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Molecular Profiling of Male Breast Cancer – Lost in Translation?

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Abbreviations: aCGH, array based comparative genomic hybridization; BCSS, breast cancer specific survival; DFS, disease free survival; EMT, epithelial-mesenchymal transition; EORTC, European Organization for Research and Treatment of Cancer; ER, estrogen receptor; IHC, immunohistochemistry; MBC, male breast cancer; NHG,

Nottingham histological grade; OS, overall survival; PR, progesterone receptor; TCGA, The Cancer Genome Atlas; TMA, tissue microarray

Abstract

Breast cancer is the most common cancer form in women and it has been extensively studied on the molecular level. Male breast cancer (MBC), on the other hand, is rare and has not been thoroughly investigated in terms of transcriptional profiles or genomic aberrations. Most of our understanding of MBC has therefore been extrapolated from knowledge of female breast cancer. Although differences in addition to similarities with female breast cancer have been reported, the same prognostic and predictive markers are used to determine optimal management strategies for both men and women diagnosed with breast cancer. This review is focused on prognosis for MBC patients, prognostic and predictive factors and molecular subgrouping; comparisons are made with female breast cancer. Information was collected from relevant literature on both male and female breast cancer from the MEDLINE database between 1992 and 2014.

MBC is a heterogeneous disease, and on the molecular level many differences compared to female breast cancer have recently been revealed. Two distinct subgroups of MBC, luminal M1 and luminal M2, have been identified which differ from the well-established intrinsic subtypes of breast cancer in women. These novel subgroups of breast cancer therefore appear unique to MBC. Furthermore, several studies report inferior survival for men diagnosed with breast cancer compared to women. New promising prognostic biomarkers for MBC (*e.g.* NAT1) deserving further attention are reviewed. Further prospective studies aimed at validating the novel subgroups and recently proposed biomarkers for MBC are warranted to provide the basis for optimal patient management in this era of personalized medicine.

1. Introduction

Male breast cancer (MBC) is similar to breast cancer in women in some aspects; for instance invasive ductal carcinoma is the most common histological type (Fentiman et al., 2006; Korde et al., 2010), and it is often detected as a painless subareolar lump and may also involve nipple retraction or bleeding from the nipple (Giordano, 2005; Ruddy and Winer, 2013). However, there are also many differences between breast cancers occurring in men *vs.* women. Most notably, breast cancer is much less common in men (only 1% of all breast cancers in the US (Siegel et al., 2013) and 0.5% in the Nordic countries (Engholm et al., 2013) occur in men), men are often older at diagnosis (67 *vs.* 62 years) (Giordano et al., 2002), their tumors are more often hormone receptor positive (estrogen receptor (ER) positivity 91-95% *vs.* 76-78% and progesterone receptor (PR) positivity 80-81% *vs.* 67%, in men and women, respectively) (Anderson et al., 2010; Giordano et al., 2002; Nilsson et al., 2013b). Lobular carcinoma is also much less common in men (Giordano et al., 2002; Weigelt et al., 2010).

A family history of breast and ovarian cancer is a risk factor for developing breast cancer in men, as in women; germline *BRCA2* mutations have been reported in 4-14% of MBC patients, while *BRCA1* mutations are less frequent, occurring in up to 4% of MBC patients (Basham et al., 2001; Chodick et al., 2008; Couch et al., 1996; Ding et al., 2010; L. S. L. Friedman et al., 1997; Ottini et al., 2008; Struewing et al., 1999). *BRCA1* and *BRCA2* mutations confer an estimated increased lifetime risk of developing breast cancer of 1-6% and ~7%, respectively (Levy-Lahad and E. Friedman, 2007; Liede et al., 2004; Tai et al., 2007), while the general lifetime risk in the male population is 0.1% (Engholm

et al., 2013; Liede et al., 2004). Among other germline mutations that confer a moderately increased risk of developing breast cancer in women, data for men are mixed for *PALB2*, *CHEK2* and *CYP17* (Blanco et al., 2011; Ding et al., 2010; Falchetti et al., 2007; kConFab et al., 2009; Ohayon et al., 2004; Silvestri et al., 2010b; Syrjkoski et al., 2003; Wasielewski et al., 2008; Young et al., 1999), while no increased risks have been found for *BRIP1* and *RAD51C* with regards to MBC (Silvestri et al., 2010a; 2011). A large genome-wide association study of MBC has identified *TOX3* and *RAD51B* to confer increased risks for MBC, the *RAD51B* locus being a novel breast cancer susceptibility locus (Orr et al., 2012). Other risk factors for men are associated with changes in the hormonal balance of estrogen to androgen, such as in Klinefelter's syndrome (resulting in a 50-fold increased risk) (Brinton et al., 2009; Hultborn et al., 1997), testicular abnormalities that result in testosterone deficiency (Brinton et al., 2009; Thomas et al., 1992), liver diseases (Sørensen et al., 1998), obesity (Brinton et al., 2009; 2008; Ewertz et al., 2001; Hsing et al., 1998) and exogenous estrogen exposure (Medras et al., 2006; Thellenberg et al., 2003).

The number of breast cancer diagnoses among women has increased over the past decades (Ly et al., 2012; Socialstyrelsen, 2012), while the incidence of MBC has not risen in most countries (Ly et al., 2012), with the exception of a slight increase that has been reported from England, Scotland, Australia and the USA (Giordano et al., 2004; Speirs and Shaaban, 2008; Stang and Thomssen, 2008; White et al., 2011).

Research into the etiology and tumor biological properties of MBC has been limited due to the rareness of the disease, and most data are derived from retrospective studies covering long time periods and geographical regions. Therefore, MBC patients are

currently being managed according to guidelines developed for female patients; there is however currently insufficient knowledge to determine whether this is the most optimal strategy.

2. Prognosis of male breast cancer

The outcome of men diagnosed with breast cancer compared to women is currently debated. Many recent studies have shown worse survival for MBC patients (Chen et al., 2013; Gnerlich et al., 2012; Greif et al., 2012; Miao et al., 2011; Nilsson et al., 2011; Scott-Conner et al., 1999; Yildirim and Berberoğlu, 1998); however this difference becomes less apparent when the cohorts are stratified on various prognostic factors (Giordano et al., 2004; Miao et al., 2011; Shaaban et al., 2012). Table 1 summarizes the largest studies comparing survival for male and female breast cancer patients to date (Chen et al., 2013; Giordano et al., 2004; Gnerlich et al., 2012; Greif et al., 2012; Miao et al., 2011; Nilsson et al., 2011; Scott-Conner et al., 1999; Shaaban et al., 2012).

