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## **Gene expression analysis reveals a different transcriptomic landscape in female and male breast cancer**

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## **ABSTRACT**

**Purpose** - Male breast cancer (MBC) is a poorly characterized disease because of its rarity. Clinical management is based on results obtained from randomized trials conducted in women notwithstanding data in the literature suggesting relevant gender associated differences in terms of biological and clinical behavior. However, a genome wide characterization of MBC on a transcriptional level is lacking.

**Methods** - In this study, gene expression profiles of 37 estrogen receptor positive (ER+) MBC specimens were compared to that of 53 ER+ Female Breast Cancer (FBC) samples similar for clinical and patho-biological features.

**Results** - Almost 1000 genes were found differentially expressed (FDR<1%) between female and male patients and biological interpretation highlighted a gender associated modulation of key biological processes ranging from energy metabolism to regulation of translation and matrix remodeling as well as immune system recruitment. Moreover, an analysis of genes correlated to steroid receptors and ERBB2 suggested a prominent role for the androgen receptor in MBC with a minor relevance for progesterone receptor and ERBB2, although, similarly to FBC, a genomic amplification could be observed.

**Conclusions** - Our findings support the idea that breast cancer is a quite different disease in male and female patients and the underlying gender related biological differences are likely to have clinical implications connected with different susceptibility to treatment.

## INTRODUCTION

Male breast cancer (MBC) is a rare disease accounting for less than 1% of all cases of breast cancer diagnosed yearly, but its incidence rate continues to increase by 1.1% annually. Research on male breast cancer has been limited by its rarity and most information on this disease has been extrapolated from results in female patients.

The etiology of male breast cancer is unclear but, similarly to women, steroid hormones levels may play a role in the development of the disease while a family history confers a relative risk of 2.5. As in women, mutations in BRCA1 and BRCA2 genes increase the risk of developing breast cancer; BRCA1 mutations are relatively rare (occurring in up to 4% of the cases), while those in BRCA2 gene are more frequent accounting for 4-16% of men with breast cancer. The major genetic factors associated with an increased risk of breast cancer in men also include mutations within the DNA-binding domain of androgen receptor (AR), polymorphism in the CYP17 gene coding for an enzyme involved in steroid synthesis, mutation of CHEK2 and PTEN tumor suppressor genes; however none of these genes has been demonstrated to have a causal association with male breast cancer [1-2].

The worse prognosis observed for male compared to female breast cancer (FBC) is mainly due to the delay in diagnosis and to a more advanced stage at presentation, since after adjustment for age at diagnosis and stage of disease, the gender-associated differences disappear [3]. The standard management of MBC derives from clinical data obtained in randomized trials conducted in women, and includes radical mastectomy plus axillary lymph node dissection, recently substituted by less invasive surgical procedures with no detectable decline in survival [2]. As for FBC, adjuvant chemotherapy is used to treat male patients who are at high risk of recurrence while the antiestrogen tamoxifen is considered the standard adjuvant hormonal therapy in male patients with steroid receptor-positive tumors [4].

Up to date, the characterization of MBC at molecular level has mainly focused on immunohistochemical analysis of a limited set of well know FBC biomarkers. As summarized in [1], a higher positivity rate of estrogen and progesterone receptors

(ER and PgR) in MBC respect to FBC is well established. However, Munoz de Toro et al [5] remarkably found that in MBC proliferation rate was higher in ER+/PgR+ compared to ER-/PgR- tumors, an opposite finding respect to FBC which strongly support the hypothesis that these receptors may play a different role in males compared to women. Controversial data were reported for AR positivity as well as for ERBB2 amplification rate, with positivity rates ranging from 0 to 95% among different studies [1-2, 6-7]. Furthermore, an immunohistochemical analysis of ER, AR and functionally related proteins indicates a reduced functionality of ER but a significant role for AR in MBC [8]. Finally, a higher percentage of BCL2 positive tumors in male was also reported [8], suggesting a role for this antiapoptotic gene during carcinogenesis.

Globally, these data indicate many differences between the biology of MBC and FBC, an observation which might imply the need of distinct therapeutic approaches and management of the disease according to the gender.

During the last decade, the application of a number of high-throughput genomic technologies refined the taxonomy of breast cancer and identified genomic fingerprints likely to impact future clinical treatment approaches [9]. However, while FBC has been intensively investigated by comprehensive molecular analysis, at present no gene expression data are available for male breast cancer. Recently, Fassan et al reported a set of microRNA as differentially expressed by comparison of 23 male and 10 female breast cancer samples [10].

Taking advantage of the availability in our Tumor Tissue Bank of a series of ER+ primary breast cancer specimens from male patients, we compared their gene expression profiles with the profile of breast cancer from female patients with similar clinical and patho-biological features, to investigate whether a gender-related biological difference does exist at transcriptomic level in breast cancer.

