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Molecular Characterization of Male Breast Cancer

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Molecular Characterization of Male Breast Cancer

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Abstract

Cancer is today a major healthcare problem worldwide. There are many forms of cancer, which is a genetic disease, believed to result from a multistep process with genetic and epigenetic changes accumulating over time. Breast cancer is one of the most common forms of cancer in women, while it is much more rare in men. Male breast cancer (MBC) exhibits both similarities and differences with female breast cancer (FBC). However, in contrast to FBC, MBC is poorly characterized on the molecular level. Multiple global profiling studies of FBC have been performed on the genetic and epigenetic levels, while only a few genetic studies have been performed on MBC.

The aim of the research presented in this thesis was to study MBC on the genomic, transcriptomic and epigenomic levels, in an attempt to reveal some of the mechanisms of tumorigenesis in MBC, and to provide a detailed molecular landscape of the disease, allowing comparisons with FBC. The various studies were carried out using high-resolution microarrays and immunohistochemistry on a well-annotated cohort of MBC patients from whom 83 fresh frozen and 220 formalin-fixed paraffin-embedded samples were available.

It was found that MBC is heterogeneous on a genomic, transciptomic and epigenetic level, consisting of various kinds of tumors, each of which exhibits its own aberrations, as is the case in FBC. Male and female breast cancer tumors had a similar appearance on a global level, but when studied in greater detail, many genetic and epigenetic differences were revealed. Furthermore, the landscapes of candidate drivers in male and female breast cancer appear to show considerable differences. Attempts were made to subclassify the fresh frozen MBC tumors with regard to copy number, and levels of mRNA and DNA methylation. Two stable subgroups were consistently identified, showing differences in the biological features of the tumors, where one of the subgroups seemed to consist of a number of more aggressive MBC tumors. The subgroups from the three datasets analyzed were significantly associated with each other, however, they differed from the known subgroups of FBC. As such, they may constitute two novel subgroups of breast cancer, occurring exclusively in men. This indicates that breast cancer in men may require different management and treatment strategies from those used in women with breast cancer.

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List of Papers

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

I. **Johansson** I, Nilsson C, Berglund P, Strand C, Jönsson G, Staaf J, Ringnér M, Nevanlinna H, Barkardottir RB, Borg A, Olsson H, Luts L, Fjällskog ML, Hedenfalk I. *High-resolution Genomic Profiling of Male Breast Cancer Reveals Differences Hidden Behind the Similarities with Female Breast Cancer.* Breast Cancer Research and Treatment, 129(3), 747–760, 2011.

II. **Johansson I**, Nilsson C, Berglund P, Lauss M, Ringnér M, Olsson H, Luts L, Sim E, Thorstensson S, Fjällskog M-L, Hedenfalk I. *Gene Expression Profiling of Primary Male Breast Cancers Reveals Two Unique Subgroups and Identifies N-acetyltransferase-1 (NAT1) as a Novel Prognostic Biomarker.* Breast Cancer Research, 14(1), R31, 2012.

III. **Johansson I**, Ringnér M and Hedenfalk I. *The Landscape of Candidate Driver Genes Differs Between Male and Female Breast Cancer.* PLoS One, 8(10), e78299, 2013.

IV. **Johansson I**, Lauss M, Holm K, Staaf J, Nilsson C, Fjällskog ML, Ringnér M, Hedenfalk I. *Whole Genome Methylation Patterns in Male and Female Breast Cancer – Identification of an Epitype with Hypermethylation of Polycomb Target Genes.* Manuscript.

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Related publications not included in this thesis:

- **Johansson I**, Aaltonen K E, Ebbesson A, Grabau D, Wigerup C, Hedenfalk I, Rydén L. *Increased Gene Copy Number of KIT and VEGFR2 at 4q12 in Primary Breast Cancer is Related to an Aggressive Phenotype and Impaired Prognosis*. Genes, Chromosomes & Cancer, 51(4), 375–383, 2012.

- Kimbung S, Biskup E, **Johansson I**, Aaltonen K, Ottosson-Wadlund, A, Gruvberger-Saal S, Cunliffe H, Fadeel B, Loman N, Berglund P, Hedenfalk I. *Co-targeting of the PI3K Pathway Improves the Response of BRCA1 Deficient Breast Cancer Cells to PARP1 Inhibition.* Cancer Letters, 319(2), 232–241, 2012.

- Nilsson C, **Johansson I**, Ahlin C, Thorstenson S, Amini R-M, Holmqvist M, Bergkvist L, Hedenfalk I Fjällskog M-L. *Molecular Subtyping of Male Breast Cancer Using Alternative Definitions and its Prognostic Impact.* Acta Oncologica, 52(1), 102–109, 2013.

- Nilsson C, Koliadi A, **Johansson I**, Ahlin C, Thorstenson S, Bergkvist L, Hedenfalk I, Fjällskog M-L. *High Proliferation is Associated with Inferior Outcome in Male Breast Cancer Patients.* Modern Pathology, 26(1), 87–94, 2013.

- Deb S, **Johansson I**, Byrne D, Nilsson C, kConFab Investigators, Constable L, Fjällskog M-L, Dobrovic A, Hedenfalk I, Fox S B. *Nuclear HIF1A Expression is Strongly Prognostic in Sporadic but not Familial Male Breast Cancer*. Accepted for publication in Modern Pathology.

Abbreviations

aCGH	Array comparative genomic hybridization
AR	Androgen receptor
BAC	Bacterial artificial chromosome
cDNA	Complementary DNA
CGH	Comparative genomic hybridization
CMF	Cyclophosphamide, methotrexate and 5-fluorouracil
CONEXIC	COpy Number and EXpression In Cancer
CpG	5-cytosine-guanine-3 dinucleotide
DAVID	Database for annotation, visualization and integrated discovery
DNMT	DNA methyltransferase
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
FBC	Female breast cancer
FFPE	Formalin-fixed paraffin-embedded
FGA	Fraction of genome altered
FGFR2	Fibroblast growth factor receptor 2
FISH	Fluorescence in situ hybridization
GEO	Gene expression omnibus
GEX	Gene expression
GISTIC	Genomic Identification of Significant Targets in Cancer
GO	Gene ontology
H3K27me3	Trimethylation of lysine 27 on histone H3
H3K4me3	Trimethylation of lysine 4 on histone 3
HE	Hematoxylin and eosin
HOX	Homeobox
IHC	Immunohistochemistry
MBC	Male breast cancer
NAT1	N-acetyltransferase-1
NHG	Nottingham histological grade
PARP	Poly(ADP-ribose) polymerase
popLowess	Population-based intensity-based lowess
PR	Progesterone receptor
PRC2	Polycomb repressive complex 2
RPMM	Recursively partitioned mixture model

SAM	Significance analysis of microarray
SEER	Surveillance Epidemiology and End Results (Program)
SNP	Single nucleotide polymorphism
TMA	Tissue microarray

Abstract

Cancer is today a major healthcare problem worldwide. There are many forms of cancer, which is a genetic disease, believed to result from a multistep process with genetic and epigenetic changes accumulating over time. Breast cancer is one of the most common forms of cancer in women, while it is much more rare in men. Male breast cancer (MBC) exhibits both similarities and differences with female breast cancer (FBC). However, in contrast to FBC, MBC is poorly characterized on the molecular level. Multiple global profiling studies of FBC have been performed on the genetic and epigenetic levels, while only a few genetic studies have been performed on MBC.

The aim of the research presented in this thesis was to study MBC on the genomic, transcriptomic and epigenomic levels, in an attempt to reveal some of the mechanisms of tumorigenesis in MBC, and to provide a detailed molecular landscape of the disease, allowing comparisons with FBC. The various studies were carried out using high-resolution microarrays and immunohistochemistry on a well-annotated cohort of MBC patients from whom 83 fresh frozen and 220 formalin-fixed paraffin-embedded samples were available.

It was found that MBC is heterogeneous on a genomic, transciptomic and epigenetic level, consisting of various kinds of tumors, each of which exhibits its own aberrations, as is the case in FBC. Male and female breast cancer tumors had a similar appearance on a global level, but when studied in greater detail, many genetic and epigenetic differences were revealed. Furthermore, the landscapes of candidate drivers in male and female breast cancer appear to show considerable differences. Attempts were made to subclassify the fresh frozen MBC tumors with regard to copy number, and levels of mRNA and DNA methylation. Two stable subgroups were consistently identified, showing differences in the biological features of the tumors, where one of the subgroups seemed to consist of a number of more aggressive MBC tumors. The subgroups from the three datasets analyzed were significantly associated with each other, however, they differed from the known subgroups of FBC. As such, they may constitute two novel subgroups of breast cancer, occurring exclusively in men. This indicates that breast cancer in men may require different management and treatment strategies from those used in women with breast cancer.

Aims

The overall aim of the studies presented in this thesis was to improve our understanding of male breast cancer (MBC) on the genomic, transcriptomic, and epigenomic levels, in order to be able to reveal the mechanisms of tumorigenesis in MBC on multiple levels, as well as to provide a detailed molecular landscape of the disease.

The specific aims were as follows:

- to obtain a detailed molecular landscape of MBC on the genomic, transcriptomic and epigenomic levels,
- to identify and characterize subgroups of primary MBC on the genomic, transcriptomic and epigenomic levels,
- to compare male and female breast cancer and
- to identify novel prognostic and/or predictive biomarkers for MBC.

Background

Introduction to Cancer

Cancer is today a major problem worldwide; in the United States one in four deaths is due to cancer [1]. In Sweden, 55,000 people developed cancer in 2011, and it is estimated that one person in three will develop cancer at some time during their life. Cancer is not just one disease, but about 200 different forms, affecting various organs and types of cells [2]. Furthermore, different forms of cancer have been shown to be extremely heterogeneous; each tumor being unique. Nonetheless, all forms of cancer arise from normal cells that have started to divide uncontrollably. There is evidence that tumorigenesis is a multistep process involving genetic and epigenetic changes (*e.g.* point mutations, chromosomal translocations, copy number abbreviations, DNA methylation and histone modifications) that accumulate over time. For a normal cell to be transformed into a cancer cell it must acquire several new capabilities. In 2000, Hanahan and Weinberg suggested six capabilities, which they called the hallmarks of cancer, believed to be shared by all types of cancer [3]. In 2011 they published an updated list including two new hallmarks of cancer and two enabling characteristics [4]. These hallmarks of cancer are described below and illustrated in Figure 1.

- *Sustainment of proliferative signaling*: Normal cell proliferation is strictly controlled by growth signals, however, tumor cells have found ways of becoming self-sufficient in growth signals. Generally, this can be achieved in three ways; autocrine stimulation by the synthesis of growth factors, alterations in the components of downstream signaling pathways leading to constitutive proliferation and overexpression of growth factor receptors, or the expression of structurally altered receptors, such as mutations in the epidermal growth factor receptor (EGFR) in human gliomas [5].
- *Evasion of growth suppressors*: Normal cell proliferation is also controlled by anti-proliferative signals, which maintain cellular quiescence and tissue homeostasis; cancer cells acquire insensitivity to these suppressors. One way of doing this is by disruption of the retinoblastoma protein pathway, which is one of the major transmitters of anti-proliferation signals [6].
- *Resistance to cell death*: Cellular homeostasis is also controlled by apoptosis, which is a form of programmed cell death. Cancer cells can avoid this by changes in proapoptotic and/or antiapoptotic signaling. One common way is

inactivation of the TP53 tumor suppressor gene that responds to many stress stimuli, and can respond by inducing apoptosis and cell cycle arrest [7].

- *Enablement of replicative immortality*: Normal cells can only undergo a certain number of replications, after which they stop growing and enter senescence. This is due to the shortening of telomeres, the repetitive sequences at the end of the chromosomes, following each cell division. When the telomeres are shortened they can no longer protect the chromosomes, and the free ends of the chromosome can take part in end-to-end fusions that eventually lead to cell death. Telomerase, the enzyme that can elongate the telomeres, is frequently upregulated in tumor cells, leading to endless replicative potential [8].
- *Induction of angiogenesis*: For a normal cell to be able to grow, it needs nutrients and oxygen, which are supplied through the blood vessels, thus tumors need to develop new blood vessels to be able to grow beyond a few millimeters. This is done by changing the balance of pro- and anti-angiogenic factors that regulate blood vessel development, *e.g.* by upregulating the pro-angiogenic factor, vascular endothelial growth factor (VEGF) [9].

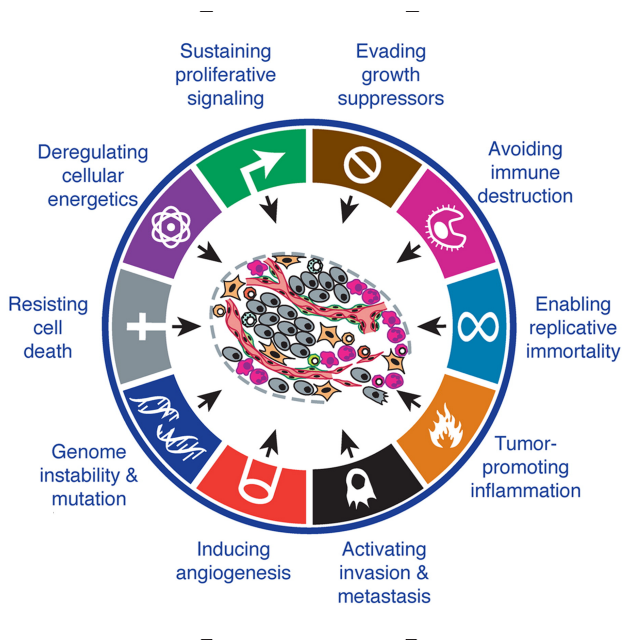

Figure 1. The hallmarks of cancer. The capabilities that a normal cell must acquire if it is to be transformed into a cancer cell. Reprinted with permission from Elsevier.

- *Activation of invasion and metastasis*: Metastasis is the cause of most cancer-related deaths. For a cancer cell to metastasize it must first become detached from the primary tumor, enter the blood circulation and lymph vessels, migrate through these vessels and finally settle at a new site, to found a new tumor colony. The invasion–metastasis cascade involves a large number of proteins in cell–cell and cell–matrix adhesion, as well as matrix remodeling. [10].

Two emerging hallmarks of cancer are *deregulation of cellular energetics* and *avoidance of immune destruction*. Tumors require large amounts of energy to grow rapidly, and the energy metabolism must thus be deregulated. It is important for a tumor to be able to avoid destruction by the immune system, in particular by T and B lymphocytes, macrophages and natural killer cells. The two enabling characteristics are *genome instability* and *tumor promoting inflammation* [4].