Many of the studies in Table 1 cover long periods of time, are based on small sample sizes, and/or include patients from many different hospitals and sometimes also countries. This is an unavoidable consequence of the rarity of the disease and limits the interpretation of the results. Moreover, when comparing overall survival (OS) between the genders, it needs to be taken into consideration that women have a slightly longer expected survival than men; *e.g.* in Sweden, life expectancy is 84 years for women and 80 years for men (Centralbyrån, 2014). Nevertheless, Table 1 includes two single center studies: one from Sweden including 99 MBC patients and one from China with 150 MBC patients, and both these studies showed inferior outcome for MBC patients (Chen et al.,

2013; Nilsson et al., 2011). The Swedish study matched on age and date of diagnosis, and contrary to what has been anticipated from the literature, found no differences in disease stage between the genders. Despite this, a significantly worse relative survival was observed for men (Nilsson et al., 2011). The Chinese study matched patients for age, date of diagnosis and stage, and found a significantly inferior disease-free as well as OS for men (Chen et al., 2013). We know today that breast cancer is a very heterogeneous disease in general and that it can be divided into comprehensive subgroups associated with differences in response to treatment and outcome. The question therefore arises on which factors one should match when comparing outcome for men *vs.* women diagnosed with breast cancer. Notwithstanding, when male and female breast cancer patients are compared on a population based level, the relative overall and breast cancer specific survival appears to be worse for male patients (Cancerfonden, 2013; Chen et al., 2013; Greif et al., 2012; Miao et al., 2011; Nilsson et al., 2011). For example, in Sweden the relative 5-year OS rates for all male and all female patients are 79.6% and 90.0%, respectively, while the corresponding relative 10-year OS rates are 67.1% and 83.5% (Cancerfonden, 2013). Furthermore, a clear trend toward increased survival rates for women with breast cancer has been seen in Sweden (Cancerfonden, 2013) and in the US, while only a small trend toward increased survival was found among men in the US (Anderson et al., 2010). Taken together, these findings suggest that there may be underlying differences in tumor biology between breast cancers arising in men and women, and that these may affect outcome.

3. Molecular subtyping of breast cancer

Breast cancer has been extensively studied on the transcriptomic, genomic and even epigenomic levels and the development of high throughput technologies and bioinformatics tools have made it possible to investigate large numbers of tumors. Much has been learned over the past decades regarding how to subclassify breast cancer into comprehensive subgroups with different biological and clinical features, leading to a paradigm shift in how we understand and study breast cancer in women and how breast cancer patients are managed. In 2000, Perou *et al*. published the first paper suggesting a subclassification of breast cancer by using gene expression profiles, coining the concept of intrinsic subtypes (Perou et al., 2000). These groups have been further refined and validated by them and others over the years. The five main intrinsic subtypes of breast cancer are (Eroles et al., 2012; Goldhirsch et al., 2013; Hu et al., 2006; Parker et al., 2009; Sorlie et al., 2001; Sørlie et al., 2003):

- Luminal A (50-60%): The majority are ER positive, they display low proliferation, and are considered more endocrine sensitive than luminal B tumors.
- Luminal B (10-20%): The majority are ER positive, they are often highly proliferative and are less endocrine responsive than luminal A tumors. Many *BRCA2* mutated tumors belong to this group.
- Basal-like (10-20%): The majority are triple-negative (ER, PR and HER2 negative) and they may express cytokeratins 5, 6 and 14. The majority of *BRCA1* mutated tumors belong to this group.

- HER2-enriched (10-15%): The majority exhibit amplification and/or overexpression of *HER2* as well as other genes in the *HER2* amplicon. An overrepresentation of ER negative tumors is found in this group.

- Normal-like (5-10%): Genes defining this group are expressed in normal breast tissue, but this is not a well-defined subgroup. It is not clear whether tumors classified as normal-like represent a true subtype of breast cancer, or whether they reflect tumors with a high degree of normal epithelial cells.

The intrinsic subtypes have distinct clinical features, with the basal-like and HER2 subgroups displaying the worst prognosis and luminal A the best, while luminal B tumors have an intermediate prognosis (Hu et al., 2006; Parker et al., 2009; Sorlie et al., 2001; Sørlie et al., 2003). Through the years, additional subgroups have been identified, including the claudin-low subgroup, which is characterized by being triple negative (negative for ER, PR and HER2). These tumors have cancer stem cell like features, express high levels of epithelial-to-mesenchymal transition (EMT) genes, display low expression of luminal genes, claudins 3, 4 and 7 and express high levels of genes expressed by lymphocytes (Herschkowitz et al., 2007; Prat et al., 2010). During recent years it has further been shown that triple negative breast cancers are a heterogeneous group, and Lehman *et al.* identified as many as six subgroups of the triple-negative tumors (B. D. Lehmann et al., 2011).

Female breast cancers have also been subclassified based on DNA copy number and DNA methylation levels. These subgroups correlate to some extent with the transcriptionally derived intrinsic subtypes, but have also further refined the classification of breast cancer and contributed to the understanding of the biology of the intrinsic

subtypes (Chin et al., 2006; Dedeurwaerder et al., 2011; Fridlyand et al., 2006; Holm et al., 2010; Jönsson et al., 2010; Russnes et al., 2010). The Cancer Genome Atlas (TCGA) network combined five types of genomic data (DNA copy number arrays, DNA methylation arrays, exome sequencing, mRNA arrays, miRNA sequencing and reverse-phase protein arrays) to identify subgroups of breast cancer. They identified four subgroups, which correlated well with the intrinsic subtypes; moreover the integrated information across platforms helped to further explain the underlying biology of the intrinsic subtypes (Koboldt et al., 2012). Curtis *et al.* also combined genomic and transciptomic data, thereby identifying ten distinct subgroups associated with different clinical outcomes and candidate driver genes, further refining the classification of breast cancer (Dawson et al., 2013; METABRIC, 2012).