Our data suggest a profound biomolecular difference between male and female breast cancer and highlight a set of differently regulated biological processes, ranging from energy metabolism to regulation of translation and matrix remodeling as well as

immune system recruitment. Furthermore, correlation analysis supports a prominent role for AR in MBC with a minor relevance for PgR and ERBB2, although, similarly to FBC, a genomic amplification may be observed for the latter also in males.

## **MATERIALS AND METHODS**

### ***Patients and tumors features***

We consecutively collected 37 snap-frozen ER+ primary tumor specimens from men submitted to radical mastectomy or conservative quadrantectomy plus axillary lymph nodes dissection for a primary breast cancer at the Istituto Nazionale dei Tumori of Milan during the period 1991-1997. All patients signed an informed consent to donate to the Istituto Nazionale dei Tumori the leftover tissue after completing diagnostic procedures. Fifty-three FBC with ER+ primaries for whom gene expression profiles were available were selected to obtain a distribution of the main clinical and patho-biological variables (age, tumor size, PgR status) comparable to that of MBC samples, limiting in this way confounding effects on comparison of gene expression data between the two genders. Clinical and patho-biological data are summarized in Table 1.

ER and PgR content was routinely evaluated at time of diagnosis according to the EORTC recommendations and within national and international quality control programs by a ligand binding assay as previously described [11] and results were expressed as fmol mg<sup>-1</sup> of protein. Tumors with an ER concentration higher than 10 fmol mg<sup>-1</sup> of protein or with a PgR concentration higher than 25 fmol mg<sup>-1</sup> of protein were defined as ER-positive or PgR-positive, respectively.

### ***RNA isolation and microarray hybridization***

Total RNA extraction, probe labeling and hybridization were performed as previously described [12]. Briefly, total RNA was extracted using Trizol reagent (Life Technologies, Inc. Grand Island, NY) following the manufacturer's instructions and treated with DNaseI (Qiagen, Valencia, CA). Each sample was both directly and indirectly labelled with Cy-dCTPs (Amersham Biosciences; GE Healthcare, Piscataway, NJ) and with 3DNA Array900 detection kit (Genisphere, Montvale, NJ), respectively. Samples were co-hybridized together with a common RNA reference (Universal Human Reference RNA, Stratagene, La Jolla, CA) on custom made cDNA

microarray slides containing 16457 sequence-verified I.M.A.G.E. clones (Research Genetics/Invitrogen) spotted in triplicate. cDNA microarrays were scanned using the GenePix 4000B microarray scanner at a resolution of 5 $\mu$ m and the images were analyzed using GenePix Pro v.5.0 software (Axon Instruments, Union City, CA).

### ***Data analysis and statistics***

*Array data* - Microarray raw data were processed using *marray* package (version 1.22.0). After a quality control, a print-tip loess normalization was applied to each array, followed by an inter-array scale normalization. Intensities from flagged spots were removed, probes with more than 10% of missing values were filtered out and replicate probes were averaged. After this filtering, 13285 distinct clones remained corresponding to 7792 distinct genes according to annotation using the Stanford SOURCE Search website. The entire dataset is available at Array Express database (ID: E-TABM-810).

All class comparisons on gene expression data were performed applying the Significance Analysis of Microarrays (SAM) [13] to data at clone level, considering as threshold an FDR<1% unless otherwise specified.

To aid the biological interpretation, Gene Ontology Analysis using the *TopGO* package (version 1.12.0) [14] using the Fisher's exact test statistics was carried out.

*Correlation analysis* - Gene expression values of single clones of interest were correlated (Spearman correlation) to gene expression values of all the other clones, separately for MBC and FBC. Only probes with a  $p < 0.01$  were considered significantly correlated. After array data filtering, no clones were found referring to the PgR gene, consequently in subsequent analysis PgR expression data were evaluated by qPCR.

*Clustering analysis* - Hierarchical clustering was performed using Euclidean distance and a complete linkage method. A Fisher's exact test was used to evaluate difference in PgR status distribution in the two clusters identified by considering the first split in the hierarchical three.

All analysis were performed employing the R software (version 2.9.0).



### ***Quantitative RT-PCR***

The RNA expression of PgR was evaluated by quantitative RT-PCR on the same stored frozen tissue used for microarray analysis. Total RNA was reverse-transcribed using the High-Capacity cDNA Archive Kit and a TaqMan reaction was carried out in duplicate on an ABI PRISM 7700 machine, using Assays-on-Demand Gene Expression Product (Hs00172183\_m1, Applied Biosystems, Foster City, CA). To normalize data, 18S ribosomal subunit was used as housekeeping (4319413E, Applied Biosystems, Foster City, CA, USA). Data analysis was performed using the Sequence Detector v1.9 software.

## RESULTS AND DISCUSSION

### ***Biological interpretation of differentially expressed genes***

From the class comparison between male and female breast cancer, we obtained 1273 clones differentially expressed at an FDR<1%, corresponding to 920 distinct annotated genes.

Not surprisingly, the most significant gene was XIST, expressed exclusively from the X inactivation center of the inactive X chromosome.