The Male and Female Breast

Male and female breasts are anatomically similar until puberty, with small ductal structures in the surrounding stroma and fatty tissue. However, the female breast starts to change during puberty, and develops lobules due to changes in the levels of sex hormones (androgens, estrogens and progestogens), while the male breast stops developing. The female breast is also exposed to cyclic changes in sex hormones through the menstrual cycle, and undergoes considerable changes during pregnancy, lactation and menopause due to changes in sex hormone levels [11,12]. These constant changes in the female breast under the influence of sex hormones makes it susceptible to carcinogenic events.

The female breast consists of milk lobules (which produce milk), milk ducts (which lead the milk to the nipple), fatty tissue, stroma, blood vessels and lymph ducts, as illustrated in Figure 2A. The male breast does not normally develop milk lobules as illustrated in Figure 2B [11,12]. The milk ducts and lobules consist of two types of cell layers: the outer basal myoepithelial and the inner luminal epithelial layers [11]. The most common benign breast disease in men is gynecomastia, which is abnormal growth of the breast, due to sex hormone imbalances, and is often reversible. Thus, the male breast, like the female breast, can start to grow under the influence of sex hormone changes [13].

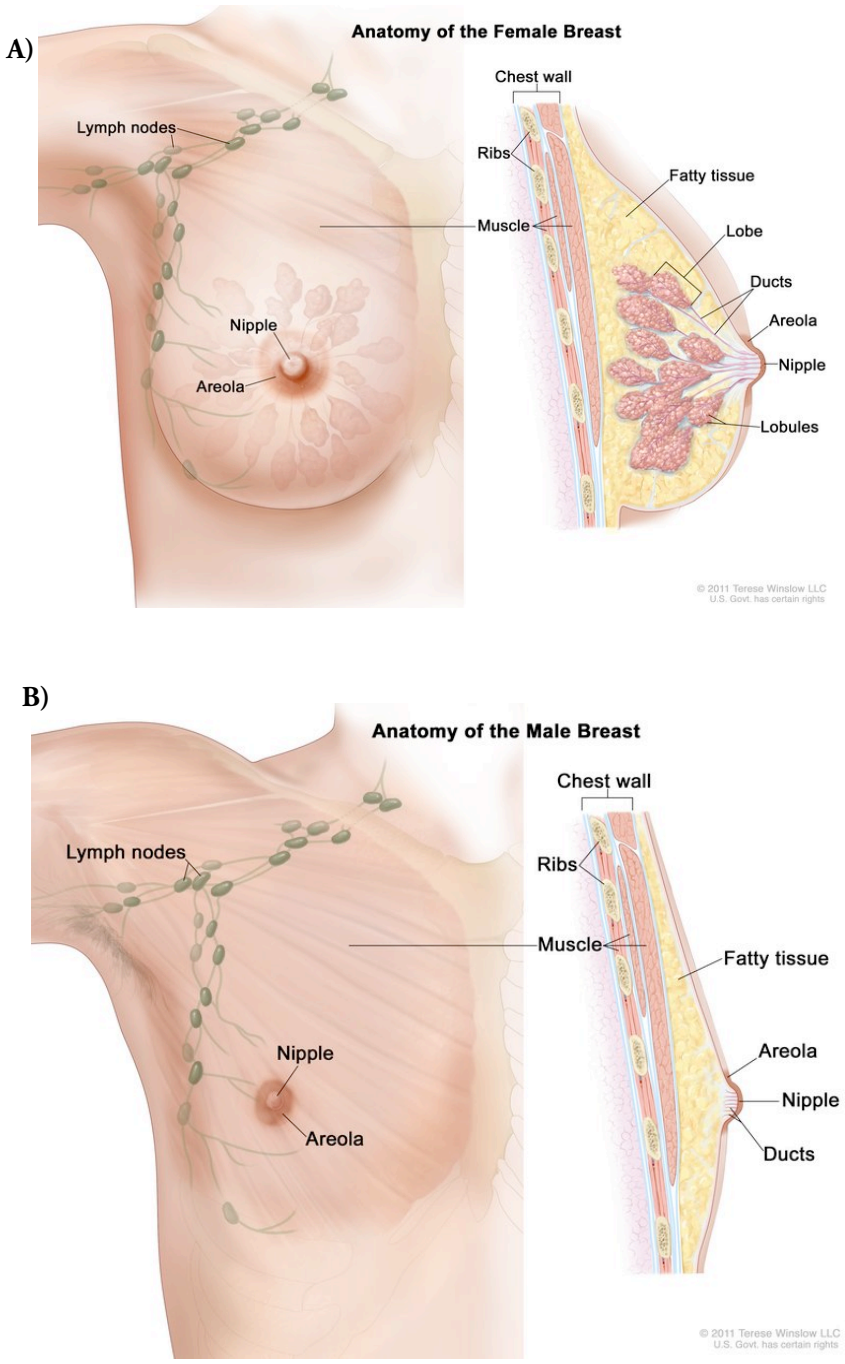

Figure 2. Female (A) and male (B) breast anatomy showing the nipple, areola, fatty tissue, ducts and lobes. Nearby lymph nodes, ribs and muscle are also shown. Reprinted with permission from Terese Winslow.

Male Breast Cancer

Epidemiology

Breast cancer is the most common form of cancer in women, in both the developed and the developing countries [14], and accounts for 30% of all cancers in women in Sweden [1,2]. Breast cancer is also seen in men, but is a rare form of cancer, constituting only 0.5% of all breast cancers in the Nordic countries [15], and 1% in the US [1]. Higher incidence rates of MBC have been observed in some African countries and in men of Jewish ancestry [16,17]. The higher incidence in Africa may be due to the higher incidence of infectious diseases that cause liver damage, which leads to higher estrogen levels [16], while the cause in Jewish men is most likely a higher frequency of germline mutations in the *BRCA1* and *BRCA2* genes [17]. The incidence of breast cancer in women has increased by 1.2% annually over the past 10 years in Sweden [18], while the incidence in men has not increased in the Nordic countries [15]. A recently published study on the incidence of male and female breast cancer in five continents showed that the incidence of female breast cancer (FBC) is increasing in the majority of the countries studied, while the trends in the incidence of MBC are more variable, with only a minority of countries showing evidence of an increased incidence [19]. However, a slight increase in the incidence of MBC has been seen in England, Scotland, Australia and the US [20-22].

Risk Factors

When discussing the risk factors associated with cancer it is important to bear in mind that aging is the main risk factor associated with the development of solid tumors [23]. Establishing the precise risk factors for MBC has proved challenging due to the rarity of the disease. However, the risk factors identified to date include a family history of breast and ovarian cancer, and a known genetic predisposition; mutations in the *BRCA2* gene being the most common, followed by *BRCA1*. *BRCA1* and *BRCA2* are tumor-suppressor genes that play a key role in the repair of damage to the DNA and cell cycle checkpoint control [24]. Male *BRCA* mutation carriers have a higher risk of developing cancer in the breast, prostate, colorectal region, and pancreas [25]. In studies on groups of MBC patients ranging from 50 to 261 individuals, a *BRCA2* germline mutation was found in 4-14% and a *BRCA1* germline mutation in 0-4% [26-32]. Men carrying a *BRCA* mutation have an estimated increased lifetime risk of developing breast cancer of ~7% for *BRCA2* and 1-6% for *BRCA1* [25,33,34], which should be compared with the risk in the general male population of 0.1% [15,25]. For other mutations associated with a moderate risk of FBC, such as those in *PALB2*, *CHEK2* and *CYP17* [35,36], results regarding the relevance for the risk of MBC are conflicting [32,37-44]. Regarding other germline mutations, *e.g. BRIP1* and

RAD51C, which have also been shown to be associated with a moderately increased risk of FBC [35], no increase in the risk of developing MBC has been found [45,46]. However, it must be borne in mind that the studies on germline mutations associated with a moderate risk of FBC are very small, and more and larger studies are needed to obtain a clear picture of the effect in men. A large genome-wide association study of MBC has identified one new breast cancer susceptibility locus in *RAD51B*, and one known FBC cancer susceptibility locus within *TOX3* as being significantly associated with the risk of MBC [47].

As in FBC, hormonal imbalances that change the ratio of estrogen to androgen are important risk factors. The hormonal imbalance most strongly associated with MBC is caused by Klinefelter's syndrome, which is characterized by at least one additional X chromosome, and it gives rise to a 50-fold increase in risk [48,49]. Other factors causing hormonal imbalance are obesity [48,50-52], liver disease [53], testicular abnormalities that result in testosterone deficiency [48,54] and exogenous estrogen exposure [55,56]. Men who work in high-temperature environments, such as those encountered at steelworks, blast furnaces and rolling mills, also have a higher risk of developing breast cancer, probably due to testicular failure resulting from long-term exposure to high ambient temperatures, resulting in a hormonal imbalance [57,58]. There is no convincing evidence of an increased risk of breast cancer in males with gynecomastia [16]. Furthermore, in a recent large study by Gooren *et al.* no increased risk of MBC was found in male-to-female transsexuals undergoing androgen deprivation and estrogen therapy, which has previously been regarded as a risk factor for MBC [59]. The effects of occupational exposure to polycyclic aromatic hydrocarbons and other chemicals [60,61] and radiation to the chest [16,62] have been evaluated as possible contributors to the risk of developing MBC. None of the lifestyle factors smoking, alcohol intake, or physical activity has been found to be associated with a higher risk of developing MBC [48,50-52].

Clinical and Tumor Biological Characteristic

The degree of similarity between breast cancer in males and females has not been well established. There are, however, some clear differences, for example, MBC is much more rare than FBC. One of the main reasons why MBC is such a rare disease is probably due to the physiology of the male body, with small amounts of breast tissue and no cyclic changes in sex hormones. Breast cancer is a hormonal disease, which is highly influenced by sex hormones, but MBC still tends to be hormone-receptor-positive more often than FBC (estrogen receptor (ER) positivity 91-95% *vs.* 76-78% and progesterone receptor (PR) positivity 80-81% *vs.* 67%, in MBC and FBC, respectively) [63-65]. Little data are available on *HER2* amplification in MBC, but four studies have been carried out using fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC) of HER2, in which it was found that 6-11% of MBC tumors were HER2-positive [66-68] and one Chinese study found 35% HER2-

positive MBC tumors [69], while 15-30% of FBC tumors were HER2-positive [70-72]. HER2-positive disease in FBC was previously associated with one of the worst outcomes, however, these patients are now given anti-HER2 treatment, and their outcome has improved considerably [73,74]. In a recent retrospective study including 197 MBC patients conducted in Sweden, it was found that 11% of MBC tumors were HER2-positive. These patients had not received any chemotherapy or trastuzumab, and HER2 positivity was not associated with a worse prognosis [67]. The mean age at diagnosis of breast cancer in men is approximately five years older than in women (67 *vs.* 62 years, respectively) [63]. The pattern in age distribution at diagnosis differs between males and females. The distribution in women is bimodal, with one peak corresponding to early-onset of the disease and one to later age at onset, while in men the curve has only one peak at ~75 years of age [75]. The distribution of histological types also varies between male and female breast cancers. The majority of MBCs are invasive ductal carcinomas (85-95%), followed by ductal carcinoma *in situ* (5-10%) [16,75]. Invasive ductal carcinomas are also the most common among FBCs (50-80%) [76], while papillary carcinomas are more common in males than females (2-4% and 1%, respectively) [75,76], and lobular carcinoma is rarer in males (1-2% and 5-12%, respectively) [63,76]. The diagnosis of breast cancer *in situ* is rarer in males than in females (9% *vs.* 12%, respectively) and a Surveillance Epidemiology and End Results (SEER) database study revealed that the rate of *in situ* tumors rose by 123% in men between 1973-2001 (despite the absence of screening mammography in men), and by 555% in women [77].

Prognosis

As in the case of all cancers, the earlier MBC is detected the better the prognosis, because the less time the cancer has had to accumulate genetic aberrations and become more aggressive. The survival rate for men with breast cancer in relation to women with breast cancer is still the subject of debate. Some studies have shown higher mortality rates in men [2,77-79], while others have found that the mortality rates are similar in men and women with breast cancer [80,81]. Due to the rareness of MBC, many of the studies suffer from small sample size, cover long periods of time, and include patients from many different hospitals and sometimes also countries, limiting the interpretation of the results. The treatment of breast cancer patients has improved over recent decades, but may vary between different hospitals and different countries. In a single-center register study conducted in Uppsala, Sweden, 99 MBC patients were each matched with four FBC patients (n=396) of a similar age at diagnosis, and time of diagnosis between 1993 and 2007. It was seen that the male and female patients were given the same treatment, and that the tumors were of the same stage, but the relative overall survival of the male patients was worse [82]. Another recently published single-center study in China included only invasive ductal carcinomas, and matched age at diagnosis, date of diagnosis and stage, for patients

diagnosed between 1980 and 2012. Disease-free survival and overall survival were also significantly worse among the men, but no corrections were made for life expectancy. Furthermore, it was concluded that the male patients received the same amount of chemotherapy but less adjuvant endocrine treatment, despite the fact that the MBC tumors were more often ER-positive. However, when comparing the MBC patients who received endocrine treatment with those who did not, no difference in survival was seen [83]. Others have reported that the difference in survival between male and female breast cancer patients disappeared when the patients were matched for least age and stage of the disease [63,81]. In another, older study it was concluded that the poor prognosis for men was due to a combination of higher age at diagnosis and that diagnosis was usually delayed [84]. However, no delay in the diagnosis of MBC was seen in the Swedish study [82]. In the largest register study of MBC to date, comparing 13,000 male and 1,440,000 FBC cases between 1998 and 2007 in the National Cancer Data Base in the US, the overall survival rate was found to be significantly worse for MBC; however, no corrections were made for life expectancy. Survival for those with stage I and II breast cancer was also found to be worse for men, while the survival was similar for males and females with stage III and stage IV breast cancer [85]. Significantly worse relative survival and disease-specific survival were found for male patients in the second largest register study on the outcome of breast cancer in many countries including 2,665 MBC patients and 459,846 FBC patients diagnosed between 1970 and 2007. However, when the men and women were matched regarding region, age, year of diagnosis, follow-up time, stage of disease and treatment (analysis including ~800 MBC patients) a slightly better survival was seen in the male patients. The authors claimed that the poorer survival observed for male patients before matching could be explained by their higher age and the more advanced stage of the disease at diagnosis, and the fact that men received less standard locoregional treatment [86]. In another study comparing breast cancer specific survival according to stage in males and females using SEER data from 1988 to 2003, the specific survival of males diagnosed with stage I breast cancer was found to be worse than that of females. However, the authors suggested that this was probably due to in-stage migration, rather than having any clinical relevance [87]. The relative 5-year overall survival rates for all MBC and FBC patients in Sweden are 79.6% and 90.0%, respectively, and the relative 10-year overall survival rates are 67.1% and 83.5% [2]. Bearing in mind the fact that ~15% of FBCs are triple-negative breast cancers (*i.e.*, negative for ER, PR and HER2), associated with poor prognosis [88,89], while only 0-6% of MBC tumors appear to be triple-negative [67,69,81,90], it is remarkable that the relative overall survival rate for all MBCs is worse than that for all FBC patients.