3.1. Surrogate IHC based definitions of the intrinsic breast cancer subtypes

Due to costs and tissue handling requirements associated with transcriptional profiling, a need to develop assays more applicable to large numbers of tumors has led to the translation of the intrinsic subtypes to an immunohistochemistry (IHC) based surrogate assay. An approximation of the classification into the intrinsic gene expression derived subtypes can thereby be accomplished based on expression levels of a small number of proteins. This allows for analysis of paraffin embedded archival tumor material to a fraction of the cost associated with mRNA based subtyping. Several definitions have been proposed, and the most commonly used surrogate IHC based definitions for classifying breast cancers into the intrinsic subtypes are (Goldhirsch et al., 2013; Kaufmann et al., 2011; Prat et al., 2013):

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

Definition II improved the distinction between luminal A and luminal B tumors by incorporating the proliferation marker Ki67. However, during the years there have been many concerns about the considerable lack of reproducibility across laboratories for the assessment of Ki67. Ki67 has proven difficult to evaluate; however a recent trial showed that Ki67 scoring is reproducible when tissue microarray (TMA) sections were stained with a standardized method, a common scoring method was used and the evaluators had been trained using a web based calibration tool (Polley et al., 2013). This report however highlights the difficulties associated with comparing non-standardized Ki67 results across

different laboratories or studies. Definition III is the most recent, and it has further improved the distinction between luminal A and luminal B tumors by also requiring luminal A tumors to be PR positive (Prat et al., 2013), and it was included in the 2013 guidelines from the St Gallen consensus conference (Goldhirsch et al., 2013). This is of importance for treatment decision making, as patients with luminal B tumors are generally recommended chemotherapy based regimens, while patients with luminal A tumors are generally not (Goldhirsch et al., 2013). Although the degree of concordance between the IHC derived subgroups and the intrinsic transcriptional subtypes is relatively high at 75-90% (Kaufmann et al., 2011), the St Gallen consensus recommended whenever possible to use gene expression based subtyping over the surrogate IHC based approach for decisions regarding chemotherapy for patients with luminal tumors (Goldhirsch et al., 2013).

3.2. IHC based classification of MBC

A number of independent research groups have attempted to subclassify MBCs into the intrinsic subtypes using the same IHC based definitions as those described above for female breast cancer. A summary of the results is shown in Table 2, with a recent large study of female breast cancer included as reference (Blows et al., 2010; Ge et al., 2009; Kornegoor et al., 2011; Nilsson et al., 2013a; Shaaban et al., 2012; Yu et al., 2013).

In common between these reports, the majority of the MBC tumors were classified as luminal A (60-98%), the subgroup of female breast cancer associated with the best survival. When definition I was used to classify female breast cancers, 71% were

classified as luminal A (Blows et al., 2010), while gene expression based classification generally results in about 50-60% luminal A tumors (Eroles et al., 2012). These data suggest that distinguishing between luminal A and B in general is problematic when using definition I. While Ki67 is applied in definitions II and III for separating luminal A from luminal B tumors, studies without standardized protocols for scoring of Ki67 are difficult to compare as discussed above. Nevertheless, when studies using definition I were compared, MBCs were more often classified as luminal A compared to female breast cancers (83-98% *vs.* 71%) (Blows et al., 2010; Ge et al., 2009; Nilsson et al., 2013a; Shaaban et al., 2012), with the exception of the Chinese study which reported very different distributions of the subgroups compared to the other studies (Yu et al., 2013). Fewer basal-like (0-2% *vs.* 16%) and HER2 enriched (0% *vs.* 6%) MBC tumors were reported in these studies than what is seen in female breast cancer. These subgroups are associated with the worst prognosis among women (Blows et al., 2010; Ge et al., 2009; Nilsson et al., 2013a; Shaaban et al., 2012). The report by Ye and colleagues is therefore surprising in light of the inferior relative OS as well as BCSS among male patients compared to females (Cancerfonden, 2013; Chen et al., 2013; Greif et al., 2012; Miao et al., 2011; Nilsson et al., 2011). Based on the studies reported to date, it appears as though a subgroup of aggressive MBC is not captured when the traditional IHC proxy markers are used for classification. These data further suggest that men and women diagnosed with breast cancer of the same IHC based subtype do not have similar outcomes and therefore most likely respond differently to standard therapies. Taken together, this indicates the need for additional biomarkers to successfully identify and

classify all cases of aggressive MBC, and furthermore that they may require different treatment strategies.

4. Global profiling of MBC

In contrast to female breast cancer, MBC is not well studied on the genomic and epigenomic levels and there have only been a few array based studies investigating DNA copy number aberrations (Johansson et al., 2011; Tommasi et al., 2010), gene expression profiles (Callari et al., 2010; Johansson et al., 2012) and microRNA profiles (Fassan et al., 2009; U. Lehmann et al., 2010), summarized in Table 3. All these global array based studies revealed that MBC, like breast cancer in females, is a heterogeneous disease. Moreover, many differences between male and female breast cancers, hidden behind the overall similarities, were discovered (Callari et al., 2010; Fassan et al., 2009; Johansson et al., 2012; 2011; U. Lehmann et al., 2010; Tommasi et al., 2010). When we combined the gene expression data (Johansson et al., 2012) with the array comparative genomic hybridization (aCGH) data (Johansson et al., 2011) to identify potential candidate driver genes of male and female breast cancer, the landscapes of candidate drivers were vastly different between the genders, with only two candidate drivers in common, *TAF4* and *CD164* (Johansson et al., 2013). MBC thereby appears to differ from female breast cancer on the molecular level, potentially suggesting different mechanisms of tumorigenesis.

4.1 MBC miRNAs and epigenetics

In both microRNA studies mentioned above, MBCs were compared with (non-malignant)

gynecomastia samples; Fassan *et al.* identified 43 microRNAs and Lehman *et al.* identified 54 microRNAs that were differentially expressed. Furthermore, when the MBCs were compared with female breast cancers, both studies identified several microRNAs differentially expressed between the genders (Fassan et al., 2009; U. Lehmann et al., 2010). In another study, analyzing RASSF1A and RARβ promoter methylation status and the expression of miRNAs miR17, miR21, miR124, and let-7a in familial breast cancers from 27 males and 29 females, miR17 and let-7 expression was lower in breast cancers from men than women, while RASSF1A was more frequently methylated in the MBCs (Pinto et al., 2013). Hypermethylation of tumor suppressor genes has been shown to play a key role in tumor progression in breast cancer (Baylin and Herman, 2000; Jones, 2002; Jones and Baylin, 2007). DNA methylation has not yet been studied on a global level in MBC, although Kornegoor *et al.* studied promoter methylation of 25 tumor suppressor genes in 108 MBC tumors and compared them to 33 female breast cancer tumors. Several hypermethylated genes were shared between the genders; however many of the genes investigated were less frequently methylated among the MBC tumors, notably *ESR1*, *BRCA1* and *BRCA2* (Kornegoor et al., 2012).