Differentially expressed genes were subjected to Gene Ontology analysis using TopGO. Sixty-one terms of the Biological Process ontology, 20 of the Molecular Function and 16 of the Cellular Component ontology were found enriched at a significance level of  $p<0.01$  (Supplementary files 1-3).

Using the TopGo results as starting point, we identified a set of biological categories each involving a large number of differentially expressed genes, which were used to attempt an unbiased biological interpretation of the class comparison results. We report below a detailed description of the identified biological functions and of their role in cancer disease, highlighting key genes differentially expressed for each function. An overview of biological differences between genders is reported in Table2. Instead, the complete lists of genes referred to a specific category are available as supplementary file (Supplementary file 4). Genes mapping on the Y chromosome have not been considered.

### **Energy metabolism**

A set of genes (e.g. CYCS, COX6C, NDUFs) coding for enzymes involved in different steps of mitochondrial oxidative phosphorylation (OXPHOS) were found down-regulated in FBC, suggesting a reduced OXPHOS compared to MBC. The observation of an impaired respiration and increased glycolysis in tumor cells dates to many decades ago [15] and is known as the Warburg's hypothesis. An explanation on why tumor cells switch to a less efficient metabolism has been proposed [16], suggesting that malignant cells have important metabolic requirements that extend beyond ATP. The Warburg's hypothesis was recently verified in breast cancer cells

related to normal mammary cells [17], but interestingly our data suggest that this effect is at least reduced in MBC compared to FBC.

Strictly related to OXPHOS is ATP metabolism, a biological function that we also found modulated according to gender. In particular we noticed in MBC an increased expression of subunits of the mitochondrial ATP synthase complex and of the *ATPIF1* gene, an inhibitor of the ATPase function of such a complex. Coherently with an increased availability of ATP, some ATPases were overexpressed, among them the vacuolar ATPase complex, which has important physiologic roles, but was also reported to be associated to tumor invasion as it is able to create a pH gradient across the membranes, mediating extracellular matrix acidification [18].

Furthermore, OXPHOS is the major source for ROS production, that is therefore expected to be higher in MBC. It was not surprising to find 4 out of 6 human peroxiredoxins up-regulated in male tumors (*PRDX1-4*). Peroxiredoxins are antioxidant enzymes which reduce hydrogen peroxide and can indirectly affect cell proliferation: their role in cancer has been in fact extensively studied [19]. Similarly, the metabolism of glutathione, a potent reducing agent, seems to be affected. The GSH:GSSG ratio is critical for cell survival and influences S-glutathionylation, a chemical modification catalyzed by glutaredoxins, important for regulation of key cellular processes like the proteasomal protein degradation [20]. A rate limiting enzyme of glutathione synthesis (*GCLC*), a glutaredoxin (*GLRX3*), two glutathione peroxidase (*GPX1*, *GPX2*) and finally the glutathione reductase enzyme (*GSR*) were among differentially expressed genes in our data.

## **Translation**

Protein synthesis is a fundamental process of eukaryotic cells and translation initiation represents a main regulatory step. Key molecules (e.g. EIF4 family members and poly(A) binding proteins) mediating this step were mostly found up-regulated in MBC. Particularly intriguing is EIF4E overexpression in MBC. This protein is frequently up-regulated in cancer and selectively enhances translation of key genes involved in tumorigenesis and cancer progression like *MYC* and *BCL2*

[21]. Consistently, these two genes are up-regulated in our data. EIF4E is a downstream effector of PI3K/AKT/mTOR mediated signals. Among signals acting on PI3K/AKT/mTOR pathway, there are many growth factors, but also the cell energy status, as a decreased AMP/ATP ratio promotes mTOR activity; mTOR itself, also overexpressed in male tumors, mediate phosphorylation of 4EBP, leading to EIF4E activation [22] (Fig. 1).

Another result suggesting an increased protein synthesis in MBC is the upregulation of many ribosomal proteins of both the 40S (RPS8, RPS12, RPS16, RPS21) and 60S subunits (RPL18, RPL30, RPL35) and associated proteins.

Globally, an increased protein synthesis has been related to cell growth and proliferation, linking this process with malignancy [23]. As corollary, a group of DEAD-Box proteins (DDX3X, DDX5, DDX11, DDX12, DDX49, DDX55), involved in many aspects of RNA metabolism are also differentially expressed.

In eukaryotic cells, translation takes place also in mitochondria where 13 polypeptides that constitute the central core of the OXPHOS complex are translated [24]. Up-regulation of an elongation factor (GFM1) and 5 mitochondrial ribosomal proteins (MRPL14, MRPL23, MRPL30, MRPL34, MRPL42), indicates an enhanced translation of these mitochondrial genes in male breast cancer, in agreement with what stated above.