The 10-year relative overall survival rate for FBC patients in Sweden has increased during the past 40 years, probably due to early detection, as a result of mammographic screening, and modern systemic adjuvant treatment [2]. A significant decrease in mortality has also been found among FBC patients in the SEER database

during recent decades, however, no significant decrease was seen for men, although a small trend towards decreased mortality was found [65]. This is probably attributable to a lack of progress in the understanding and treatment of MBC.

In conclusion, the relative overall, and breast cancer specific survival seem to be worse for MBC patients than FBC patients, however, when the patients are matched regarding different prognostic factors, the difference sometimes disappears. It is well known that breast cancer is a very heterogeneous disease, and the question is which factors should be matched when comparing the survival of men and women.

Diagnosis for Prognosis

Male breast cancer is often detected as a suspicious painless subareolar lump, but it can also involve nipple retraction or bleeding from the nipple [91,92]. The diagnosis of breast cancer is based on a triple approach that includes [93]:

- clinical examination of the breast and loco-regional lymph nodes,
- radiological examination combined with ultrasound and, in some cases, magnetic resonance imaging (MRI) and
- histological examination of fine-needle aspirates, often supplemented with a core biopsy.

Prognostic and Predictive Factors

Prognostic and predictive factors are of great importance in the clinic to guide the choice of therapeutic strategy. A prognostic factor indicates the inherent aggressiveness and the natural history of the disease. A predictive factor indicates the likelihood of response to a given therapy. The prognostic and predictive factors that are established in FBC, and are used in clinical routine today in Sweden are age at diagnosis, tumor size, lymph node status, presence of distant metastases, histological classification, Nottingham histological grade (NHG), ER, PR, HER2 and Ki67 [93]. These factors have been evaluated in MBC in different retrospective studies with varying results, thus it has not yet been definitively established whether all these factors are equally prognostic and predictive in men and women. However, the current recommendations for treating men with breast cancer in Sweden are that they should be treated like women with breast cancer, with the exception that treatment with aromatase inhibitors is probably not optimal, and therefore tamoxifen should be the first choice for treating ER-positive MBC [93].

Age at Diagnosis

Young age (<35) in females with breast cancer is associated with aggressive tumors and an unfavorable prognosis [94,95]. However, young age in patients with MBC does not appear to be correlated with disease-free survival [96,97]. In contrast, several studies have found high age to be a prognostic factor for worse overall survival [63,97,98], which can probably be explained by comorbidity and a lower tolerance to therapy. The number of MBCs among young men is very small, making it difficult to study, or to draw any conclusions from the findings.

The TNM Classification System

The clinical stage according to the TNM classification system is based on tumor size (T), lymph node metastasis (N) and distant metastasis (M), and is a well-established prognostic tool for FBC [99]. Tumor size and lymph node status have been shown to be independent but additive prognostic factors in FBC [100]. A large study on 2,537 MBC patients revealed an independent significantly poorer survival for patients with tumors >20 mm and with lymph node involvement [63]. Both large tumor size and lymph node involvement were found to be associated with poor survival in a univariate analysis of the 220 MBC tumors included in the present studies. However, only lymph node involvement remained significant in a multivariate analysis [96]. In a study on 489 MBC patients Cutuli *et al.* also found only lymph node involvement to be independently associated with metastatic risk [101].

Nottingham Histological Grade

The NHG is a method of grading breast cancer described by Elston and Ellis, and is a measure of the aggressiveness of the tumor, and is strongly correlated with prognosis in FBC. It is obtained by the combined evaluation of tubule formation, degree of nuclear pleomorphism and mitotic count [102]. Some studies have found grade to be an independent prognostic factor for poor prognosis in MBC [101], while others have not [63,96].

The Estrogen and Progesterone Receptors

ER is a nuclear steroid receptor protein that is activated by the three main estrogens, estrone, estradiol and estriol. It functions as an intracellular transcription factor, and is involved in cell growth and survival [103,104]. Both ER and PR are established prognostic factors in FBC [105,106], and they are also predictive markers of the response to endocrine treatment [107-109]. However, the value of PR has been questioned, and a recent meta-analysis failed to show any additional predictive value regarding tamoxifen response [110]. The prognostic value of ER and PR has not been confirmed in MBC; some studies have shown ER and/or PR to have some prognostic value [64,84,111], while others have found no prognostic value [63,112,113].

HER2

The proto-oncogene HER2 is a tyrosine kinase receptor that belongs to the human epidermal growth factor receptor family, which also includes HER1 (EGFR), HER3 and HER4. Amplification of HER2 in FBC leads to proliferation, increased invasive capacity, increased angiogenesis, and independence of growth signals [114,115], and it is associated with poorer prognosis in FBC [71,116]. Some studies in men showed a lack of prognostic power for HER2-positive breast tumors [68,117-119], while others found that HER2 positivity predicted a shorter disease-free or overall survival [111,120,121].

Ki67

Ki67 is a protein and a proliferation marker that is expressed by all proliferating cells in the body, in all phases of the cell cycle, except the quiescent phase, however, its function is still relatively unknown [122]. The prognostic value of Ki67 in FBC has been demonstrated in several studies [122-124], nonetheless, there is still no international consensus on the assessment of Ki67. Few studies have been performed to assess the prognostic value of Ki67 in MBC, and some found it to have no prognostic value [64,111], while a poorer progression-free survival was found in one study on patients with Ki67-positive tumors, however, no such effect was seen on overall survival [125].

Management

Due to the rarity of MBC there is a lack of prospective studies, and MBC is an incompletely characterized and understood malignancy. Therapeutic approaches for the management of MBC are hence extrapolated from small retrospective trials and prior knowledge of FBC. MBC patients are today treated according to the same guidelines as FBC patients. More molecular studies are needed to improve our understanding of the pathobiology of the disease, and prospective randomized trials are required to optimize patient management. One of the great challenges in cancer treatment today is customizing treatment to each patient's individual needs. In determining individual treatment strategies it is important to bear in mind that it is as essential to identify the patients that should do well with less treatment, as the patients that would benefit from more aggressive treatment. Many cancer therapies are aggressive and can cause severe side effects, which may lead to more suffering than benefit. A large proportion of breast cancer patients in Sweden today are actually cured by primary surgery alone, and further treatment is unnecessary. Nonetheless, better biomarkers are needed to be able to identify more of these patients [93].

Surgery

The surgical options for MBC patients in use today are mastectomy or breast-conserving therapy, followed by radiation. Several randomized prospective trials have been performed comparing mastectomy with breast-conserving therapy followed by radiation in FBC patients, showing no difference in survival between the two techniques. Breast-conserving therapy is thus the first choice for FBC patients with early stage breast cancer [126,127]. However, breast-conserving surgery is considered less appropriate in MBC patients mainly due to the comparative scarcity of breast tissue, the central location of many of the tumors, and more often advanced staging at diagnosis. Therefore, the most common surgical treatment of MBC is mastectomy [75]. Only a few small studies have been carried out on MBC to compare the outcome of patients undergoing mastectomy to those undergoing breast-conserving therapy. Higher recurrence rates were found in the patients who had undergone breast-conserving surgery [128,129].

Lymph node involvement is a strong predictor of both local recurrence and metastatic risk, and sentinel node biopsy, which uses the techniques of blue dye and radioisotope localization to identify the sentinel node, is recommended as the initial procedure for determining the lymph node status [93]. Sentinel node biopsy has been proven to be a safe and effective technique in FBC [130], and a few studies on MBC have shown it also to be a reliable tool in the management of MBC [131,132].

Radiotherapy

Postoperative radiotherapy is administered in order to eliminate possible residual microscopic disease. According to international guidelines, radio-therapy should be administered if the risk of developing a local recurrence with-in the next 10 years after surgery is greater than 20%, and includes patients undergoing partial mastectomy with tumors larger than 50 mm, and with more than three lymph node metastases [93]. One study on the pattern of local recurrence in MBC revealed that it was similar to the pattern in FBC, and thus postoperative radiotherapy was recommended in both male and female breast cancer patients [133]. This is further supported by the findings of another study on whether gender was a significant prognostic factor for loco-regional relapse post-mastectomy, when taking known prognostic factors and radiotherapy into consideration, but no evidence of any difference between men and women was found [134].

Systemic treatment

The aim of systemic treatment in an adjuvant setting is to target possible micro-metastases, thus reducing the risk of recurrence. In a neoadjuvant setting the aim is to shrink the tumor to make it operable, and in a palliative setting the purpose is to shrink the tumor and metastases to improve the quality of life for the patients and to prolong life. Systemic treatment includes endocrine treatment, chemotherapy and targeted drugs [93].

Endocrine treatment

Many invasive breast tumors are dependent on estrogen for their survival, and it has long been known that blocking the ER pathway can be used to treat ER-positive tumors [135]. This can be done by inhibiting the ER with tamoxifen or by removing the ligand estrogen with aromatase inhibitors such as anastrozole, letrozole and exemestane. Over 90% of MBC tumors are ER-positive, which strongly supports the use of adjuvant endocrine therapy as a cornerstone of treatment, as in FBC therapy. Endocrine treatment has been shown to reduce recurrence rates and improve the survival of FBC patients with ER-positive tumors, while no effect was seen in patients with ER-negative tumors [110]. The response of FBC patients to endocrine treatment seems to increase gradually with increasing levels of ER expression [108]. The recommended cutoff in Sweden for ER-positive tumors is >10% positive tumor cells [93], however, a cutoff as low as 1% has been recommended in other countries [136,137]. No clinical trials involving endocrine treatment have been carried out in MBC, however several retrospective studies have shown benefit to patients using adjuvant tamoxifen [128,138,139], while others have not [83,111]. Tamoxifen is the treatment of first choice for adjuvant and metastatic endocrine treatment in men, since aromatase inhibitors are unable to prevent testes-derived estrogen synthesis, which is the source of 20% of endogenous estrogen in men [140,141]. One population-based retrospective study in Germany, including 257 MBC patients compared the overall survival of patients who received adjuvant tamoxifen with those given adjuvant aromatase inhibitors, showing a significantly worse survival for the patients treated with aromatase inhibitors. Treatment with aromatase inhibitors has been linked to a 1.5-fold increase in the risk of mortality, compared to tamoxifen, after adjusting for age at diagnosis, tumor size, lymph node status and grade [142]. However, the use of tamoxifen therapy in men may be limited by the high incidence of adverse effects, often leading to poor compliance. Common adverse side effects include weight gain, hot flashes, sexual dysfunction, neurocognitive deficits and thromboembolic events, and in one study 20% of the MBC patients discontinued their tamoxifen therapy due to side effects [143]. Another study showed that as many as 63% of the MBC patients who received tamoxifen experienced side effects [144]. In one retrospective study it was found that the aromatase inhibitor letrozole in combination with gonadotropin-releasing hormone was effective and safe in 19 men with metastatic MBC [145]. A phase-II clinical trial, SWOG-S0511, of 56 MBC patients with recurrent or metastatic disease is ongoing, in which goserelin will be administered subcutaneously on day 1, followed by oral anastrozole for 28 days for 12 cycles. Recruitment to the trial has been completed, and it is hoped that the results of the trial will show whether more frequent use of aromatase inhibitors in combination with a gonadotropin-releasing hormone agonist is suitable in the treatment of MBC [146].

Chemotherapy

Chemotherapy is another type of systemic treatment that, for the treatment of breast cancer, includes cyclophosphamide, methotrexate and 5-fluorouracil (CMF), anthracycline-containing regimens and taxanes. Chemotherapy is today given as poly-chemotherapy due to the improved efficiency compared to single-agent regimens; the reason for this being the potential synergetic effects and the different toxicity profiles, thus allowing more intense treatment [147]. Several studies have shown the superiority of anthracycline-containing regimens over CMF [148-150], and the addition of taxanes to anthracycline-containing therapy further improved the outcome [151]. Standard adjuvant anthracycline-containing regimens, or in combination with taxanes, has been shown to reduce 10-year overall mortality by about one-third, independently of age (up to 70 years), nodal status, tumor size, histological grade, ER status or tamoxifen use. The improvement depends on the absolute risk; the greatest gain being found in patients with high absolute residual risk [152]. In Sweden, adjuvant chemotherapy is recommended for breast cancer patients with one or more of the following risk factors: ER-negative tumor, low age, high proliferation rate, lymph node positivity and an HER2-positive tumor. Normally, if the tumors are strongly ER-positive and only 1-3 lymph nodes are positive, one more risk factor is needed to qualify the patient for chemotherapy, otherwise endocrine treatment is considered sufficient [93]. Only a few retrospective studies have been conducted on chemotherapy in MBC, and in the two largest studies, including 135 and 121 MBC patients, a trend towards better outcome was seen in men treated with chemotherapy [79,140]. However, the small sample sizes and the lack of randomized clinical trials make it difficult to draw any definitive conclusions regarding the benefit of chemotherapy in the treatment of MBC.

Targeted therapy

Targeted therapies make use of drugs that target specific molecules involved in tumor growth and progression, with the aim of blocking the growth and spread of the tumor. One example is the monoclonal antibody trastuzumab, which is directed against HER2. Trastuzumab has been shown to significantly reduce mortality and improve recurrence-free survival in HER2-positive FBC [73,153,154]. Its role in MBC is less clear, due to the fact that no data have been published on the efficacy of trastuzumab in HER2-positive MBC, with the exception of one case report, in which the successful use of trastuzumab in a single metastatic MBC patient was described [155]. Another example of a drug used in targeted therapy that is currently being investigated and used in FBC is the tyrosine kinase inhibitor lapatinib [156].

A promising drug for targeted therapy of FBC is everolimus, an mTOR inhibitor. Addition of everolimus to hormonal treatment or anti-HER2 treatment has been shown to improve the outcome of FBC patients in several clinical trials. Activation of the mTOR pathway could be one mechanism responsible for the development of resistance to endocrine treatment and chemotherapy in FBC, and the addition of

everolimus to standard treatments of FBC is a promising strategy to overcome resistance and improve outcome [157]. Poly(ADP-ribose) polymerase (PARP) inhibitors are another example of drugs being investigated for targeted therapy in connection with FBC, and it has shown that they selectively kill cells with defective double-stranded DNA break repair mechanisms, and are thus interesting for the treatment of breast cancer tumors with a *BRCA1* or *BRCA2* mutation [158,159].