4.2 MBC Genomics

On the copy number level, two studies applying metaphase CGH to 39 and 26 MBCs, respectively, reported that the most common aberrations were the same in male and female breast cancers (Rudlowski et al., 2006; Tirkkonen et al., 1999). In our aCGH studies of MBC, based on high resolution tiling BAC arrays, we also recognized that the

most common aberrations were the same in male in female breast cancers. However, when studied in more detail, many differences between male and female breast cancer were revealed (Johansson et al., 2011). Tommasi *et al.* found that the MBC tumors had an overall lower frequency of copy number aberrations compared to female breast cancers. However, the eleven cases of female breast cancer used in their study were all ER negative basal-like tumors, an aggressive type of breast cancer harboring the largest number of copy number aberrations among all female breast cancers (Jönsson et al., 2010). This makes it difficult to draw any conclusions regarding copy number differences between male and female breast cancer in general (Tommasi et al., 2010). In contrast, in our study, where 56 MBC tumors were compared to 359 FBC tumors, run on the same platform and normalized in the same manner, we identified more gains and fewer losses among the MBC tumors. The MBC tumors also harbored more whole chromosome arm gains and fewer high-level amplifications than the FBC tumors (Johansson et al., 2011). Furthermore, in a gene expression profiling study, Callari *et al.* reported several genes to be differentially expressed between the genders. However, they performed a direct comparison between male and female breast cancers even though they were run at different time points, making it difficult to distinguish true findings from potential technical artifacts (Callari et al., 2010). We reported a global gene expression profiling study based on 66 MBCs with 359 female breast cancers for comparison; however they were not run on the same platform and therefore only indirect comparisons between the genders could be performed (Johansson et al., 2012). The results are discussed in the following sections.

4.3 Global subgrouping of MBC

Accurate subtyping of MBC into comprehensive subgroups is essential for developing an appropriate therapeutic strategy. Today, treatment decisions for male patients are based on biomarkers developed for breast cancer in women, although we do not know if they sufficiently capture the heterogeneity of breast cancer arising in men. The sub-classification of MBC into the IHC based subgroups of female breast cancer indicates that these markers do not adequately identify the aggressive forms of MBC (Ge et al., 2009; Nilsson et al., 2013a; Shaaban et al., 2012; Yu et al., 2013). We therefore recently sought to subclassify MBC into different subgroups in an unsupervised manner using different types of genome scale data and then compared these MBC subgroups with the previously described intrinsic subtypes of female breast cancer. Two subgroups were identified both on the copy number and transcriptional level, and these groups were highly correlated, indicating fundamental underlying differences (Johansson et al., 2012; 2011).

A large cohort of 359 female breast cancers, in which Jönsson *et al*. had previously identified six subgroups (7q12, basal-complex, luminal-simple, luminal-complex, amplifier and mixed), was used as a reference dataset in our aCGH study of MBC. Four of these subgroups were correlated with the intrinsic, transcriptionally derived subtypes; basal-complex with basal-like, 17q12 with HER2 enriched, luminal-complex with luminal B and luminal-simple with luminal A, respectively (Jönsson et al., 2010). In an attempt to subclassify the MBC tumors in our cohort, we performed hierarchical clustering based on 133 commonly aberrant genomic regions identified among the female

breast cancers (Johansson et al., 2011). The clustering revealed two subgroups with significantly different aberrant copy number levels, which we named male-complex (80% of tumors) and male-simple (20% of tumors), respectively. The male-simple subgroup appeared to be comprised of less aggressive tumors, as they were found to have a lower overall fraction of the genome altered, lower S-phase fractions and the tumors were smaller. This subgroup was remarkably different from the previously described six subgroups of breast cancer, and seemed to represent a new subgroup of breast cancer occurring only in males. Tumors in the more aggressive subgroup of MBC, male-complex, were overall similar to the luminal-complex subgroup of female breast cancer. When the groups were studied in more detail, however, differences in whole chromosome aberrations and common aberrant regions were revealed (Johansson et al., 2011).

Gene expression profiling is a common method used for defining the phenotypes of various tumor types at the transcriptional level; the most extensively studied tumor type in this context is breast cancer (Hu et al., 2006; Parker et al., 2009; Sorlie et al., 2001; Sørlie et al., 2003). Since MBCs make up a very small fraction of all breast cancers, they have not previously gained much attention in this context. To date, we are the only group who has focused specifically on subclassifying MBC tumors using global gene expression profiles. Hierarchical clustering was performed on 66 MBCs using the top most varying genes across the dataset; two subgroups, luminal M1 (70% of tumors) and luminal M2 (30% of tumors), were thereby identified. The stability of these subgroups was validated by hierarchical clustering of the tumors on co-clustering frequencies from 10,000 bootstrapped datasets, and furthermore the subgroups were also validated (Johansson et al., 2012) in an external dataset of MBC (Callari et al., 2010). Due to the

limited sample size no further subdivision was possible, but it is possible that additional subgroups of MBC may exist. Importantly, when the novel MBC subgroups were compared with each of the intrinsic subtypes of FBC, no resemblance to any subtype was observed. Classification of MBC tumors into (female) intrinsic breast cancer subtypes according to the gene expression centroids published by Hu *et al.* (Hu et al., 2006) resulted in >50% of the MBC tumors being unclassified. Conversely, >60% of the female breast cancers in the reference dataset were not classifiable into the MBC (luminal M1/M2) subgroups. Recognizing that >90% of MBC tumors are ER positive, we therefore also classified the MBCs using only genes for ER positive female breast cancers *(i.e.* luminal A/B); still 36% remained unclassified (Johansson et al., 2012). To further study the biological differences between the new MBC subgroups and the intrinsic subtypes of female breast cancer, we applied seven gene expression modules representing key biological processes involved in breast cancer tumorigenesis (Desmedt et al., 2008) to the datasets. The luminal M2 subgroup of MBC demonstrated higher scores for the immune response and ER modules, while luminal M1 MBC tumors displayed higher scores for the tumor invasion and metastasis, proliferation and HER2 modules, indicating that MBCs of the luminal M1 subgroup display more aggressive features than other MBC tumors. When comparing the patterns of the module scores across the intrinsic subtypes of female breast cancer and the MBC subgroups, neither of the MBC subgroups displayed patterns resembling any of the intrinsic subtypes (Figure 1). Instead, the MBC subgroups shared different features with different intrinsic subtypes. These findings suggest that the two MBC subgroups appear to be unique in terms of underlying biology and may occur only in males (Johansson et al., 2012). In order to

firmly establish the relation between male and female breast cancer, and to establish the distribution of subtypes, global unsupervised transcriptional profiling should be applied to a large cohort representing the whole spectrum of both male and female breast cancers, enabling direct comparisons.