### **Cytoskeleton and GTPase**

Reorganization of the cytoskeleton is the primary mechanism of cell motility, is essential for most types of cell migration and is regulated by Rho family small GTPase. The tubulin/microtubule system is an important target for anticancer therapy with vinca alkaloids and taxanes, but also with newly developed drugs (e.g. epothilones [25] or eribulin [26]). Therefore, the observed gender divergence at level of cytoskeleton organization is particularly interesting. In fact, genes of both alpha (TUBA1A, TUBA3D, TUBA4A) and beta (TUBB, TUBB2C) tubulin families were up-regulated in MBC while two microtubule-associated proteins (MAP1B, MAP2), able to regulate the assembly-disassembly ratio, were down-regulated.

Actin reorganization is the underlying mechanism of cell migration both in physiological and malignant conditions. In MBC the actin polymerization process was altered by modulation of the ARP2/3 complex components and overexpression of the capping protein CAPZA1. This process is mainly regulated by Rho family small GTPase [27-28]. RND2, a member of this family is more expressed in FBC. Two Rho GTPase activating proteins (SRGAP2, ARHGAP23) and a guanine nucleotide exchange factor, key regulators of Rho activity, are differentially expressed. Additionally, overexpression of TNK2 occurs in MBC. This gene is a tyrosine kinase that binds Cdc42 (a Rho family GTPase) and has been causally related to migration of cancer cells by enhancing cell surface localization of EGFR [29].

Rho GTPase are only one of 5 protein families composing the RAS GTPase superfamily: Rho, Ras, Rab, ARF, RAN family. Members belonging to all of these families showed a different expression pattern in male and female breast cancers. In detail, we observed an increase in MBC of ARF (ARF6, ARL1, ARL8B) and RAB (RAB1A, RAB6A, RAB14, RAB22A, RAB31) GTPase as well as regulator molecules (respectively ARFGEF1 and GDI2, RAB11FIP1). These GTPase are main actors in intracellular trafficking and their role in cancer is well documented as they are implicated in modulating growth factor receptor localization and supporting invadopodia and filopodia formation [30]. Specifically, ARF6, overexpressed in MBC, was shown to mediate MDA-MB-231 breast cancer cells invasive activities [31].

RAN and the regulatory RANBP1, also up-regulated in MBC, are instead involved in a wide range of cellular processes ranging from DNA synthesis and microtubules organization at mitosis to DNA and protein translocation through the nuclear pore [32]. Interestingly RAN is also an important androgen receptor coactivator as later discussed.

Finally also RAS family GTPase (RAP2C, RASL10A) and specific regulators of their activity were found transcriptionally altered comparing the two genders.

## Tumor environment

Many recent studies are pointing out the role of the microenvironment during breast cancer development, growth and distant spreading. As a matter of fact, a prognostic role has been reported for some matrix metalloproteinases [33] and other ECM genes [34] as well as for stromal derived signatures [35].

Extracellular matrix is mainly constituted by two major components: the basement membrane and the interstitial matrix. Different types of collagen form a scaffold for both these components, laminins are the most abundant proteins of the basal membrane while among the proteins constituting the interstitial matrix it is worth to cite tenascin, proteoglycans and other glycoproteins. All these molecules strictly interact with a plethora of adhesion molecules expressed on cell surface, not only with a structural function but also to mediate signals regulating primary cell processes. Notably, genes belonging to all of cited extracellular matrix protein families and adhesion molecules as well were found as significantly both up- and down-regulated in our dataset. More in detail, various structural proteins like COL5A3, LAMC1, TNC, ECM2, FBN2, SPON1, THBS1 and the proteoglycans BGN, GPC3 as well as the UGDH, an enzyme which participate in glycosaminoglycan biosynthesis (e.g. hyaluronan) were up-regulated in MBC, although some were more expressed in FBC (FN1, MTN2, MGP, TMBS3).

Furthermore, extracellular matrix is not a static structure and is instead constantly remodeled by well regulated matrix protease [36]. MMPs (matrix metalloproteinases), ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), TIMPs (tissue inhibitors of metalloproteinases) along with the SERPIN superfamily of serine protease inhibitors constitute an important proteolytic axis. Many genes from the above families whose dysregulation in breast cancer is well documented, were either up-regulated in MBC (MMP11, TIMP3, SERPINA5, SERPINA6, SERPINAB2, SPINT2) or in FBC (ADAMTS3, ADAMTS7, ADAMTSL4, MMP7).

Therefore, while it clearly appears that there are differences in the complex regulation of extracellular matrix remodeling, it is difficult to trace a gender related

model using only gene expression data, although it can be suggested that the gender differences at the level of the extracellular matrix may have a relevant impact on cell survival, growth and migration due to the central role of tissue remodeling in tumor biology.