Breast Cancer Genetics

A reference genome is required to be able to study all the somatic changes leading to cancer, and in 2003 the sequencing of the Human Reference Genome was completed [160]. This led to the rapid development of powerful new tools, such as microarrays and massively parallel sequencing technologies, for the investigation of cancer genomes. As the resolution and scope of these techniques have improved, the complexity and heterogeneity of tumors is becoming increasingly evident. Three different high-throughput microarray-based techniques were used in the work described in this thesis to study global copy number aberrations (Papers I and III), gene expression (GEX) (Papers II and III) and DNA methylation (Paper IV) in MBC. Considerable effort has been made to characterize the biology of FBC using many different types of microarrays and sequencing techniques. In 2000 Perou *et al.* identified four intrinsic subgroups of FBC tumors using microarray-based gene expression profiling [161]. These intrinsic subtypes have been further studied, reproduced and refined by them and others [162-165]. The five main intrinsic subtypes of FBC are given below.

- Luminal A: The majority of these are ER-positive, often with low proliferation.
- Luminal B: The majority of these are ER-positive, and often highly proliferative. *BRCA2*-mutated tumors are often classified as luminal B.
- Basal-like: The majority of these are triple-negative, and express cytokeratins 5, 6 and 14. *BRCA1*-mutated tumors are often classified as basal-like.
- HER2-enriched: These show an overrepresentation of ER-negative tumors, and most exhibit amplification and/or overexpression of HER2.
- Normal-like: This is not a well-defined subgroup, and the expression of the genes is similar to that in normal breast tissue. It is not clear whether they represent a true subtype of FBC tumors, or reflect a tumor sample with a high degree of normal cells.

These subtypes are correlated to prognosis and treatment response: basal-like and HER2-enriched subtypes have the worst prognosis and the luminal A subtype the best

prognosis, while the luminal B subgroup has an intermediate prognosis [162-165]. FBC has also been classified into subgroups on the DNA level by several research groups [166-169]. Jönsson *et al.* identified seven DNA subgroups of FBC, four of which were highly related to the intrinsic subtypes of FBC based on gene expression [166].

MBC, on the other hand, is not well characterized on the genomic level. Only a few small microarray-based studies of MBC have been reported, showing similarities to, and differences between, FBC and MBC on the genomic [170], transcriptomic [171] and microRNA levels [172,173]. Callari *et al.* performed a direct comparison between MBC and FBC tumors, but because the samples were not run together, it is difficult to determine which of their findings are true differences and which could be due to potential batch effects [171]. In the array comparative genomic hybridization (aCGH) study by Tommasi *et al.* MBC tumors were only compared with basal-like FBCs, making it difficult to draw any conclusions about the whole group of FBC compared to MBC [170]. No attempts were made to subclassify the MBC tumors in any of these microarray studies of MBC [170-173].

FBC can also be classified into transcriptionally derived subgroups using standard IHC markers, and there is a relatively high concordance of 75-90%, between the intrinsic gene expression subtypes and IHC subgroups [174]. The following IHC markers are commonly used in FBC as substitutes for the classification of the four main intrinsic gene expression subtypes:

- Luminal A: Definition 1: ER- and/or PR-positive, HER2-negative and low Ki67. Definition 2: ER- and/or PR-positive, HER2-negative.
- Luminal B: Definition 1: ER- and/or PR-positive, HER2-positive and/or high Ki67. Definition 2: ER- and/or PR-positive, HER2-positive.
- Triple-negative (Basal-like): ER- and/or PR-negative, HER2-negative and sometimes also CK5/6- and/or EGFR- and/or CK14-positive.
- HER2-enriched: ER- and/or PR-negative, HER2-positive.

MBC has been classified into the same IHC-based subgroups as FBC by several authors: the results are summarized in Table 1.

Table 1. Classification of MBC into the IHC-based FBC subgroups

Study (Number of tumors)	Luminal A N (%)	Luminal B N (%)	Triple-negative (basal-like) N (%)	HER2-enriched N (%)
Ge, 2009# (42)	35 (83%)	7 (17%)	0 (0%)	0 (0%)
Shaaban, 2011# (203)	199 (98%)	0 (0%)	4 (2%)	0 (0%)
Kornegoor, 2011* (129)	98 (76%)	27 (21%)	4 (3%)	0 (0%)
Nilsson, 2013# (143)	160 (81%)	21 (11%)	2 (1%)	0 (0%)
Yu, 2013# (68)	41 (60%)	17 (25%)	4 (6%)	6 (9%)

* Using definition 1. # Using definition 2.

In all five studies, the majority of the tumors were classified as the luminal A (60-98%) subtype. The luminal B subtype (0-25%) seemed to be the second most common subtype, while triple-negative tumors (0-6%) were absent or rare [67,69,81,175,176], and none of the tumors was classified as HER2-enriched in four of the studies [67,81,175,176] while the Chinese study classified 9% as HER2-enriched [69]. It should, however, be pointed out that there were HER2-positive MBC tumors in the four studies, but they were also hormone receptor positive, and thus not classified as belonging to the IHC-based HER2-enriched subgroup [67,81,175,176].

Cancer Epigenetics

Cancer is indeed a heterogeneous disease that involves multiple pathways and genetic aberrations. However, epigenetic changes are also crucial for the development and progression of cancer, thus adding an additional dimension to the way in which the genome can be regulated [177,178]. Epigenetic changes can arise during development and cell proliferation and result in alterations in gene expression. They are crucial for development and differentiation, although they can also arise through random

changes or under the influence of the environment [179,180]. Epigenetic mechanisms determine where and when the transcriptional machinery can access the primary DNA sequences, and these mechanisms are regulated by a large number of proteins that establish, read and erase specific epigenetic modifications [181]. There are different types of epigenetic changes, none of which involve mutations of the DNA, nonetheless, they are stable and heritable during cell division [178]. Two types of epigenetic changes, DNA methylation and histone modification, were studied in the present work (Paper IV).

DNA Methylation

DNA methylation is currently one of the best studied epigenetic mechanisms, and occurs in mammals by the addition of a methyl group (CH3) to the 5′ position of the cytosine ring in a 5-cytosine-guanine-3 dinucleotide (CpG) [181]. Once DNA methylation has been established by the *de novo* DNA methyltransferase (DNMT) enzymes DNMT3a and DNMT3b, DNA methylation is maintained after DNA replication through mitosis, primarily by the DNMT1 enzyme [182]. CpGs occur less frequently than expected throughout the genome, probably due to the fact that methylated cytosines are prone to mutate to thymine [183,184]. However, CpG-rich regions are found in more than half of the human promoter regions, and are called CpG islands. Hypermethylation of CpG islands can lead to silencing of the gene by making the DNA inaccessible, and is a common mechanism of silencing tumor suppressor genes in cancer [184]. However, tumors often gain global hypomethylation, which causes genomic instability [185]. CpG methylation is heritable during cell division, and is also reversible, which makes CpG methylations highly promising targets for cancer drugs [186,187].

Histone Modification

Histones, the main components of chromatin, package and order the DNA and provide structural stability. The tails of the histones can be post-translationally modified in many different ways, *e.g.* through methylation, acetylation or phosphorylation, and these modifications can regulate gene expression by either altering the chromatin structure or by recruiting other regulatory proteins [179]. Histone methylation can be carried out by the polycomb repressive complex 2 (PRC2), which catalyzes the trimethylation of lysine 27 on histone H3 (H3K27me3) and leads to chromatin compaction, thus it is a mark of transcriptionally silent chromatin [188]. It has been shown that genes that have H3K27me3 have an increased frequency of *de novo* methylation [189]. Chromatin can also have a bivalent state, with the silencing H3K27me3 and the activating trimethylation of lysine 4 on histone 3 (H3K4me3), which is common in developmental genes in embryonic stem cells. It keeps the genes

silenced, although they are poised for activation, thus facilitating rapid changes in gene expression [190]. The main components of PRC2 are EZH2, EED and SUZ12, however, EZH2 is the core member that catalyzes H3K27me3 [191]. PRC2 target genes are involved in deciding the fate of the cell, embryonic development and differentiation [192]. EZH2 is overexpressed in many cancers and overexpression is correlated with a poorer prognosis in breast, prostate and urothelial cancer [193-196]. A promising strategy for the treatment of cancer is to specifically block EZH2 expression or activity in tumor cells [193]. In fact, McCabe *et al.* showed in a recent study that GSK126, a small-molecule inhibitor of EZH2 methyltransferase activity, decreased global H3K27me3 levels, reactivated silenced PRC2 target genes in lymphoma, decreased proliferation in lymphoma cell lines and inhibited growth in lymphoma xenografts in mice [197].

Tumor Material

We have collected a unique consecutive retrospective cohort of primary MBC patients, consisting of 83 fresh frozen samples and 220 formalin-fixed paraffin-embedded (FFPE) tumors, from all cases of MBC diagnosed in the Lund and Uppsala-Örebro regions of Sweden between 1990-2007, when sufficient tumor material was available, plus additional cases in the periods 1983-1990 and 2007-2009 for which frozen material was available. Clinicopathological data were collected from the patients' charts, and survival data were obtained from the National Population Register. The patients had received different combinations of adjuvant treatment, including endocrine therapy, chemotherapy and radiation treatment. Most of the patients included were not screened for *BRCA1/2* mutations. Histopathological re-evaluation according to current pathological standards was performed by a breast pathologist; all histological grades were represented. The FFPE tissues have been arranged in a tissue microarray (TMA) and evaluated regarding the expression of ER, PR, HER2, Ki67, cyclin A, EGFR and CK5/6 [64,67]. Figure 3 shows a flow chart of the number of MBC tumors included in the different studies.

The studies were approved by the regional Ethics Committee in Lund (2012/89) and Uppsala (2007/254), waiving the requirement for informed consent for the present study.

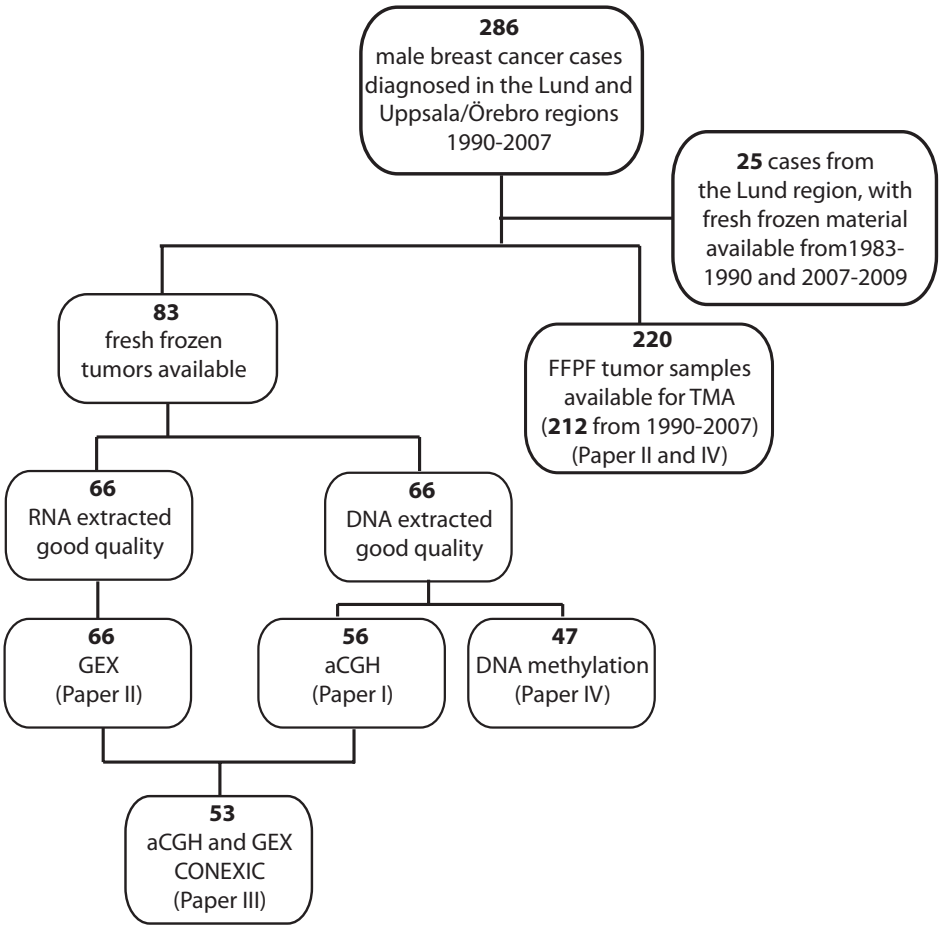

Figure 3. Flow chart of the male breast cancer samples included in the different studies.

Overview of the Main Methods

Immunohistochemistry

IHC is used to analyze the protein expression in tissue, and has the advantage that FFPE samples arranged in a TMA can be used, and thus very little material is needed for the analysis. The principle of IHC is the interaction of target antigens with specific antibodies tagged with a visible label, making it possible to visualize the distribution and localization of the specific antigen within cells and in the context of the tissue.

A TMA containing 1 mm cores from the 220 FFPE MBC tumors, in duplicate, was used, as described previously [82]. In the study presented in Paper II the TMA was stained with an N-acetyltransferase-1 (NAT1) antibody (a kind gift from Prof. E. Sim) [198,199] and the HC10 antibody (a monoclonal mouse antibody to the polymorphic heavy chain of human MHC Class I, with preferential binding to HLA-B and HLA-C alleles and some HLA-A, generously provided by Prof. Dr. J. Neefjes) [200,201]. In a later study (Paper IV), the TMA was stained with an EZH2 antibody (clone 11, BD Transduction Laboratories, Franklin Lakes, NJ, USA). A Dako Autostainer (DakoCytomation, Glostrup, Denmark) was used for the staining procedure. The main steps used in IHC are: tissue sectioning, antigen retrieval, blocking of endogenous enzymes, addition of the primary antibody, addition of the labeled secondary antibody, addition of 3,3'-diaminobenzidine (DAB) (the chromogen substrate), counterstaining with hematoxylin and eosin (HE), mounting and evaluation of the intensity and percentage of positive tumors cells with an Olympus light microscope (Olympus, Hicksville, NY, USA).

Nucleic Acid Extraction

Tumor cellularity was determined on HE-stained fresh-frozen sections, and only of tumors with high (>70%) tumor cell content were included. RNA was extracted manually from fresh frozen tissue using the Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA), and DNA using a modification of a back-extraction protocol from the organic phase as follows: 1 M Tris-buffer containing 4 M guanidine thiocyanate and 50 mM sodium citrate, followed by glycogen precipitation

[202]. To ensure high quality of the RNA and DNA, all samples were analyzed using the NanoDrop ND-1000 (NanoDrop Products, Wilmington, DE, USA) for quantification and assessment of RNA and DNA purity. The RNA samples were also run on the 2100 Bioanalyzer to assess RNA quality, and only samples with an RNA integrity (RIN) ≥7 were hybridized to the gene expression arrays.