5. ER activity in MBC

An unexpected finding from the gene expression profiling study was that the more aggressive MBC tumors of the luminal M1 subgroup displayed a very low score for the ER module, despite the majority of the tumors being ER positive by IHC (Figure 1). By comparison, both luminal subtypes of female breast cancer within which the vast majority of the tumors are ER positive, displayed equally high module scores for ER, while the basal-like subtype, mainly harboring ER negative tumors, had the lowest score for ER. Hence, even if luminal M1 MBC tumors are ER positive they appear to share some features with ER negative female breast cancers. Given the data above, this may indicate that luminal M1 MBC tumors might not have an active ER pathway, leading to the question whether these patients respond to endocrine treatment in the same way as women with an ER positive tumor (Johansson et al., 2012). Two other reports on hormone receptors in MBC have indicated different hormonal dependencies in male and female breast cancer (Shaaban et al., 2012; Weber-Chappuis et al., 1996). Shaaban *et al.* studied 251 male and 263 female breast cancers matched on age, grade, and lymph node status. They investigated the hormone receptors ERα, ERβ1, -2, -5, PR, PRA, PRB and AR by IHC on a TMA and hierarchically clustered the male and female tumors separately based on the expression of these proteins. Among female breast cancers, the PR isoforms

and ERα clustered together, while the PR isoforms formed a separate cluster and ERα clustered together with the ERβ isoforms and AR in MBC (Shaaban et al., 2012). Weber-Chappuis *et al.* studied paraffin-embedded tumor material from 66 male and 190 female breast cancer patients by IHC, stained for hormone receptors and antigens under estrogen and androgen control. They found that although a larger fraction of the male tumors were ER positive compared to the female tumors, they were only weekly associated with antigens under estrogen control and more often positive for antigens under androgen control, while the opposite was true for female breast cancer (Weber-Chappuis et al., 1996). Furthermore, in a large genome-wide association study of MBC, no association was found to the rs2981582 SNP in the fibroblast growth factor receptor 2 (*FGFR2*) gene (Orr et al., 2012) known to have the strongest known association with ER positive breast cancer in women (García-Closas et al., 2008). Also, in the investigation of candidate driver genes in male *vs.* female breast cancer, *GATA3* was identified as one of the top candidate drivers among all female breast cancers as well as within the luminal A and luminal B subtypes, while it was not identified as a candidate driver for MBC. *MAP2K4*, which is also highly connected to ER positive breast cancer in women was however identified as a candidate driver also among MBCs (Johansson et al., 2013). This further supports the notion that not all ER positive MBC tumors behave in the same way as ER positive tumors in women, but rather seem to share features with both ER positive and ER negative female breast cancer.

6. Prognostic and predictive biomarkers in MBC

Many prognostic and predictive factors been investigated in breast cancer, and some are

established and used in the clinic today to guide treatment decisions. The current St Gallen consensus guidelines recommend age at diagnosis, tumor size, lymph node status, presence of distant metastases, histological classification, Nottingham histological grade (NHG), ER, PR, HER2 and Ki67 to be used in the clinical setting (Goldhirsch et al., 2013). These factors are not as a well established for MBC and have only been evaluated in a small number of retrospective trials; there is thus little evidence that they provide the same prognostic and predictive information as for women. In fact, many of the studies have shown contradicting results: some studies have found high NHG to be an independent prognostic factor for poor prognosis (Cutuli et al., 2010), while others have not (Giordano et al., 2004; Johansson et al., 2012). This is also true for Ki67, where some studies found it to have no prognostic value (Kanthan et al., 2010; Nilsson et al., 2013b; Wang-Rodriguez et al., 2002), while another study found Ki67 to be prognostic (Rayson et al., 1998). As mentioned above, however, the difficulties associated with evaluation of Ki67 limit the ability to draw any firm conclusions based on non-standardized Ki67 results (Polley et al., 2013). Both Ki67 and NHG are strongly associated with proliferation and proliferation is highly prognostic in female breast cancer, particularly in patients with ER positive cancers. One would therefore expect it to be prognostic even for men diagnosed with breast cancer, among whom >90% of the tumors are ER positive. Other markers have been used to study proliferation in female breast cancer, including the cyclins (Agarwal et al., 2009; Klintman et al., 2013; Michalides et al., 2002; Niméus-Malmström et al., 2010). We assessed cyclins A, B and D1 in a cohort of 197 MBC tumors, and while Ki67 was not found to be prognostic, cyclins A and B were prognostic for poor prognosis and cyclin D1 predicted a better outcome (Nilsson et al., 2013b).

Cyclin D1 was also found to predict better outcome in MBC in two other studies (Kanthan et al., 2010; Rayson et al., 1998). The study by Kanthan *et al.* also reported other cell cycle protein markers in a cohort of 75 MBCs, and found that c-myc positive tumors were also linked with favorable outcome while overexpression of p21, p57, and PCNA was associated with worse outcome. No correlation with outcome was however found for Ki67, p27 or p16 (Kanthan et al., 2010). Notably, Lacle *et al.* did not find mitotic index, one of the three components of NHG, to be prognostic in a series of 151 MBCs. The expression of bcl2 was also investigated, which neither alone nor in combination with mitotic index was prognostic (Lacle et al., 2013). In contrast, the combination has been proven to be of strong prognostic value in female breast cancer (Abdel-Fatah et al., 2010). Furthermore, one of the seven transcriptional modules representing key biological processes in breast cancer tumorigenesis was involved in proliferation (Desmedt et al., 2008), and the more aggressive luminal M1 MBC tumors showed a higher score for this module compared to the luminal M2 MBCs. No differences in Ki67 or cyclin A levels were however observed between the subgroups (Johansson et al., 2012). *SPAG5*, which regulates a gene module involved in the mitotic checkpoint control and progression, was identified as a candidate driver gene in MBC. However, no difference in survival was observed between MBCs positive and negative for *SPAG5*, respectively (Johansson et al., 2013). *SPAG5* has on the other hand been shown to be prognostic in ER positive but not in ER negative female breast cancers (Abdel-Fatah et al., 2012; Johansson et al., 2013). Another interesting candidate driver identified in MBC tumors was *THY1*, which regulates a gene network involved in invasion and is related to EMT. Of interest, men with *THY1* positive breast cancers had

significantly inferior survival compared to those with negative tumors (Figure 2) (Johansson et al., 2013). It may therefore be possible that processes other than proliferation are more important for the aggressive behavior of a subset of MBCs. Another protein recently found to be prognostic for disease-specific survival in sporadic but not familial MBC was HIF1A (Deb et al., 2014).