The tumor microenvironment is also strongly affected by effectors of immune response. The myeloid and lymphoid tumor infiltrating cells has been reported for breast cancer with a frequency around 40% of tumors [37]. Moreover, chronic inflammation has recently been linked with an increased risk of distant metastases [38], while other studies suggest a protective role exerted by immune cells [39-40]

Surprisingly, we found among differentially expressed genes many immune cells specific genes globally indicating a clear reduction of immune response in MBC. For instance, a numerous set of chemokines essentially produced during immune response (CCL25, CXCL1, CXCL9, CXCL10, IL23A, IL17B, IL32) were more expressed in female tumors. A similar pattern was observed for two members of the MHC II complex expressed by APC cells (HLA-DQB1, HLA-DRB1), a lymphocyte-specific protein tyrosine kinase (LCK), a member of the T-cell receptor complex (CD3D), a natural killer specific receptor (KLRK1) and a chitotriosidase secreted by activated macrophages (CHIT1). An exception to this is represented by the two B-cell specific genes found in our list (BCAP31, IGHG1) that are more expressed in male samples.

### **Membrane transport**

Genes involved in membrane transport displayed differences between females and males. Two ATP-binding cassette transporters (ABCC2 and ABCC5) which have been linked to chemo-resistance in human cancer [41-42] are more expressed in FBC. On the contrary, 21 members of the solute carrier family are either up- or down-regulated. This is however a highly heterogeneous family, including active transporters as well as proteins which facilitate transport, ion channels and aquaporins. Here a detailed analysis of single transporters would be useless, but it is worth to remember that they can regulate drug uptake as well as many cancer related

processes [43]. For example changed expression of two glucose transporters (SLC2A1 and SLC2A3) could impact on glucose metabolism, consistently with what described about metabolic differences.

### **Growth factors response and apoptosis**

Failure of inhibition of proliferation and overcome of apoptosis signals are crucial for cancer cell growth and survival. These two crucial aspects of breast cancer biology also appeared to be different according to the gender indirectly suggesting that male and female breast tumors adopt different survival strategies. For example, concerning growth signaling, ERBB2 and NRG1, a glycoprotein that enhances ERBB2 phosphorylation [44], GRB2, an EGFR interacting protein and MET, a growth factor receptor with a well established role in breast cancer [45] are downregulated in MBC. On the opposite, FGF receptor 2 is more expressed in MBC.

Focusing on apoptosis related genes, we found differentially expressed genes belonging to several apoptotic pathways: BCL2 with its interacting proteins BAG4 and BNIP3L, Fas-related proteins (FAIM3, FASTK), TNF mediated apoptosis genes (TNFRSF21, TNFRSF25, TNFSF13, TRADD). BCL2 was overexpressed in MBC, according to data reported elsewhere by IHC [8], supporting the role of this antiapoptotic gene in carcinogenesis.

### **Response to steroid stimulus**

As expected, genes related to response to steroid hormones were quite different in MBC and FBC. Some genes coding for proteins functionally related to the androgen receptor as RAN, a GTPase able to enhance AR transactivation [46], RNF14, a coactivator interacting with AR signaling in prostate cancer [47] and PAK6, an AR and ER signaling inhibitor [48-49] were overexpressed in MBC.

### **Correlation and clustering analysis**

To further highlight similarities and differences between breast cancer in the two genders, we performed a correlation analysis as described. Given the well established role of steroid hormones in breast cancer, we separately searched in male and female



breast tumors profiles for genes significantly correlated with AR expression values obtained from array data and PgR expression value obtained by qPCR. The same type of analysis was carried out for the ERBB2 gene, usually amplified in about 20% of FBC with a strong impact on prognosis and therapeutic strategy.

From the number of correlated genes and the overlap between genes correlated in the two genders, we could indirectly extrapolate some hints on how relevant and how similar the biological functions of these three genes were in the two genders. As shown in Table 3, huge differences were found in the numbers of receptor-correlated genes and few of them were common in the two genders.

2288 clones were significantly correlated with ERBB2 in the female samples dataset and only 243 were so in the male dataset with 45 common genes. This strongly suggests a reduced relevance of ERBB2 in MBC biology compared to FBC. Moreover, 12 out of the 45 common clones map near the ERBB2 locus (Table S1), with increasing correlation values for genes closer to ERBB2, in both genders (Fig. 2). Hence, similarly to what happen in females, also in male breast tumors ERBB2 overexpression is the consequence of a genomic amplification of its locus, according to literature results of FISH analysis in MBC samples [50-51].

An inverse behavior was observed for AR correlated genes, where only 86 clones were correlated to AR in FBC, against 441 clones in MBC. Interestingly, two genes up-regulated in MBC, BCL2 and GATA3, which are known to be associated to ER in female breast cancer, were slightly inversely correlated with AR in FBC ( $S=-0.25$ ,  $p=0.1486$  and  $S=-0.22$ ,  $p=0.1374$  respectively), but significantly correlated to AR expression in MBC ( $S=0.43$ ,  $p=0.0194$  and  $S=0.41$ ,  $p=0.0347$ ).