Microarray Technology

The analysis of chromosomal aberrations has been improved by the development of the powerful molecular cytogenetic technique comparative genomic hybridization (CGH) by Kallioniemi *et al.* in 1992 [203]. In this technique, normal metaphase chromosomes are hybridized with differentially labeled tumor DNA and reference DNA, and fluorescent signals in paired samples are measured along the longitudinal axis of each chromosome. This gives a fluorescence ratio (tumor sample/reference) that indicates whether there are gains, losses or no differences between the tumor sample and the reference sample. Balanced translocations, inversions and structural aberrations that do not change the overall copy number cannot be detected. A drawback of the method is that it can only provide a limited resolution of 10-20 Mbp. To enable measurements at higher resolution, microarray techniques were introduced to allow measurements of chromosomal aberrations [204].

DNA-based microarray technology constitutes a flexible tool for studying thousands of nucleic acid probes simultaneously, and has revolutionized the field of molecular biology. The technique facilitates high-throughput analysis of copy number alterations, methylation profiles, single nucleotide polymorphism (SNP), gene expression, micro-RNA expression and alternative splicing patterns, and many different types of microarray platforms are available today. The principle of microarray technology is to hybridize the labeled RNA or DNA of the samples to complementary probes at fixed locations or self-assembled in microwells on a glass or polymer slide, which is then scanned to measure the intensity of each probe on the array. In the initial method, introduced by Schena *et al.* in 1995, 600-2,400 base-pair complementary DNA (cDNA) was used as hybridization probes, which was spotted onto glass in a matrix pattern [205]. Short oligonucleotide probes were developed simultaneously with cDNA probes. Oligonucleotide microarrays are more cost-efficient, management-efficient and flexible than cDNA microarrays, and can be generated in different ways: using photolithography [206], ink-jet synthesis [207] or by robotic deposition of pre-synthesized sequences [208,209]. Microarrays can either be single- or dual-channel platforms, as illustrated in Figure 4. Dual-channel microarrays are typically co-hybridized with cDNA prepared from two samples that

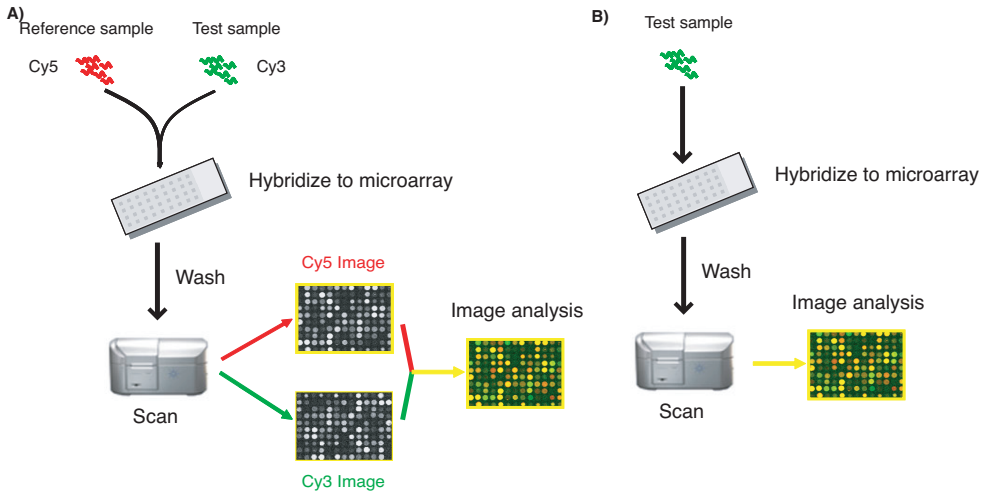

Figure 4. Overview of dual- and single-channel microarrays. A) In a dual-channel microarray, the test and reference samples are labeled with different colors and are competitively hybridized to the microarray with the probes. After washing, the slides are scanned with lasers of different wavelengths, resulting in two images, which are merged for identification and the extraction of intensity ratios (test/reference) for each probe. B) In single-channel microarrays, the test sample is labeled and hybridized to the microarray, which is then washed, scanned and analyzed. The microarray provides intensity data for each probe, providing a relative level of hybridization with the labeled target rather than the actual level. The values can be compared to values obtained with other samples or conditions when processed in the same experiment. Modified from an illustration courtesy of Johan Staaf, Lund University.

are to be compared, which are labeled with two different fluorescent dyes. The intensity of each dye on each spot is measured and combined to give an intensity ratio for each probe, reflecting the differences between the two samples. In a single-channel microarray only one sample is hybridized to the microarray, and the intensity of each probe indicates the relative abundance compared to other samples processed in the same experiment.

Array Comparative Genomic Hybridization

A high-resolution, tiling bacterial artificial chromosome (BAC) aCGH containing ~32,000 BAC clones (Gene expression omnibus (GEO) platform GPL4723) with a resolution down to ~80 kbp [210], produced at the SCIBLU Genomics Resource Center, Lund University, Sweden, was used in this work (Paper I). The method has been described elsewhere [211]. Each spot on the BAC array represents the specific part of the genome that is contained in the BAC, and a whole genome tiling path BAC array comprises the number of BAC clones necessary to cover the whole genome

in an overlapping manner. The tumor DNA and reference DNA were labeled with the fluorescent dyes Cy3 and Cy5, respectively, and they were competitively hybridized to the BAC arrays. After washing, the arrays were scanned to record the fluorescence intensity of the hybridized sample and reference DNA separately, using an Agilent G2565AA microarray scanner (Agilent Technologies, Santa Clara, CA, USA). Normal genomic DNA from Promega (Madison, WI, USA) was used as the reference DNA.

Global Gene Expression

The global gene expression profiles presented in Paper II were analyzed using Illumina's HumanHT12 V3.0 bead array (Illumina Inc., San Diego, CA, USA) (GEO platform GPL13534), which contains 48,803 probes. It is a single-channel oligonucleotide microarray, based on randomly arranged beads with an oligonucleotide sequence specific to each bead type, which is replicated about 30 times on the microarray to provide internal technical replication. The oligo-nucleotides consist of an address connected to the 50-base gene-specific probe. Total RNA was extracted from the fresh frozen tumors and sent to the SCIBLU Microarray Facility at Lund University, Sweden [212], where biotinylated cRNA was prepared and the samples hybridized to the bead chips in three batches. The bead chips were then washed and scanned.

Whole Genome Methylation

In the study described in Paper IV, the methylation status over 480,000 CpG sites of the whole genome of the fresh frozen MBC samples was analyzed with Illumina's Infinium Methylation 450K BeadChips (GEO platform GPL6947), at the SCIBLU Microarray Facility at Lund University, as described elsewhere [213]. Briefly, the DNA is bisulfite-converted, during which unmethylated cytosine is deaminated to produce uracil, while the methylated cytosines are protected from conversion to uracil and remain unchanged. The bisulfite-converted DNA is then whole-genome amplified, enzymatically digested and hybridized to the arrays. The arrays were then washed and the captured DNA underwent single nucleotide extension, which incorporates detectable labels. Finally, the BeadChips were scanned with the two-color laser Illumina HiScan SQ scanner.

Computational Analyses

Array Comparative Genomic Hybridization

TIFF images were analyzed using the Gene Pix Pro software (Axon Instruments, Foster City, CA, USA), and the quantified data matrix was loaded into the BioArray Software Environment (BASE) [214]. Median spot pixel values were used to calculate background-corrected intensities. Spots were removed and regarded as missing values if flagged during image analysis. Log2 ratios were calculated for each spot as log2(intensity sample/intensity reference sample). Normalization was performed by applying population-based intensity-based lowess (popLowess), which is a method that considers copy number populations when using lowess [215]. Normalization of microarray data is crucial, and several reports have shown that partitioning algorithms taking copy number imbalances into account are better for the normalization of aCGH data than conventional normalization methods developed for gene expression, which are based on the assumption that the majority of investigated genes in a large gene set are not differentially expressed [215-217].

Gene Expression

The key assumption used in standard normalization methods for gene expression arrays is that only a few genes are actually expressed at different levels in different samples, thus, the expression levels of the majority of genes should be similar in all samples. It is, therefore, possible to adjust different samples to a common baseline using a normalization algorithm. The gene expression data were normalized using quantile normalization in BASE [214], and were thereafter log2 transformed. Probes with a mean value of <5.8 were filtered out, and the remaining probes were mean centered across the entire dataset. In the studies described in Papers II and IV, the samples were handled as three batches when they were run on the microarrays. It was therefore necessary to make adjustments for systematic technical variations between the batches to allow direct comparisons. To adjust for differences between batches, the samples must be randomized across all batches, at the same time ensuring that all kinds of samples are evenly represented in each batch. If an experiment is supervised, subgroups need to be taken into consideration during batch adjustment. However, in the present studies we had no prior knowledge of any subgroups. The batches were adjusted using a supervised empirical Bayes method (ComBat) [218]. Principal component analysis was performed to ensure that batch adjustment had been successful [219]. Four samples from each of the first and second hybridization batches were re-hybridized in the third batch; these replicates were excluded after batch

correction. Finally, the Illumina probes were re-annotated using re-annotation and mapping for oligonucleotide array technologies, and only probes that bound to the gene they were annotated with were included in the subsequent analyses [220].

Copy Number and Expression in Cancer

A need to combine different types of genomic data to further unravel the complexity of cancers and increase our understanding exists today. The computational framework COpy Number and EXpression In Cancer (CONEXIC) was used in the study presented in Paper III, in an attempt to unravel the complex mechanisms responsible for cancer. This algorithm integrates copy number and gene expression data to detect candidate driver genes among all the altered passenger genes [221]. It not only identifies candidate drivers, but also associates them with several gene modules that are believed to be altered by each candidate driver. CONEXIC was inspired by Module Networks [222], and uses an integrative Bayesian scoring approach to identify the candidate drivers and their gene modules. CONEX consists of three key steps [221]:

1. candidate driver genes are selected from commonly aberrant regions among the tumors,
2. the initial association between the candidate drivers and the gene modules is identified in the single modulator step and finally,
3. the initial modules are improved in an iterative network learning step.

Whole Genome DNA Methylation

The intensities of the images were extracted using GenomeStudio Methylation Module Software (version 2011.1). GenomeStudio normalizes data using the internal controls that are present on the Human Methylation 450K BeadChip. It also normalizes the data with the aid of internal background probes, and the data are then exported to R [223]. Samples with >90,000 probes with a detection p-value >0.05 (eight FBC samples) were removed and the β-values, were calculated from the raw intensity as methylated/(methylated+unmethylated), thus ranging from 0 to 1, where 1 corresponds to completely methylated and 0 to completely unmethylated probes. The 450K methylation array contains two Infinium assays with different designs. The Infinium I assay contains two types of beads per CpG locus: one that recognizes the methylated locus and one that recognizes the unmethylated locus, as illustrated in Figure 5A. The Infinium II assay design contains only one probe per CpG locus, the methylation state is detected by single-base extension, which results in the addition of

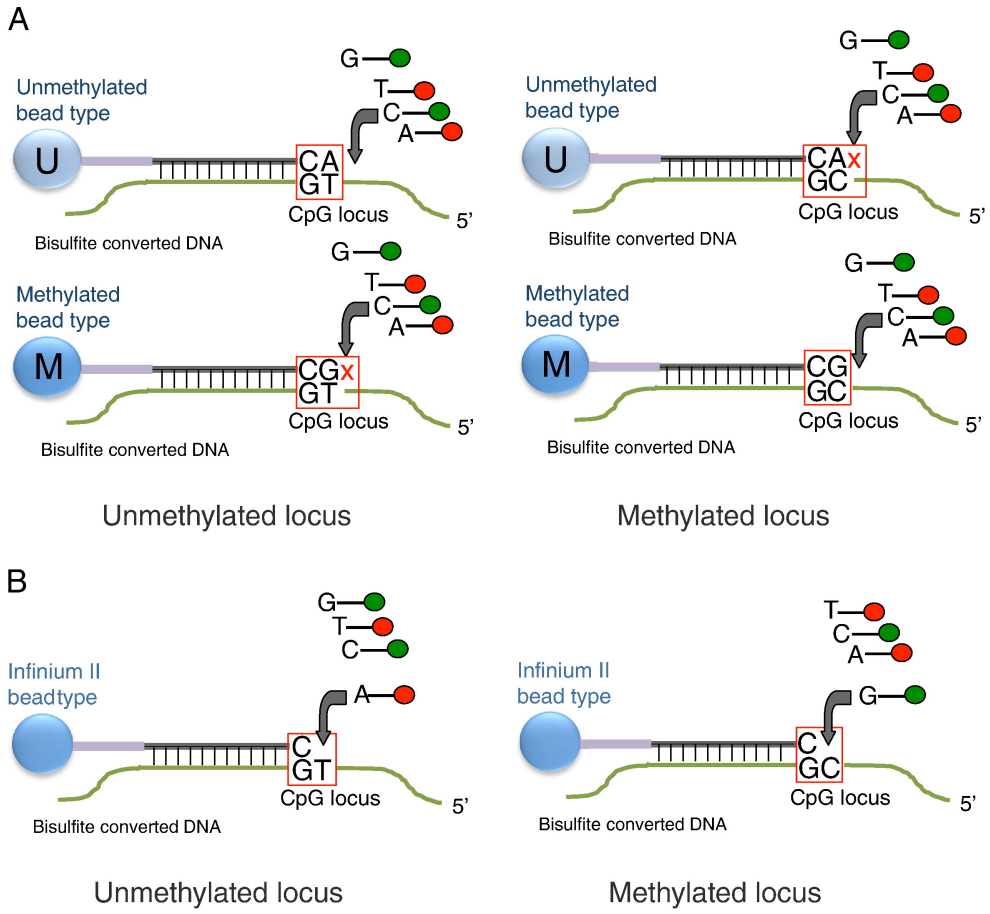

Figure 5. Overview of the Infinium type I and II assays. A) The Infinium I assay contains two types of beads per CpG locus: one that recognizes the methylated locus and one that recognizes the unmethylated locus. B) The Infinium II assay requires only one probe per CpG locus, and the methylation state is detected by single-base extension, which results in the addition of a labeled G or A base. Labeled "A" is always incorporated at unmethylated query sites ("T"), while "G" is incorporated at methylated query sites ("C"). Reprinted with permission from Elsevier.

a labeled G or A base. Labeled "A" is always incorporated at unmethylated query sites ("T"), and "G" is incorporated at methylated query sites ("C"), as shown in Figure 5B. The values of β obtained with the type I and type II Infinium assays have been found to diverge, thus corrections are required. A peak-based correction, similar to that described by Dedeurwaerder *et al.* was used [224]. Briefly, the values of β were smoothed using an Epanechnikov smoothing kernel for both assay designs to estimate the unmethylated and methylated peaks, and linear scaling was used to adjust the unmethylated peak to 0 and the methylated peak to 1, meaning that values of β

between 0 and 1 were stretched. Values of β greater than 1 were set to 1, and values below 0 were set to 0. Values of β for probes with a p-value >0.05 were defined as missing values. In the combined MBC and FBC dataset, the sex chromosome probes, probes that contained SNPs at the target CpG, and probes that cross-hybridized between autosomes and sex chromosomes were removed, resulting in 415,080 probes [225].