With the aim of identifying novel prognostic biomarkers of relevance in MBC, we assessed the genes that varied the most between the luminal M1 and M2 MBC tumors in our transcriptional profiling study. One of these genes, *NAT1*, was followed up on the protein level using a TMA of 220 MBC tumors. There was a high degree of correlation between the protein and mRNA levels of NAT1, and furthermore luminal M1 tumors displayed low levels of NAT1 on both mRNA and protein levels. Men with NAT1 negative breast tumors had significantly inferior survival compared to those with NAT1 positive tumors, a finding that remained significant in the multivariate setting (Figure 3) (Johansson et al., 2012). High levels of NAT1 have been shown to predict response to tamoxifen in women with ER positive breast cancer. NAT1 is a xenobiotic metabolizing enzyme that may be involved in metabolizing tamoxifen, thereby potentially contributing to tamoxifen activation (Bièche et al., 2004). This may further indicate that although their tumors are ER positive, men with luminal M1 breast cancer may not respond in the same way to tamoxifen as women with ER positive breast cancer.

7. Concluding remarks

Due to the fact that the relative OS and BCSS for men is inferior compared to women, and since recent studies suggest that the prognostic factors used in clinical practice today

do not accurately capture the aggressive MBCs, new biomarkers for MBC are needed. As such, NAT1 and THY1 are two new promising candidate biomarkers for MBC that deserve further attention. Stratification of MBC into molecularly based subgroups has revealed two subgroups that differ both on the copy number and transcriptional levels from female breast cancers and thereby seem to constitute two new breast cancer subgroups occurring only in males. Furthermore, the more aggressive MBC subgroup, luminal M1, might not have an active ER pathway despite the majority of cases being ER positive. MBCs clearly constitute a molecularly and clinically heterogeneous group of malignancies, which differ from breast cancer in women. Understanding this diversity is essential to be able to improve the prognosis for MBC patients and to optimize treatment strategies. Further research into MBC is required to optimize management strategies and to improve survival. The rarity of the disease necessitates international collaborations to increase sample sizes in future studies. To this end, an international EORTC sponsored consortium has been established to study MBC (ClinicalTrials.gov NCT01101425); it includes a retrospective part, collecting clinical data and archival tissue for translational investigations, as well as a prospective clinical part.

Figure captions

Fig. 1. Gene expression modules associated with key biological processes. The module scores of gene expression modules representing key biological processes involved in breast cancer tumorigenesis (Desmedt et al., 2008) in the two gene expression subgroups of MBC (A), in the intrinsic subgroups of female breast cancer (B), and in the MBC validation dataset (C), respectively. Proliferation (Wilcoxon test, P = 0.064), HER2 (Wilcoxon test, P = 0.0057), tumor invasion and metastasis (Wilcoxon test, P = 1.0 × 10-5), ER (Wilcoxon test, P = 1.3 × 10-8) and immune response (Wilcoxon test, P = 0.16) displayed a significant or borderline significant difference between the two subgroups of MBC (A). The ANOVA test was used to calculate P-values (B). Reprinted with permission from *Breast Cancer Research* (Johansson et al., 2012).

Fig. 2. Kaplan-Meier survival analysis. Distant metastasis free survival of the 66 male breast cancer patients stratified by *THY1* gene expression. The numbers below the plots indicate the number of patients at risk in each group at the given time points. Reprinted with permission from *PLoS ONE* (Johansson et al., 2013).

Fig. 3. Kaplan-Meier survival analysis. Distant metastasis free survival of the 220 male breast cancer patients included in the TMA stratified NAT1 expression. The numbers below the plots indicate the number of patients at risk in each group at the given time points. Reprinted with permission from *Breast Cancer Research* (Johansson et al., 2012).