More PgR correlated genes were found in FBC (582) than in MBC (159) with a limited overlap (26 genes). Thus the PgR gene, more expressed in FBC ( $p=0.0006$ ), also seems to play a more important role in these tumors rather than in MBC. This hypothesis was further confirmed by a clustering analysis exploiting independent public data by Chanrion et al [52]. From this dataset 145 ER+ FBC samples with clinico-pathological features comparable to our FBC samples were isolated. About

4000 clones (common to our data) were searched for genes differentially expressed between PgR+ and PgR- tumors. Fourteen clones were called as differentially expressed at FDR=0. As expected, hierarchical clustering of Chanrion's patients using this gene set revealed two main clusters significantly associated to PgR status ( $p=0.00001$ , Fig. 3A). The same clustering in our FBC samples also identified two main clusters significantly associated to PgR status ( $p=0.002$ , Fig. 3B). Whereas, clustering of MBC samples identified two clusters not at all associated to PgR status ( $p=0.413$ , Fig. 3C). This finding further supports the concept that PgR, similarly to ER, AR and ERBB2, may play a different role in MBC and FBC. The inverse approach searching for differentially expressed genes between PgR+ and PgR- in MBC was also performed. Hierarchical clustering with the 74 clones differentially expressed (FDR<5%) identified a sub-cluster containing 7 out of 8 PgR- tumors, while a quite random distribution of PgR- samples was observed both in our FBC dataset and in the Chanrion FBC dataset (Supplementary Fig. S1).

## **Conclusion**

Male breast cancer is a rare disease poorly characterized and treated similarly to female counterpart. A few studies, reported a different expression in male samples of validated breast cancer biomarkers, but a comprehensive molecular characterization of these tumors is missing. At the best of our knowledge, our study is the first reporting gene expression data of male breast cancer specimens compared to samples with similar clinical and patho-biological features of the female counterpart. The aim of the study was to give a genome wide description of these two tumors focusing on main biological relevant discrepancies in order to generate useful hypothesis to better understand MBC biology and to provide a guide for treatment strategies.

Class comparison uncovered profound differences between MBC and FBC transcriptomes, with around 1000 genes called as differentially expressed despite a stringent FDR. Biological interpretation of our results clearly pointed out the relevance of such a difference, as almost all processes we found to be differentially modulated, have a clear and well known role in tumorigenesis and cancer

progression. Furthermore in some cases, the observed differences might have strong implications on treatment choice and outcome. For example, inhibition of translation targeting either EIF4E or mTOR has been proposed as a promising new therapeutic strategy [21-22] and an increased susceptibility to such treatment in MBC could be hypothesized based on our data. Our molecular data also suggest that drugs targeting tubulin/microtubules system like the vinca alkaloids and taxanes might also be more effective in MBC due to their overexpression of various tubulin subunits compared to FBC. It might also be hypothesized that MBC could be a preferred target for the newly developed epothilones [25].

An interesting difference appeared for ERBB2. In female patients amplification of ERBB2 characterizes a subgroup of tumors with a peculiar behavior in terms of aggressiveness and which is treated in different way from tumors without this amplification [53] The strong biological role played by ERBB2 in FBC is reflected by the high number of correlated genes. In MBC a tenfold lower number of genes was found to be correlated with ERBB2, suggesting a minor role for this gene in males and raising doubts about usefulness of treatment strategies targeting ERBB2 in males.

According to our data, the AR could instead be one of the driving genes in MBC biology as also suggested in previous studies [8]. Thus, a shift towards hormonal therapy targeting AR rather than ER could be envisioned.

The metabolic features of MBC, where the anaerobic glycolysis pathway does not seem to be the preferential glucose metabolic pathway as for most tumors, is also worth to be further investigated. It has biological implication which may be linked with tumorigenesis, as strong activation of oxidative phosphorylation is responsible for production of ROS, but also represent a warning when using diagnostic procedures as positron emission tomography which rely on  $^{18}\text{F}$ -deoxyglucose uptake.

Research recommendations on MBC collected in a specific multidisciplinary meeting were recently published [54] and strongly underline that MBC is a unique

disease rather than an illness similar to postmenopausal FBC. As the majority of MBC are endocrine responsive the development of endocrine therapy options designed for MBC in specific clinical trials was considered as a priority. However, while waiting for results from large international multicenter studies to learn more on this disease, treatment cannot be simply derived from FBC, but should rely on single center studies like ours which highlight peculiar features of MBC. In this sense our results which suggest a different role of steroid receptors (and ERBB2) in males are a warning in the design of hormonal treatment in MBC.

In conclusion, our data highlighted the need to investigate the link between molecular characterization and outcome in MBC as done for FBC, in order to personalize the therapy and improve patient survival. These preliminary data may trace a path for future studies, however due to the rarity of MBC, studies with a multicenter setting with a standardized biospecimens handling are essential.