Experimental Considerations

When studying a rare kind of tumor it is difficult to collect a large number of tumor samples, and external datasets for the validation of findings are not generally available. When samples are further divided into subgroups the numbers rapidly decrease, further reducing the statistical power. The sample cohort of MBC tumors used in these studies is one of the largest that has been collected in a single country. To obtain this large number it was necessary to include patients over a long period of time, during which the treatment of breast cancer has changed. The treatment given at different hospitals also varies. Our research group is collaborating with an international consortium (EORTC) studying MBC (ClinicalTrials.gov NCT01101425), which has so far collected retrospective clinical data and archival information on 1,800 MBC tumors; Sweden has contributed 300 of these cases. A database of this size will provide sufficient statistical power to validate the IHC findings of the present work.

It is important to bear in mind that tumors are heterogeneous, and that tumor biopsies consist of different tumor subpopulations and stromal cells, including infiltrating lymphocytes, endothelial cells and normal breast epithelial cells. Non-cancer cells in the samples will affect the aCGH, GEX and DNA methylation data by "diluting" the results with the unchanged genetic profiles of the normal cells. This is why only tumors with >70% cancer cells were included in the analysis. Two sections were taken from the frozen tumor samples. One was HE stained and the percentage of tumor cells counted, and one was used for extraction. However, due to the heterogeneity of the tumors, there is no guarantee that the two sections contained the same amount of tumor cells.

Microarray technology is well established and has revolutionized studies in molecular biology by allowing measurements of thousands of probes simultaneously. Nonetheless, several technical issues must be considered when planning microarray experiments and analyzing the data, such as systematic technical variations, batch effects, the quality of the probes and measurement accuracy [220,225,226]. However, gene expression microarray technology has been shown to be a reliable technique with high inter- and intra-platform consistency across test sites and platforms when experiments are carefully designed and appropriate data transformation and analyses

are used [227]. Suitable sample preparation and assessment of mRNA quality are important to minimize the effects related to sample quality. Normalization of microarray data is crucial; the aim of which is to adjust for and minimize effects that arise from systematic technical differences rather than biological differences. Some of the biological information may be lost, depending on how well this procedure is performed. If the samples have been handled in different batches during the experiments this must, in most cases, also be adjusted for. To be able to adjust for differences between batches the samples must be randomized across all batches, ensuring that different types of samples are evenly distributed in all the batches, thus careful experimental design is essential. The quality of the probes must also be taken into consideration, to ensure that they actually quantify a specific transcript. However, in reality, many microarrays include a substantial number of incorrect probes, which may be due to cross hybridization, alternative mRNA splicing, SNPs, repeat sequences, and probes targeting genomic regions with no known transcription. For example, Illumina's Human Wg-6 v3 platform contains 28% unreliable probes [220] while the Agilent 44K human microarray contains 40% unreliable probes [228]. It is therefore crucial to re-annotate the probes to determine which ones are of good quality. Without knowing what the probes map to, no biologically meaningful interpretation can be made from functional biological analyses, such as gene ontology (GO) enrichment analysis as database for annotation, visualization and integrated discovery (DAVID) [229,230]. The GO nomenclature provides a description of gene products at three different levels: biological, cellular and molecular [231].

Another problem encountered when analyzing microarray data is the accurate extraction of the biologically relevant signal from thousands of related measurements. The problem of multiple comparisons can be dealt with in different ways. In the present work, the significance analysis of microarray (SAM) method was used to identify differentially aberrant probes [232].

Microarrays are evolving rapidly, and aCGH platforms (*e.g.* zoom-in arrays) are available today with higher resolution than those used in this work. Moreover, traditional aCGH cannot detect balanced translocations, in contrast to SNP-based CGH assays such as Illumina SNP arrays, which can provide additional valuable information on copy-number-neutral alterations.

While CONEXIC is a powerful tool, it does not identify all potential cancer drivers, since it only detects drivers in amplified or deleted regions that pass the stringent statistical tests, and will thus miss drivers arising, for example, from point mutations [221]. Recent attempts to identify mutations in driver genes in FBC have shown remarkable heterogeneity between individual tumors; only 3-7 genes being found to be mutated in more than 10% of FBCs [233,234]. The task of identifying candidate drivers in breast cancer is hence not easy. Thus, a much larger patient cohort would be required to identify all driver genes in MBC, as well as information regarding mutations and epigenetic changes.

IHC is an important tool in scientific research and is used today for the elucidation of various diagnoses. Although IHC is a relatively simple technique, the outcome is dependent on many factors, such as the method of fixing the material, the age of the material, the specificity and sensitivity of the antibody, the antigen retrieval method, the staining method and the experience of the evaluator. Hence, the results of IHC must be interpreted with caution. Before an antibody can be used as a biomarker, a standardized IHC protocol must be developed and validated in several independent cohorts by independent laboratories. TMAs offer a rapid means of studying molecular targets in tumors that requires very little material and allows high-throughput simultaneous analysis of multiple samples. One of the concerns when using TMAs is, however, that the cores used as samples only represent a very small fraction of the tumor, and may not be representative of the whole tumor due to heterogeneity. However, good concordance has been reported between TMAs and whole sections [235,236]. TMAs are not good for studying all types of proteins; for example, they are not suitable for studying proteins expressed by infiltrating immune cells in tumors.

Results and Discussion

The Landscape of Genomic, Transcriptomic and Epigenomic Aberrations in MBC

In the study described in Paper I, 56 MBC tumors were analyzed using high-resolution tiling BAC arrays, and they were compared to the results from 359 FBCs representing all intrinsic subtypes of FBC, obtained using the same aCGH platform and analyzed in the same manner. It was found that MBC, like FBC, is a heterogeneous disease on the copy number level, since the tumors exhibited a broad spectrum of aberrations. The MBC tumors also revealed a heterogeneous pattern of changes on the transcriptomic and DNA methylation levels, as reported in Papers II and IV. The male and female breast cancers investigated had the same global frequency of DNA methylation; ~40% each of the probes were hypermethylated and hypomethylated in both male and female breast cancer tumors. This overall similarity was also observed on the copy number level, as the male and female breast cancer tumors shared the most common genomic aberrations and displayed similar patterns of chromosomal imbalance [237,238], as shown in Figure 6. Similar findings have been reported by others studying MBC with metaphase CGH [239,240]. However, when studying the genomic aberrations in more detail, it was observed that the MBC tumors had more gains and fewer losses than the FBC tumors, although they had the same fraction of genome altered (FGA). The male breast cancer tumors also harbored significantly more whole chromosome arm gains than the FBC tumors, while high-level amplifications were more common among the FBC tumors. Thus, although similar genetic changes seem to be responsible for tumor progression in male and female breast cancer, the mechanisms driving the genetic aberrations leading to disease progression may in fact differ. The most common aberrations were found to be the same in another aCGH study of 25 MBC and 16 FBC tumors, which was published at the same time as Paper I. However, they found both less amounts of losses and gains in the MBC than in the FBC tumors. It should, however, be pointed out that the FBC tumors included in their study were aggressive breast cancers with basal-like features, and were thus not representative of all types of FBC [170]. In FBC, the basal-complex tumors (corresponding to the basal-like intrinsic subtype) have the highest FGA, while luminal-simple (corresponding to the luminal A intrinsic subtype) have the lowest FGA, and luminal-complex (corresponding to the luminal B intrinsic subtype) have an intermediate FGA [166].

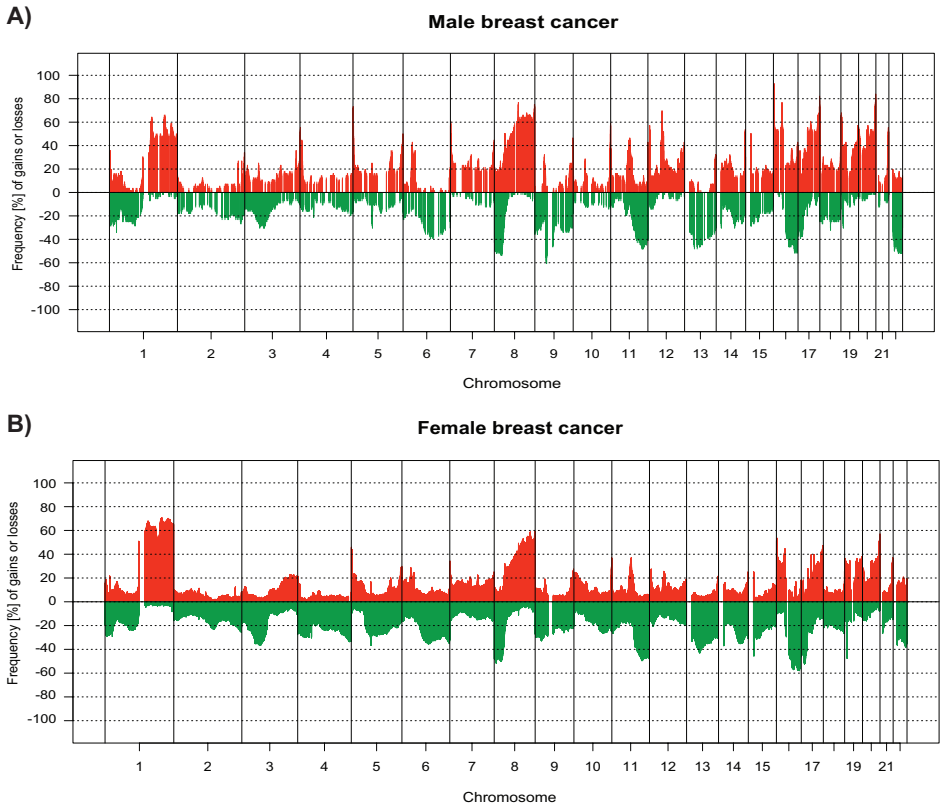

Figure 6. Frequency of copy number aberrations in the 56 male breast cancer tumors (A) and the 359 female (B) breast cancer tumors. Gains are shown in red, and losses in green. From Paper I.

When the aCGH and GEX data were combined to identify candidate target drivers in male and female breast cancer, remarkably different landscapes were observed, and they shared only two candidate drivers among the 30 found in the MBC dataset and the 67 found in the FBC dataset. This constitutes further evidence that the pathogenesis of MBC differs from that of FBC.

Two Stable Subgroups of MBC

One of the main aims of this work was to try to classify MBC into stable subgroups reflecting different biological and clinical characteristics. To the best of the author's knowledge, this has not previously been performed using high-resolution microarray data. FBC, on the other hand is well characterized on the genomic, transcriptomic and epigenetic levels, and has been classified on all these levels by several independent research groups. Today, MBC patients are given the same treatment as FBC patients, and it is thus of great interest to establish whether MBC tumors can be classified into the same subgroups as FBCs, or whether there are subgroups of breast cancer occurring only in males, thus potentially requiring different treatment strategies.

The fresh frozen MBC tumor samples were classified according to copy number, mRNA and DNA methylation levels using high-resolution microarray data. Two stable subgroups, one of which seemed to consist of a group of more aggressive MBC tumors, were consistently identified in all three MBC datasets. The subgroups identified in the three datasets were significantly associated with each other, as shown in Figure 7. Unsupervised hierarchical clustering of 56 MBC tumors was performed on the genomic level using 133 regions that had previously been identified from a large dataset of FBCs using Genomic Identification of Significant Targets in Cancer (GISTIC) (Paper I) [166,241]. This revealed the genomic subgroups male-simple (11 MBCs) and male-complex (43 MBCs); the male-complex subgroup being

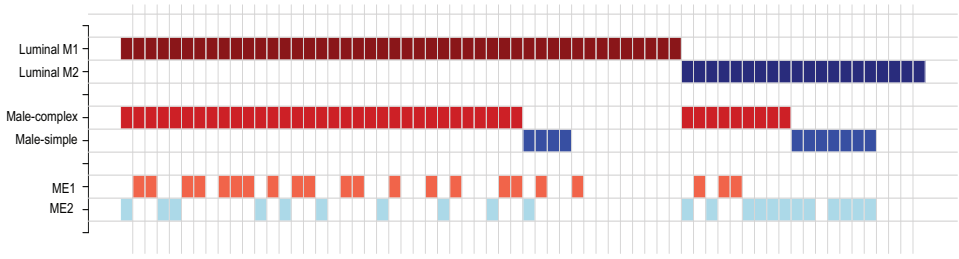

Figure 7. Annotations of the two stable male breast cancer subgroups on the genomic, transcriptomic and epigenetic levels. The subgroups from the three datasets are significantly associated with each other.

characterized by higher FGA, larger tumors and higher S-phase fractions. Unsupervised hierarchical clustering was performed on the transcriptomic level, using the 1,652 genes that varied the most among the 66 MBC tumors included in the analysis described in Paper II. Two transcriptomic subgroups, luminal M1 (46 MBCs) and luminal M2 (20 MBCs) were found, and the stability was controlled by bootstrapping methods. Importantly, it was possible to validate these subgroups in an independent MBC dataset, which is the only other MBC gene expression dataset that exists today [171]. The luminal M1 transcriptomic subgroup, which was correlated with the male-complex genomic subgroup, also displayed a more aggressive phenotype with a worse prognosis, as it showed significant up-regulation of homeobox (*HOX*) genes and the genes involved in cell migration, angiogenesis, and the cell cycle, and significantly higher proliferation and invasion scores than the luminal M2 transcriptomic subgroup. Finally, on the epigenetic level, unsupervised clustering of 47 MBCs using a recursively partitioned mixture model (RPMM) was performed on the ~10,000 most variable CpG probes (Paper IV), revealing two stable epitypes, ME1 (23 MBCs) and ME2 (24 MBCs). The ME1 epitype was correlated to luminal M1 and male-complex subgroups, and was characterized by being more proliferative and aggressive, exhibiting a higher frequency of cyclin-A-positive tumor cells, a higher FGA and a higher S-phase fraction than the ME2 tumors. Overall, the ME1 tumors displayed a higher frequency of hypermethylation and lower frequency of hypomethylation than ME2 tumors. These results are in line with the findings of Kornegoor *et al.* when studying the DNA methylation of 25 tumor suppressor genes in 108 MBCs, *i.e.*, that higher promoter methylation frequencies were associated with an aggressive phenotype and poor survival [242].