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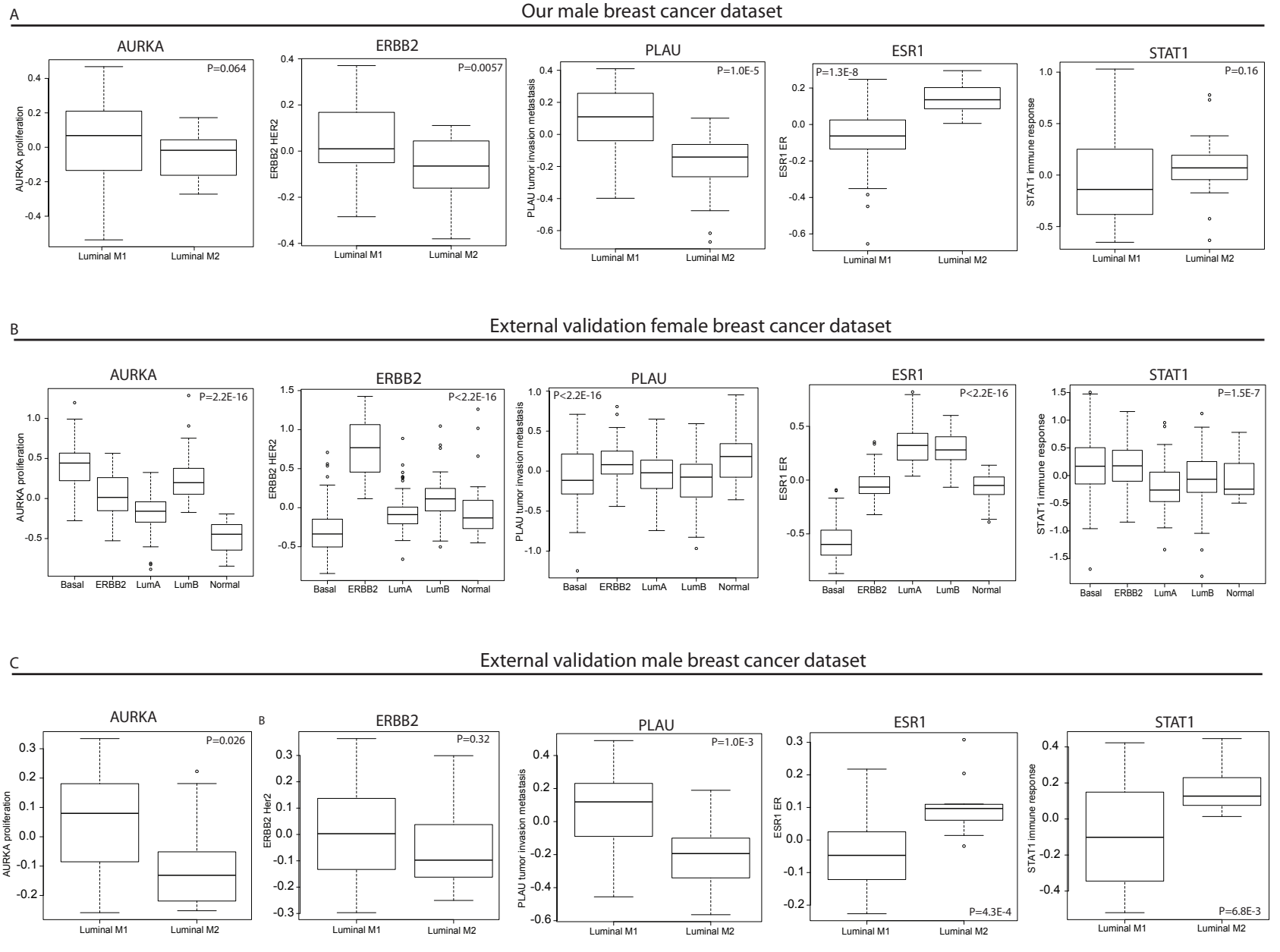
A
Our male breast cancer dataset
AURKA
P=0.064
AURKA proliferation
Luminal M1
Luminal M2
ERBB2
P=0.0057
ERBB2 HER2
PLAU
P=1.0E-5
PLAU tumor invasion metastasis
ESR1
P=1.3E-8
ESR1 ER
STAT1
P=0.16
STAT1 immune response
B
External validation female breast cancer dataset
AURKA
P=2.2E-16
ERBB2
P<2.2E-16
PLAU
P<2.2E-16
ESR1
P<2.2E-16
STAT1
P=1.5E-7
Basal
ERBB2
LumA
LumB
Normal
C
External validation male breast cancer dataset
AURKA
P=0.026
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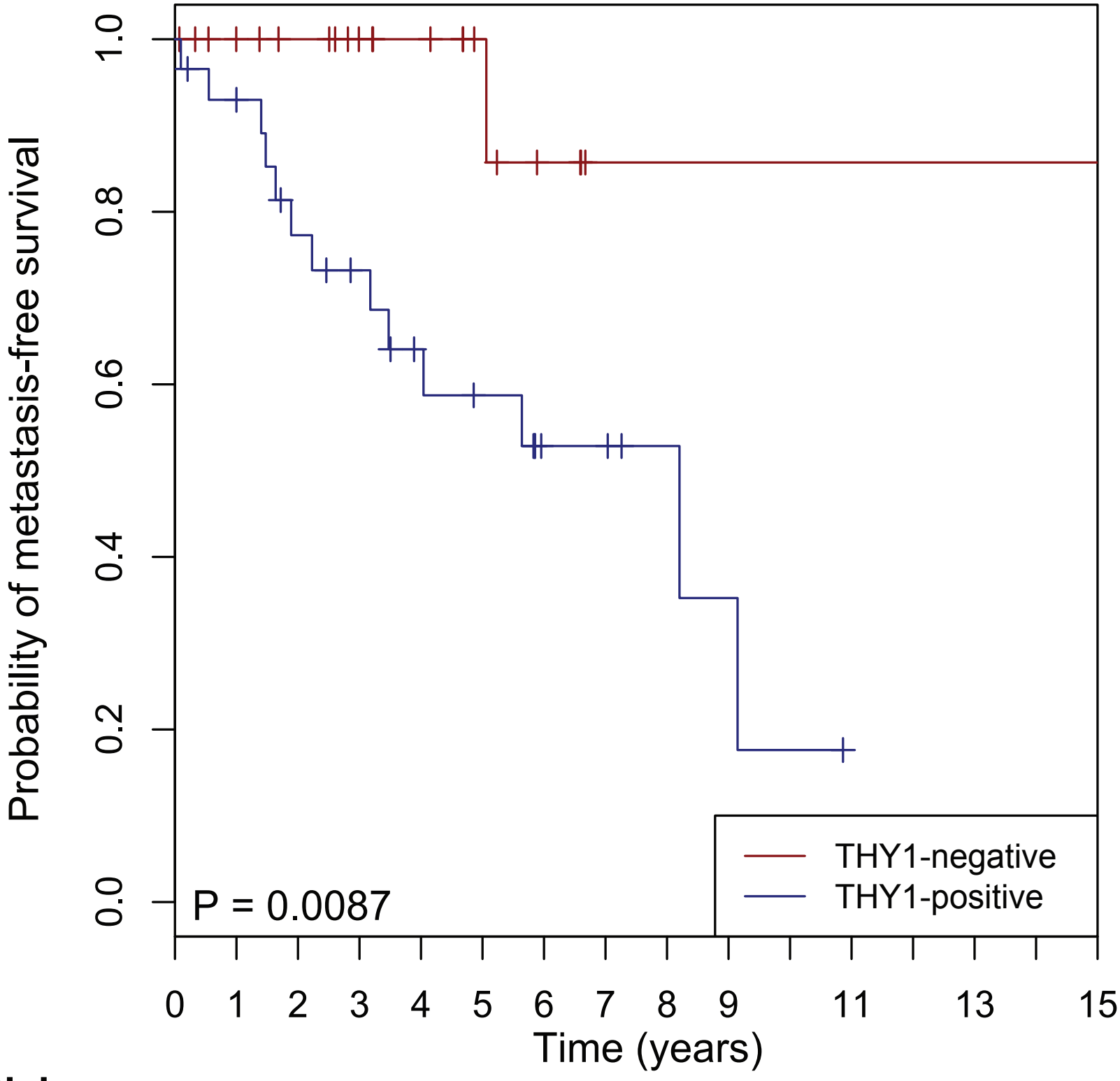
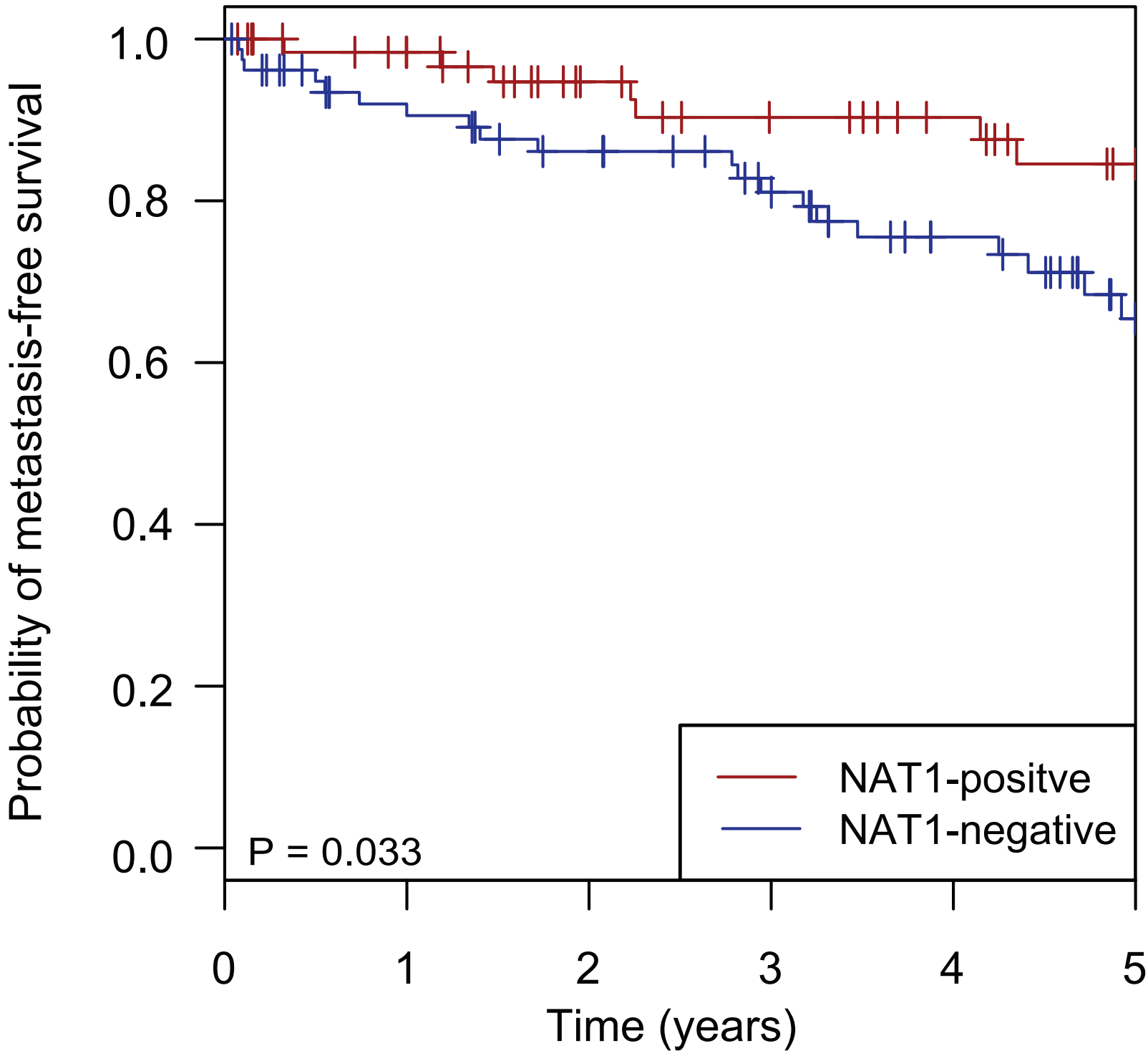
P=0.32
PLAU
P=1.0E-3
ESR1
P=4.3E-4
STAT1
P=6.8E-3



