Eftersom sjukdomen är så ovanlig har det inte gått att utvärdera effekten av olika behandlingar vid manlig bröstcancer och idag behandlas männen enligt de riktlinjer som gäller vid kvinnlig bröstcancer. Vid kvinnlig bröstcancer används olika prognostiska samt prediktiva faktorer för att välja den bästa behandlingen för varje enskild patient. En prognostisk faktor förutsäger sjukdomens naturliga utveckling medan en prediktiv faktor beskriver känslighet för en viss behandling. Idag är tumörstorlek, körtelstatus, tumörgrad, Ki-67, progesteronreceptoruttryck samt HER2 (human epidermal growth factor receptor 2) de viktigaste prognostiska faktorerna 5,2. Under senare år har det även varit en ökad användning av olika genprofiler som hjälper i den prognostiska bedömningen 70, 72, 73. Vi vet dock inte om det kan finnas avgörande skillnader i den tumörbiologiska bakgrunden mellan manlig och kvinnlig bröstcancer som i så fall också kan innebära att de idag använda prognostiska och prediktiva faktorer inte har samma betydelse vid manlig bröstcancer.

Delarbete I

kunde dock inte se några skillnader i andelen patienter som planerades för postoperativ strålbehandling efter mastektomi, dvs. borttagande av hela bröstet. Vi kunde inte heller se någon skillnad i andelen patienter som erbjöds adjuvant cytotoksmiska eller endokrin behandling. Trots att manliga och kvinnliga patienter hade jämföbar sjukdomsstadium vid diagnos och planerades för lika intensiv behandling, hade manliga bröstcancerpatienter en sämre överlevnad vilket kan tala för en mer aggressiv sjukdom.

Delarbete II

I delstudie II utvärderades den prognostiska betydelsen av olika tumör-karakteristika som idag används för att styra patienternas behandling. Vi undersökte också värdet av 3 olika modeller där immunhistokemiska markörer användes för uppdelning i molekylära subtyper. Molekylär subtypning har visats ha prognostisk betydelse vid kvinnlig bröstcancer och baseras ursprungligen på skillnader i genuttryck mellan olika bröstcancertumörer. Immunhistokemiska färgningar samt tumörgradering utfördes på tumörer från 197 manliga bröstcancerpatienter. Vi kunde visa att tumörstorlek, körtelstatus samt uttryck av östrogenreceptorer var oberoende prognostiska faktorer men vi kunde inte visa något prognostiskt värde av tumörgrad, Ki-67 eller HER2. Vi kunde inte heller visa någon statistisk säkerställd skillnad i prognos mellan olika molekylära subtyper, oavsett vilken definition som användes.

Delarbete III

överuttryck av cyclin A och B samt ett högt antal mitoser talar för en ökad risk för bröstcancerdöd. Överuttryck av cyclin D1 var istället associerat till minskad risk för bröstcancerdöd, där en möjlig förklaring kan vara att cyclin D1 utöver proliferation också har associerats till processer som styr tumörinvasivitet och metastasering. Ki-67 analyserades på nytt med den beskrivna metoden, men visade inget prognostiskt värde oavsett cut-off.

Delarbete IV

Kvinnliga bröstcancertumörer kan baserat på genuttryck delas upp i 5 olika molekylära subtyper; luminal A, luminal B, basal-typ, HER2-typ samt normal-typ. Subtyperna har visats ha prognostisk betydelse 69, 70. I delarbete IV användes frusen tumörvävnad från 66 manliga bröstcancerpatienter för att undersöka genuttrycksprofiler. RNA extraherades och hybridiserades till gen-array. Två subtyper identifierades; luminal M1 samt luminal M2. Trettio procent av tumörerna klassades som luminal M2, en subtyp som kännetecknades av ett högt uttryck av gener associerade till immunologiska processer samt till östrogenreceptorsignalering. Resterande tumörer klassades som luminal M1 vilka istället karakteriserades av ett lågt uttryck av gener associerade till östrogenreceptorsignalering trots att en majoritet av tumörerna hade ett starkt östrogenreceptoruttryck vid immunhistokemisk bedömning. Luminal M1 tumörerna var associerade till karakteristika som talade för en mer aggressiv tumör typ och hade också en tendens till sämre prognos. Vid jämförelse mot data från kvinnliga bröstcancertumörer kunde mindre än hälften av manliga tumörer klassas enligt de undergrupper som är validerade vid kvinnlig bröstcancer. Proteinuttrycket av två gener som visade stor skillnad i uttryck mellan de manliga grupperna; N-acetyltransferase-1 (NAT-1) samt class 1 humant leukocyt antigen, undersöktes senare med immunhistokemisk metod och NAT-1 visade sig vara en prognostisk faktor med överuttryck associerat till bättre överlevnad. Sammanfattningvis identifierades två undergrupper vid manlig bröstcancer med tydliga skillnader i tumör-karakteristika och prognos. De manliga undergrupperna överensstämmer inte med någon av de ofta omnämnda kvinnliga undergrupperna.

Delarbete V

Vid kvinnlig bröstcancer har omfattande forskning bedrivits på molekylär nivå. Molekylära subtyper med prognostisk betydelse har identifierats både vid analyser av genuttryck samt vid analyser av förändringar i genomet (DNA). Genom att studera förändringar av genomet kan man också få information om vilka mekanismer som ligger bakom cancercellernas genomiska instabilitet. I delarbete V analyserades frusen vävnad från
56 manliga bröstcancertumörer med högupplöst BAC-array teknik för att undersöka avvikelser på DNA-nivå och se om undergrupper med prognostisk betydelse kunde identifieras. Resultaten jämfördes sedan med 396 kvinnliga bröstcancertumörer som hade analyserats med samma teknik.

Analysmetoden Genomic Identification of Significant Targets in Cancer (GISTIC) användes för att identifiera signifikanta DNA-avvikelser som var ofta förekommande i tumörerna. Med utgång från de GISTIC regioner som hade identifierats i de kvinnliga bröstcancertumörerna utfördes en hierarkisk klustring av de manliga bröstcancertumörerna. Hierarkisk klustring är en analys metod som syftar till indelning i undergrupper med likartat mönster av DNA-avvikelser.

Vi kunde inte visa någon signifikant skillnad i den totala mängden DNA-avvikelser mellan manliga och kvinnliga bröstcancer tumörer, men vi såg skillnader i mönstret av DNA-avvikelser. Vid klusteranalysen identifierades två undergrupper av manlig bröstcancer; ”male complex” och ”male simple”. Grupperna skiljde sig från identifierade undergrupper vid kvinnlig bröstcancer. En majoritet av manliga bröstcancertumörer tillhörde ”male complex”, den tumörtyp som var associerad med karakteristika som talade för en mer aggressiv tumör. Sammanfattningsvis visade studien att det finns tydliga skillnader mellan manlig och kvinnlig bröstcancer på DNA-nivå.
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References

48. Bloom HJ, Richardson WW. Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years. Br J Cancer 1957; 11(3):359-77.

115. The R Project for Statistical Computing [www.r-project.org].


129. [www.scan.bmc.lu.se]. South Sweden Cancerome Analysis Network.

130. [www.u-can.uu.se]. Uppsala-Umeå Comprehensive Cancer Consortium.
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