MEI Tumors Show Hypermethylation of PRC2 Target Genes

The ME1 tumors displayed a higher frequency of hypermethylated probes, specifically on poised promoters, than the ME2 tumors and normal tissues. Furthermore, when the methylation level of a set of 654 PRC2 target genes [243] was analyzed (Paper III), ME1 tumors showed a significantly higher average value of β and, correspondingly, the average expression of these genes was significantly lower than in ME2 tumors. One of the core components of PRC2 is EZH2, which catalyzes H3K27me3 [193]. The levels of mRNA and the protein of EZH2 were both significantly higher in the ME1 epitype than in the ME2 epitype. All of the above results indicate that developmental processes in ME1 tumors may be repressed by histone modification by the PRC2 complex, and further repressed by *de novo* DNA methylation of PRC2 target genes. The hypermethylation of polycomb target genes is a common phenomenon in tumors, in contrast to normal cells [244-246], and it has

also been shown to occur more frequently in more aggressive forms of tumors [196,245]. Overexpression of EZH2 has also been associated with a more aggressive phenotype in many different types of tumors [193,194,196,247,248]. These results further strengthen the picture of the ME1 epitype representing a more aggressive form of MBC.

Are There More Subgroups of MBC?

Although the collection of fresh frozen MBC tumors used in this work is one of the largest and clinically most well annotated available to date, it still suffers from the problem of small sample size. While additional subgroups of MBC may exist, the sample size was too small for further subdivisions in all three studies. Interestingly, the genomic profiles within the male-complex subgroup were not as homogeneous as those in the luminal-simple subgroup, possibly indicating that the male-complex subgroup could be further subdivided. Furthermore, the same tendency was seen in the luminal M1 subgroup on the transcriptomic level.

The Subgroups of MBC Differ from the Known Subgroups of FBC

The male-complex genomic subgroup showed overall similarities with the previously reported luminal-complex FBC subgroup, while the male-simple subgroup showed features that were not associated with any of the described FBC genomic subgroups, and may therefore represent a new subgroup of breast cancer occurring only in men. However, when the complex subgroups were compared in more detail, distinct differences were found; *e.g.* male-complex tumors harbored more whole chromosome arm gains, and the frequencies of several of the GISTIC regions differed significantly between the genders. Interestingly, the findings were the reverse on the transcriptomic level: the luminal M2 subgroup showed some similarities with luminal A, while the luminal M1 subgroup was strikingly different from all intrinsic FBC subtypes. Despite that fact that it was only possible to classify 50% of the luminal M2 tumors with the applied cutoff using the Hu genes [164], all the tumors that were classified were of the luminal A subtype. When seven GEX modules associated with key biological processes in FBC (tumor invasion and metastasis, immune response, angiogenesis, apoptosis, proliferation, and ER and HER2 signaling) [249] were used to discover biologically meaningful differences between MBC transcriptomic subgroups and to compare them with the intrinsic subtypes of FBC, the luminal M2 tumors showed a significantly higher score for the immune response module than the

luminal M1 tumors. Conversely, luminal A FBC tumors showed the lowest score for this module. This, together with the significantly different DNA copy number profiles of the majority of the luminal M2 tumors indicates that it is most likely a distinct subgroup of breast cancer with unique biological and clinical features, occurring only in males. Moreover, the luminal M1 tumors displayed a pattern of module scores for the seven GEX modules that did not resemble any of the intrinsic FBC subtypes. Interestingly, the luminal M1 tumors had significantly higher module scores for the tumor invasion and metastasis, proliferation and HER2 modules, and a significantly lower ER module score than luminal M2 tumors. When the MBC tumors were classified using the Hu centroids, 55% of the tumors were unclassified. As MBCs are generally ER-positive, the MBCs were also classified using ER-positive FBC luminal subtype centroids, and 36% still remained unclassified. Furthermore, when attempts were made to subclassify the ER-positive FBCs into the MBC subgroups, the fraction of unclassified tumors was even higher, 63%. The fraction of unclassified tumors when FBCs have been classified into the intrinsic subtypes has previously been reported to be 0-20% [250]; hence 55% unclassified tumors is remarkably high, and indicates that the MBC do not belong to the intrinsic subtypes of FBC, and indeed represent two new subgroups of breast cancer most likely occurring only in males. We and others have attempted to classify MBC tumors into the intrinsic subtypes of FBC using IHC as proxy markers for the subtypes. The majority of the MBC tumors have been classified as the luminal A (60-98%) subtype, while the luminal B subtype (0-25%) appeared to be the second most common subtype. Triple-negative tumors have been found to be rare (0-6%) [67,69,81,175,176], and no MBC tumors have been classified as HER2-enriched by four groups [67,81,175,176] and the Chinese group found 9% as HER2-enriched [69]. Based on the findings of the present work, it is unlikely that almost 80% of MBCs are of luminal A subtype, bearing in mind that this is the subgroup of FBC tumors with the best prognosis, while the relative overall survival of men with breast cancer is significantly worse than that of women in Sweden [2]. This demonstrates that the IHC markers used for subclassifying FBC tumors into their intrinsic subtypes are not appropriate for the classification of MBC subgroups.

In this work, male and female breast cancer tumors were analyzed together for the first time (Paper IV), making it possible to identify clusters consisting of both MBC and FBC tumors, and to make direct comparisons. The MBC tumors formed clusters mainly with luminal A and B FBC tumors. However, the MBC tumors were grouped together within the clusters, rather than being interspersed among the FBC tumors, suggesting that they differ from FBC tumors in some respects. On the methylation level, the ME1 tumors behaved more similarly to the FBC tumors. No differences were seen in methylation frequencies between the ME1 tumors and the FBC tumors in the same cluster, although the ME1 tumors had a low ER module score while the FBC tumors had a high score for ER. The ME2 tumors exhibited a high ER score, and were different from the FBC tumors in the same cluster in that they had higher

hyper- and hypomethylation levels. Furthermore, performing SAM analysis on the male and female tumors in the two clusters containing the majority of the MBC tumors and the luminal A and luminal B FBC tumors resulted in many thousands of differentially methylated probes between the genders. This provides further evidence that male and female breast cancer are biologically different.

How Active is the ER Pathway in the Aggressive Group of MBC?

The high proportion of ER-positive tumors in men has made hormonal manipulation an attractive therapeutic intervention in MBC. However, it has not been shown that hormone receptor positivity is associated with the same prognostic and predictive implications in MBC as in the female disease. Furthermore, the importance of endocrine treatment is not as well established in MBC as it is in FBC. Reports on the response of male patients to endocrine treatment are scarce and, so far, results have only been published from retrospective studies. Male patients in the cohort studied in this work who received endocrine treatment had a tendency towards worse distant metastasis-free survival than those who did not receive any endocrine treatment. However, since the patients who received endocrine treatment had a poorer prognosis at baseline (more lymph nodes involved) it is difficult to draw any conclusions from this finding. Other research groups have seen no difference in survival between MBC patients who received endocrine treatment and those who did not [83,111]. This could indicate that MBC patients do not respond in the same manner to endocrine treatment as FBC patients. However, it should be borne in mind that these data are not derived from a prospective randomized trial.

An interesting finding in the luminal M1 tumors was that while the majority were ER-positive they had a significantly lower ER module score than the luminal M2 tumors. This was also true for the epitypes, where the ME1 tumors showed the lowest ER module score. In FBC, luminal A and B tumors have an equally high ER module score, while basal-like tumors have a significantly lower score and HER2 tumors have an intermediate score. This could indicate that the ER pathway is not as active in luminal M1 MBC tumors as in luminal M2 MBC tumors and luminal FBC tumors. These results are in line with the findings of Weber-Chappuis *et al.* when studying hormonal receptors and antigens under estrogen and androgen control in male and female breast cancer. They reported that although a higher proportion of ER-positive tumors was seen in MBC than in FBC, there was only a weak association with markers under estrogen control in FBC, while several markers under androgen control were more highly expressed in the MBC tumors [251]. Another comprehensive study of different isoforms of steroid hormone receptors in breast cancer also revealed gender-specific differences. Hierarchical clustering on the protein

expression of steroid hormone receptors for male and female breast cancer separately revealed that ERα and PR isoforms were grouped together in FBC, and that ERβ isoforms were grouped together with androgen receptor (AR) in FBC. In MBC, ERα was instead grouped together with ERβ and AR, and the PR isoforms formed their own group, further suggesting different hormonal dependencies in male and female breast cancer [81]. Moreover, no significant association was found between the rs2981582 SNP in the fibroblast growth factor receptor 2 (FGFR2) gene and MBC in a large genome-wide association study of MBC [47]. This SNP has the strongest known association with ER-positive FBC [252], further supporting the finding in the present work that not all ER-positive MBC tumors behave in the same way as ER-positive tumors in FBC. Moreover, HER2 has questionable prognostic significance in MBC. Interestingly, in contrast to FBC, there does not seem to be a negative association between HER2 and ER status. In the tumors studied in this work, 86% of the HER2-positive tumors were ER-positive. Another group has also reported that all HER2-positive MBC tumors were also ER-positive [253]. Thus, growth factor receptors other than HER2 may play more important roles in MBC. The predictive value of HER2 in MBC is also unclear. There is only one case report describing the use of trastuzumab in one metastatic MBC patient, where good response was demonstrated [155].

Moreover, when candidate drivers were investigated in male and female breast cancers, one known driver for FBC, *GATA3* [254], was identified as a candidate driver in the analyses of all FBCs, and within the luminal A and luminal B subtypes of FBC, while it was not detected among the MBC tumors. However, *MAP2K4* was detected as a candidate driver in MBC, and mutations and deletions in *MAP2K4* have been identified in luminal FBCs [255,256]. This is a further indication that MBC tumors do not behave like luminal FBC tumors, but probably share features with both ER-positive and ER-negative FBC tumors.

It is not known whether the ER is functional in MBC under the low estrogen levels in the male mammary gland. One explanation of the high percentage of hormone-receptor-positive MBCs could be aberrant steroid receptor up-regulation in this low-estrogen environment. Most FBCs are estrogen-dependent, however, if the male tumors are in fact hormonally dependent, it has yet to be determined what these hormones actually are, and which pathways they are involved in. Studies of such pathways may provide important information, useful in the revision and improvement of therapy targeted against the more vital pathways in MBC.

THY1 – A Candidate Prognostic Invasion Marker in MBC

One of the most biologically and clinically interesting candidate drivers found in MBC was *THY1* (Paper III). Significantly worse survival was observed in the THY1-positive subgroup of MBC patients, as can be seen in Figure 8. *THY1* was included as an up-regulated gene in the epithelial-mesenchymal transition (EMT) core signature by Taube *et al.* and seven of the genes included in the gene module that it regulates were also included among the up-regulated EMT core genes [257]. Furthermore, the THY1-positive MBC tumors displayed a significantly higher activity in the EMT-induced stroma module identified by Fredlund *et al.* [258]. *THY1* may thus be a driver of invasion related to EMT in MBC. The EMT-induced stroma module activity correlates with aggressive disease in the basal-like subtype of FBC [258]. The THY1-positive group was significantly associated with the luminal M1 group, thus further strengthening the finding that this subgroup of MBC does not behave like ER-positive FBC. Finally, *THY1* expression was more highly correlated to its target genes than to its copy number; in fact, deletions were more common than amplifications in this region, indicating that amplification is not the main driver of high expression in this case.

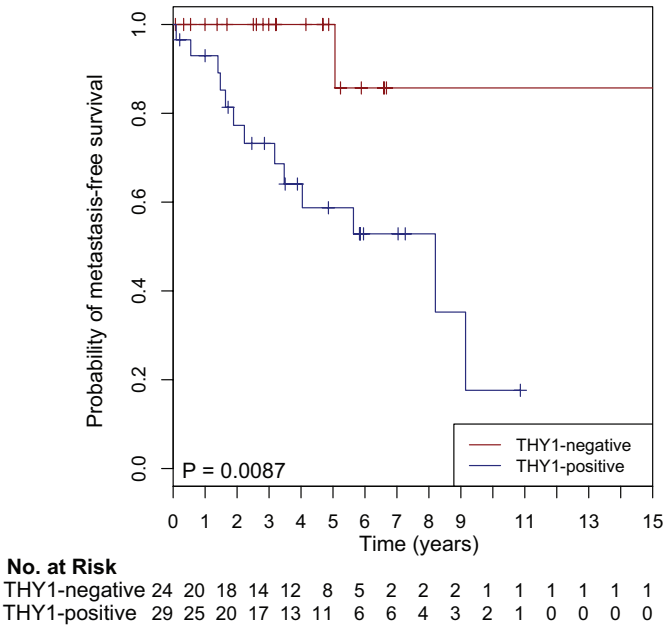

Figure 8. Distant metastasis-free survival of the 66 MBC patients stratified by THY1 gene expression. The numbers below the plots indicate the number of patients at risk in each group at the given points in time. From Paper III.

NAT1 – A Potential Biomarker for MBC

The luminal M1 subgroup of MBC was shown to be a more aggressive group of MBC, and thus associated with a worse prognosis. Bearing in mind that the ER pathway appeared to be less active in the luminal M1 tumors, the question is whether this group of MBC patients would respond to endocrine treatment, or whether they require other, more aggressive approaches. Furthermore, when NAT1 was validated (one of the genes significantly differentially expressed in the transcriptomic subgroups), good correlation was found between protein and mRNA levels, and significantly lower expression of NAT1 was seen in the luminal M1 tumors (Paper II). Analysis of the survival rate showed a significantly worse 5-year distant metastasis-free survival for patients with NAT1-negative tumors, as shown in Figure 9, and this remained significant in a multivariate analysis when adjusting for lymph node status,

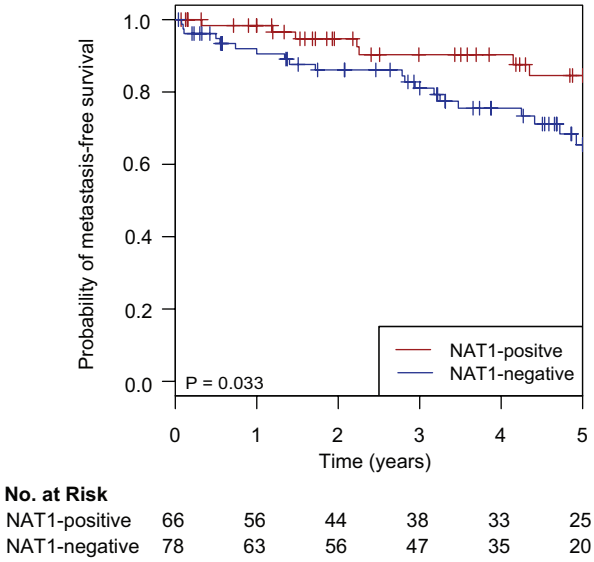

Figure 9. Distant metastasis-free survival of the 220 MBC patients included in the TMA stratified by NAT1 protein expression. The numbers below the plots indicate the number of patients at risk in each group at the given points in time. From Paper II.