No. at Risk

THY1-negative	24	20	18	14	12	8	5	2	2	2	1	1	1	1	1	1
THY1-positive	29	25	20	17	13	11	6	6	4	3	2	1	0	0	0	0



No. at Risk

NAT1-positive	66	56	44	38	33	25
NAT1-negative	78	63	56	47	35	20

Tables

Table 1.

Survival comparison of male and female breast cancer

Study (reference #)	Country/ region	Number of MBC patients	Number of FBC patients	Years of diagnosis	Matched on	Survival comparison[a]
Nilsson, 2011	Uppsala, Sweden	99	369	1993-2007	Age and date of diagnosis	Inferior relative OS for men
Chen, 2013	Tianjin, China	150	300	1980-2012	Age, date of diagnosis and stage	Inferior DFS and OS for men
Shaaban, 2011	Europe, Canada	251	263	Before 2006	Age, grade and lymph node status	Similar OS
Giordano, 2004	Surveillance, Epidemiology, and End Results (SEER) database, USA	2,524	380,856	1973-1998	Age and stage	Similar relative OS
Greif, 2012	National Cancer Data	13,457	1,439,866	1998-2007		Inferior OS for men

	Base, USA					
Scott-Connor, 1999	National Cancer Data Base, USA	4,755	624,174	1985-1994	Age, demographics, stage and hospital.	Similar relative OS, however there was a trend toward inferior survival in men with stage III/IV disease
Miao, 2011	Sweden, Denmark, Finland, Norway, Geneva and Singapore,	2,665	459,846	1970-2007	Region, age, year of diagnosis, follow-up time, stage of disease and treatment (then only included ~800 MBCs)	Slightly better relative survival for men. Inferior relative survival and BCSS for men when patients were not matched
Gnerlich, 2011	SEER database, USA	1,541	244,518	1988-2003	Controlling for confounders	Inferior BCSS for men with stage I disease and similar for stages II-IV

[a]OS, overall survival; DFS, disease free survival; BCSS, breast cancer specific survival

Table 2.

Classification of male breast cancer into IHC based subgroups

Study (Number of patients)	Luminal A N (%)	Luminal B N (%)	Triple-negative (basal-like) N (%)	HER2-enriched (%)	N
Male breast cancer					
Ge, 2009[a] (42)	35 (83%)	7 (17%)	0 (0%)	0 (0%)	
Shaaban, 2011[a] (203)	199 (98%)	0 (0%)	4 (2%)	0 (0%)	
Nilsson, 2013[a] (183)	160 (87%)	21 (11%)	2 (1%)	0 (0%)	
Yu, 2013[a] (68)	41 (60%)	17 (25%)	4 (6%)	6 (9%)	
Kornegoor, 2011[b] (129)	98 (76%)	27 (21%)	4 (3%)	0 (0%)	
Female breast cancer					
Blows 2010[a] (10,159)	7,243 (71%)	639 (6%)	1,645 (16%)	632 (6%)	

[a]Definition I; [b]Definition II

Table 3.

Array based profiling studies of male breast cancer

Study (Number of MBCs)	Type of data	Platform	FBC samples	Platform FBC
Johansson, 2011 (56)	aCGH	BAC arrays	359	BAC arrays
Tommasi, 2010 (25)	aCGH	Agilent Human Genome CGH Microarray Kit 44B and 44 K	16	Agilent Human Genome CGH Microarray 44B
Johansson, 2012 (66)	mRNA	Illumina HT12 v3	359	Spotted oligonucleotide array from Swegene
Callari, 2010 (37)	mRNA	Custom made cDNA microarray	53	Custom made cDNA microarray
Fassan, 2009 (23)	microRNA	custom miRNA microarray chip containing ~1,100 probes	10	custom miRNA microarray chip containing ~1,100 probes
Lehmann, 2010	microRNA	319 mi RNAs		Various published