NHG and tumor size. Several studies of FBC have shown that NAT1 is more highly expressed in ER-positive tumors than in ER-negative tumors on both the protein and mRNA levels [161,259-261]. Two studies of FBC have also shown that high NAT1 expression correlates with a better prognosis [262,263]. Furthermore, in a study of ER-positive FBC, in which all the patients were treated with primary surgery followed by adjuvant tamoxifen alone, a lower expression of NAT1 was found to be significantly correlated with poorer survival. NAT1 may therefore be a predictor of tamoxifen response in FBC [262], and perhaps also in MBC. NAT1 is a xenobiotic metabolizing enzyme [264], and it may alter tamoxifen metabolism and bioavailability, which could contribute to tamoxifen resistance. This provides further evidence that the luminal M1 group, with NAT1-negative tumors, might not respond to tamoxifen, and may therefore require other treatment strategies such as chemotherapy. Among the patients with NAT1-negative tumors in the present work, only 12% received chemotherapy. The relative overall survival of MBC patients is worse than that of FBC patients in Sweden [2]. Considering that FBC includes ~15%

triple-negative tumors, and the prognosis for this group is poor [88,89], while only 0-6% of MBC tumors appear to be triple-negative [67,69,81,90,175], further indicates that there is a group of MBC patients with poor prognosis that requires other treatment strategies. Ki67 is an important marker of proliferation in FBC, and is today used in the clinic to guide decisions on treatment strategies. However, Ki67 was not found to be prognostic in the cohort studied in this work [67], and was not correlated with the NAT1-negative aggressive subgroup of MBC. This illustrates that new biomarkers are needed to identify the more aggressive forms of MBC. NAT1 could be such a biomarker. It is also important to take the high average age at diagnosis of MBC patients into consideration. About 50% of the men in the NAT1-negative group were over 70 years old, and their general state of health and co-morbidity may preclude more aggressive treatment. Due to the fairly small sample size, this finding should be interpreted with caution, and more studies are needed to validate the potential role of NAT1 as a prognostic biomarker in MBC.

Conclusions

The main conclusions drawn from the research presented in this thesis are given below.

- The results of this work have shown that, like FBC, MBC is a heterogeneous disease on the genomic, transcriptomic and epigenetic levels, where each MBC tumor harbors its own specific set of aberrations.
- Although male and female breast cancers share many similarities on global genomic, transcriptomic and DNA methylation levels, many differences were revealed when they were studied in more detail.
- The landscapes of candidate drivers in male and female breast cancer show remarkable differences.
- MBC tumors were subclassified according to copy number, mRNA and DNA methylation levels using high-resolution microarray data. In all three datasets two stable subgroups were consistently identified, one of which was associated with a more aggressive phenotype. Moreover, the subgroups in the three datasets were significantly associated with each other.
- The two stable subgroups of MBC that were identified did not resemble any of the know subgroups of FBC, and are not easily identified by the IHC-based classification applied to FBC; thus they may represent two new subgroups of breast cancer occurring only in males.
- The more aggressive form of MBC may not have an active ER pathway, despite the fact that the majority of the tumors are ER-positive. They may, therefore, not respond to endocrine treatment in the same way as FBC patients with ER-positive tumors, and may require more aggressive treatment such as chemotherapy.
- The ME1 tumors may be repressed by histone modification by the PRC2 complex, and further repressed by *de novo* DNA methylation of PRC2 target genes.
- THY1 appears to be a driver of invasion related to EMT in MBC, and may be a prognostic invasion marker for MBC.

- NAT1 positivity corresponded to better outcome in MBC and may be a prognostic biomarker for MBC.

Future Perspectives

The sample cohort of MBC tumors used in this work is one of the largest and most well annotated collections of FFPE and fresh frozen MBC tumors that have been collected in a single country. However, it is still small in relation to the number of samples normally used in microarray studies. Therefore, further studies are needed to validate our findings. It would be of great value to perform gene expression profiling on the FFPE samples, which would greatly increase the sample size. The sample size could be further increased if the samples in the EORTC study could be included. However, the quality of DNA and RNA in FFPE samples is not as good as from fresh frozen tissue. To further increase the value of measurements on the FFPE samples, MBC tumors should be analyzed together with FBC tumors so that direct comparisons can be made.

An alternative approach to microarrays for studies of the whole genome and transcriptome is massively parallel sequencing techniques. These techniques, together with bioinformatics to handle the data, have developed rapidly during the decade since the end of the human genome project, and the cost of such techniques is falling rapidly, although it is still higher than that of microarray approaches. The benefits of massively parallel sequencing techniques are that they allow analyses at a far higher resolution than microarrays, and when studying DNA using information from paired end or mate pair datasets it is possible to detect all classes of structural variation in the genome. Thus, running massively parallel sequencing on MBC tumors would add further depth to the genomic and transcriptomic analyses. It would also be interesting to further study the landscape of candidate drivers in MBC by investigating the landscape of mutations in MBC.

An international EORTC-led consortium for studying MBC has been established. A retrospective study is underway, in which clinical information as well as tumor material from 1,800 MBC patients diagnosed in the past 20 years have been collected. TMAs will be constructed and the prognostic/predictive factors already established in FBC will be evaluated in relation to MBC. Molecular subtyping and DNA sequencing will also be performed. This will also provide a large cohort that can be used to validate the findings of the present studies, including the prognostic value of NAT1.

It would also be interesting to investigate the role of growth factor receptors other than HER2, for example HER3 and HER4, in MBC, and to explore whether the ER pathway is active in all ER-positive MBC tumors.

Populärvetenskaplig Sammanfattning

Bröstcancer är den vanligaste cancerformen hos kvinnor i Sverige, men även män kan drabbas. Hos män är det dock en mycket ovanlig cancerform. I Sverige insjuknar cirka 40 män per år av bröstcancer jämfört med cirka 8000 kvinnor per år. Bröstcancer hos män och kvinnor är på många sätt lika. Till exempel är sjukdomstecknen desamma och visar sig oftast som en knöl eller knuta i bröstet. Emellertid finns det även en hel del skillnader, som att män oftast är äldre än kvinnor när de drabbas av bröstcancer. De flesta män som drabbas är 60–70 år eller äldre. Risken för att män ska drabbas av bröstcancer ökar bland annat om de har en obalans av könshormonerna, något som kan uppkomma vid bland annat övervikt och leversjukdomar. En annan riskfaktor är om man har en familjehistoria med många bröstcancerfall och/eller äggstockcancerfall inom familjen, eftersom det finns vissa former av bröstcancer som är ärftliga hos både kvinnor och män.

På grund av att manlig bröstcancer är en så pass ovanlig tumörform har det inte studerats så mycket. Därför finns det stora kunskapsluckor om hur manlig bröstcancer fungerar och hur patienterna ska behandlas på bästa sätt. Många känner inte till att även män kan utveckla bröstcancer, och därför är ofta män mindre uppmärksamma på förändringar i deras bröst än kvinnor. Detta leder till att bröstcancertumörer hos män ibland upptäcks i ett senare skede än hos kvinnor och har då hunnit bli mer aggressiva. Möjligheten att bota bröstcancer är större ju tidigare en diagnos ställs. Idag får manliga bröstcancerpatienter samma behandling som kvinnliga bröstcancerpatienter. En svensk studie har visat att även om män får samma behandling som kvinnor med bröstcancer, så har männen sämre överlevnad. Det gäller också om man enbart jämför kvinnor och män i samma ålder.

Bröstcancer är inte bara en sjukdom utan många olika sjukdomar. Beroende på vilken sorts bröstcancer man har behöver man olika sorters behandling. Idag har man lyckats dela in kvinnlig bröstcancer i olika undergrupper som fungerar olika biologiskt, är olika aggressiva och där patienterna har olika chans att överleva. Bröstcancerbehandlingar idag går ut på att ge varje patient en individuell behandlingsplan beroende på patientens hälsotillstånd och vilken form av bröstcancer de har. Till sin hjälp har läkarna idag ett antal faktorer hos patienten och tumören som de kan använda sig av för att bestämma vilken behandling som skall ges i tillägg till kirurgi. Många bröstcancerpatienter blir friska idag, men tyvärr inte alla. Varje år i Sverige dör 1400 kvinnor och 15 män av bröstcancer. En av de stora utmaningarna idag är att veta vilken patient som kommer att ha nytta av vilken behandlingsplan.

Många bröstcancerpatienter idag skulle bli friska med enbart kirurgi, men på grund av att vi inte vet vilka och att vi vill förhindra återfall hos så många som möjligt behandlas många bröstcancerpatienter i onödan med tuffa tilläggsbehandlingar. Tilläggsbehandlingar kan bestå av strålning eller vara någon form av medicinska tilläggsbehandlingar som endokrin behandling, cytostatika och/eller målinriktade behandlingar. Det finns ett behov av att hitta nya faktorer som bättre kan identifiera vilka patienter som har hög risk för återfall och som därför är i störst behov av mer omfattande tilläggsbehandlingar.

Cancer är ett samlingsnamn för flera hundra olika sjukdomar och de är genetiska sjukdomar som uppkommer genom att kroppens egna celler har samlat på sig många genetiska förändringar vilket slutligen leder till att de börjar dela sig okontrollerat och inte följer kroppens signaler längre. När kroppens kontroll över cancercellerna är satt ur spel kan de dela sig obegränsat antal gånger och deras dotterceller ärver deras genetiska förändringar. När en cancercell har uppnått förmågan att växa invasivt och kan sprida sig är det en malign tumör. Tumörer som inte kan sprida sig kallas benigna. Maligna tumörer kan till slut sprida sig till andra platser i kroppen och bilda nya tumörer (metastaser).

I de fyra delarbetena som ingår i denna avhandling har vi studerat ett stort material av 220 paraffininbäddade och 83 färskfrysta manliga bröstcancertumörer som vi har samlat in från södra Sverige och Uppsala-regionen mellan 1983 och 2009. Dessutom har vi samlat in information om tumörerna och patienterna. De färskfrysta tumörerna använde vi för att studera hur manliga bröstcancertumörer fungerar biologiskt och jämförde dem med kvinnliga bröstcancertumörer. Vi har studerat hela arvsmassan av tumörerna på tre olika nivåer:

- Mönster av ökningar eller förluster av arvsmassan (DNA). Arvsmassan innehåller all information om kroppens alla celler och finns normalt i två kopior i varje cell.
- Mönster av genaktiviteten (mRNA). Arvsmassan innehåller gener och olika celler i kroppen använder sig av olika gener. Generna i sin tur utgör ritningar för proteiner som utför allt arbete i cellerna och bygger upp kroppen.
- Mönster av en specifik epigenetisk förändringar (DNA metylering). Epigenetiska förändringar är ett av kroppens system för att bestämma vilken gener som ska användas.

De paraffininbäddade manliga bröstcancertumörerna använde vi för att studera proteiner.

Genom dessa studier har vi kunnat se att manlig bröstcancer liksom kvinnlig bröstcancer är en mycket heterogen sjukdom med mycket förändringar på de tre nivåerna. Rent generellt liknar förändringarna i manlig bröstcancer förändringarna i kvinnlig bröstcancer på alla tre nivåerna. När förändringar studerades mer i detalj hittade vi dock många skillnader mellan manlig och kvinnlig bröstcancer. Som

exempel innehåller de manliga bröstcancertumörerna fler ökningar och färre förluster av arvsmassan än de kvinnliga bröstcancertumörerna.

Ett av huvudmålen i avhandlingen har varit att dela upp de manliga bröstcancertumörerna i olika undergrupper, precis som man tidigare gjort med kvinnlig bröstcancer. Genom våra mätningar på de tre olika nivåerna har vi hitta två stabila undergrupper. De två identifierade undergrupperna av manlig bröstcancer fungerar olika biologiskt, är olika aggressiva och patienterna har olika chans att överleva. Dessa undergrupper verkar inte vara representerade bland undergrupperna för kvinnlig bröstcancer. Troligen finns därför dessa två nya grupper av bröstcancer bara hos män, och därför kan män med bröstcancer tänkas behöva andra behandlingsstrategier än kvinnor.

Mer än 90% av de manliga bröstcancertumörerna har receptorer för det kvinnliga könshormonet östrogen, jämfört med de kvinnliga bröstcancertumörerna där cirka 75-80% av dem har östrogenreceptorer. Man vet att hos kvinnor med bröstcancertumörer med östrogenreceptorer så är tumörerna ofta beroende av östrogen för sin tillväxt och överlevnad. En mycket effektiv behandlingsmetod är att blockera tillförseln av östrogen genom endokrin behandling, till exempel med läkemedlet tamoxifen. Om risken för återfall bedöms som hög erbjuds bröstcancerpatienter även mer intensiva tilläggsbehandlingar som cytostatika och/eller målinriktade behandlingar. För manlig bröstcancer vet man inte säkert om endokrin behandling är lika effektivt, men några små studier har visat att en del manliga bröstcancertumörer med östrogenreceptorer svarar på behandling med tamoxifen. I den mer aggressiva undergruppen av manlig bröstcancer fann vi något oväntat, att även om de har östrogenreceptorer så verkar tumörerna inte fungera på samma sätt som kvinnliga bröstcancertumörer med östrogenreceptorer. Detta kan innebära att tumörerna inte är beroende av östrogen för sin tillväxt och överlevnad, och att de därför inte svarar lika bra på endokrin behandling. Dessa manliga bröstcancerpatienter skulle därför kanske behöva andra behandlingsstrategier än endokrin behandling. Bland de manliga bröstcancerpatienterna vars tumörer tillhörde den mer aggressiva gruppen, fick bara 12% tilläggsbehandling med cytostatika och det är mycket troligt att fler patienter i den gruppen skulle ha behövt tillägg av cytostatika. De faktorer som idag används för att bestämma behandlingsplan för manlig bröstcancer räcker inte till för att hitta alla patienter som behöver de mer intensiva tilläggsbehandlingarna, och nya faktorer behöver identifieras. Vi har hittat en ny lovande faktor, NAT1. I vårt material såg vi att om tumörerna har NAT1-proteiner så har patienterna en mycket lägre risk för återfall. Majoriteten av de manliga bröstcancertumörerna i den aggressiva undergruppen har inte NAT1-proteiner. Detta gör NAT1 till en mycket intressant faktor att studera vidare och i framtiden eventuellt kunna använda den för att bestämma vilken behandling som skall ges i tillägg till kirurgi till män med bröstcancer.

Sammanfattningsvis så har vi visat att manlig bröstcancer skiljer sig mer från kvinnlig bröstcancer än vad man tidigare trott. Vi har också identifierat två undergrupper av

manlig bröstcancer med olika biologiska egenskaper och olika överlevnads chans för de drabbade patienterna. Dessa undergrupper verkar inte vara representerade bland undergrupperna för kvinnlig bröstcancer. Våra resultat visar att män med bröstcancer kan tänkas behöva andra behandlingsstrategier än kvinnor med bröstcancer. Vi har identifierat NAT1 som en lovande faktor att använda sig av för att bestämma behandlingsstrategi för män med bröstcancer. Om de manliga bröstcancertumörerna inte har NAT1 proteiner så har patienterna en mycket högre risk att utveckla återfall.

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