### **Competing interests**

The Authors declared that they have no competing interests

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## LEGENDS

**Fig.1** Regulation of translation initiation mediated by PI3K/AKT/mTOR pathway.

The translation initiation factor EIF4E specifically promotes translation of cancer associated genes. Its activity is regulated by the PI3K/AKT/mTOR pathway, in turn regulated by AMP/ATP ratio. Genes in red were found up-regulated in MBC compared to FBC, and our data suggest a lower AMP/ATP ratio in MBC.

**Fig.2** Correlation pattern of ERBB2 neighbor genes

Genes mapping near the ERBB2 locus on chromosome 17 are reported according to their correlation with ERBB2 in FBC (A) and MBC (B) samples. Genomic coordinates (Mb) are reported on x-axis. Correlation values increase for genes closer to ERBB2, in both MBC and FBC samples.

**Fig.3** Clustering analysis with PgR associated genes

Using a set of clones differentially expressed between PgR+ and PgR- FBC samples in the Chanrion dataset [52], a hierarchical clustering analysis was performed on the Chanrion dataset itself (A) as well as on our dataset, separately for FBC (B) and MBC (C) samples. Significant associations between the two main clusters (red and blue) and the distribution of PgR status were observed in FBC datasets but not in the MBC dataset.

**Fig.S1** Clustering analysis with PgR associated genes

Using a set of clones differentially expressed between PgR+ and PgR- MBC samples, a hierarchical clustering analysis was performed on the MBC dataset itself (A) as well as on our FBC dataset (B) and on the Chanrion FBC dataset (C) samples. A sub-cluster containing 7/8 PgR- tumors was noticed in the clustering of MBC samples, while a quite random distribution of PgR- tumors was observed in FBC datasets.



**Tab. 1 – Patients characteristics**

	<b>MBC</b>	<b>FBC</b>
<b>Age</b>		
Range (year)	32-87	45-81
Median (year)	66	66
≤50 year	4 (11%)	5 (9%)
>50 year	33 (89%)	48 (91%)
<b>Size</b>		
Range (cm)	1-5	1-7
Median (cm)	2	2.2
≤ 2 cm	12 (32%)	17 (32%)
>2 cm	25 (68%)	36 (68%)
<b>ER</b>		
pos	37 (100%)	53 (100%)
neg	0 (0%)	0 (0%)
<b>PgR</b>		
pos	29 (78%)	41 (77%)
neg	8 (22%)	12 (23%)
<b>Positive lymph nodes</b>		
>3	10 (27%)	15 (28%)
0-3	27 (73%)	38 (72%)

**Tab. 2 – Summary of differences in biological characteristics of male and female tumors**

<b>BIOLOGICAL CATEGORIES</b>	<b>MBC</b>	<b>FBC</b>	<b>Key genes</b>
OXPHOS and ATP metabolism	↑	↓	<b><u>Cytochrome C, ATP synthase</u></b>
Redox homeostasis	↑	↓	<b><u>Peroxiredoxins</u></b>
Translation	↑	↓	<b><u>Ribosomal proteins, Eukaryotic translation initiation factor Tubulin <math>\sigma/\beta</math>,</u></b>
Cytoskeleton and cell motility	↑ ↓	↑ ↓	Microtubule-associated proteins, <b><u>ARFs</u></b>
ECM composition and remodeling	↑ ↓	↑ ↓	<b><u>LAMC1, TNC, MMP11, MMP7, TIMP3, SERPINS,</u></b> FN1, ADAMTSs
Immune response	↓	↑	CCL25, CXCLs, CXCL10, IL23A, MHC II, LCK, CD3
Membrane transport			ABCC2, ABCC5
ABC transporters	↓	↑	
Solute carrier family	↑ ↓	↑ ↓	<b><u>SLC2A1, SLC2A3, SLC16A3</u></b>
Growth factors response and apoptosis	↑ ↓	↑ ↓	<b><u>FGFR2, ERBB2, MET</u></b>

Legend:

**GENE**: up-regulated in MBC

GENE: down-regulated in MBC

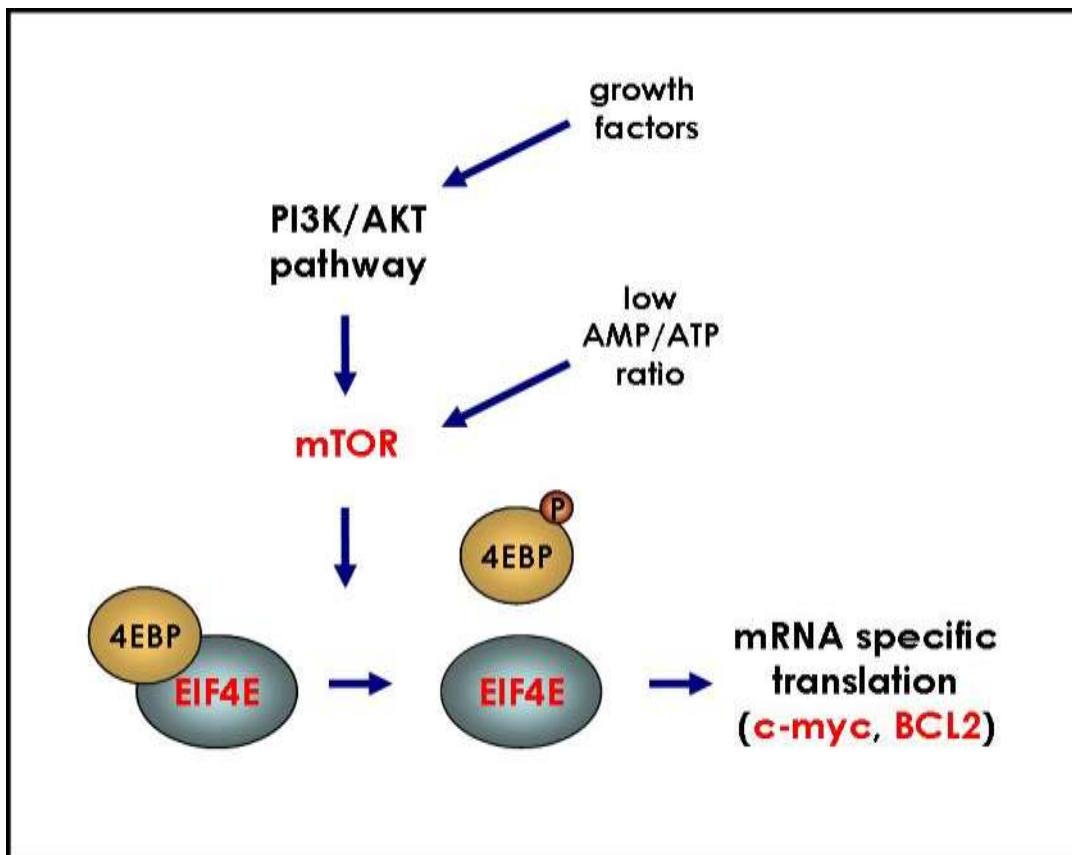
↑ = all DE genes are up-regulated

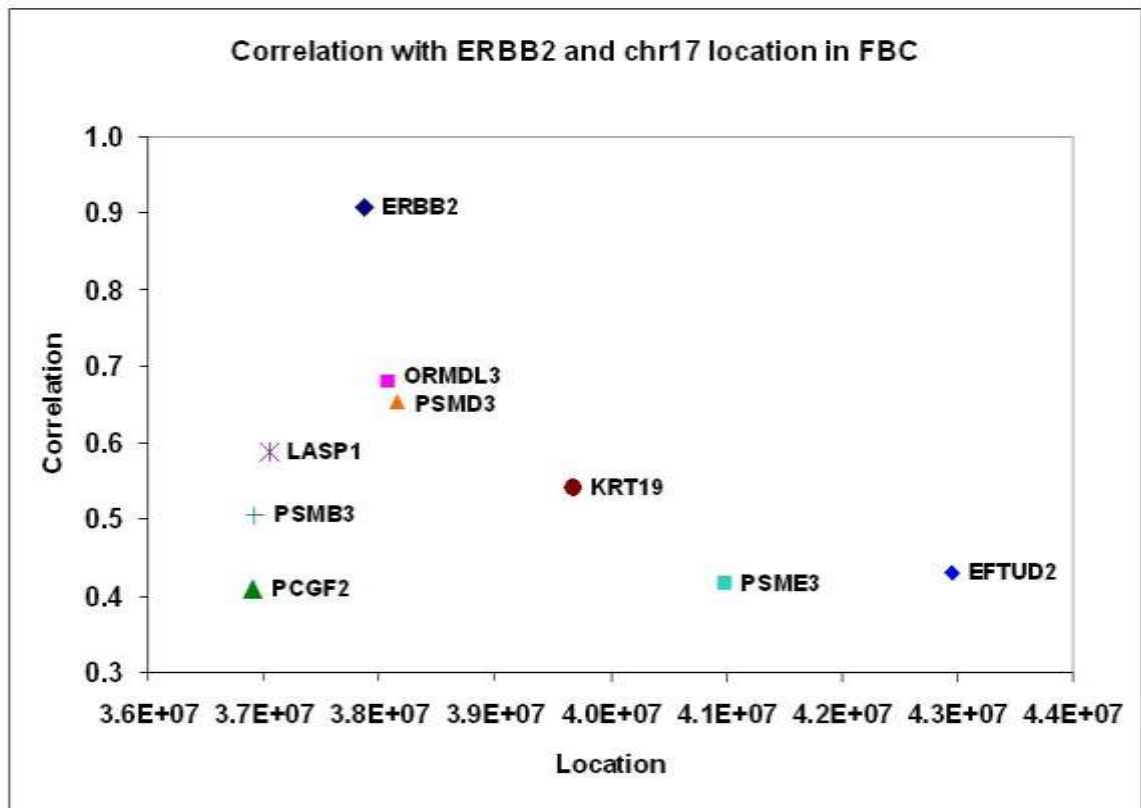
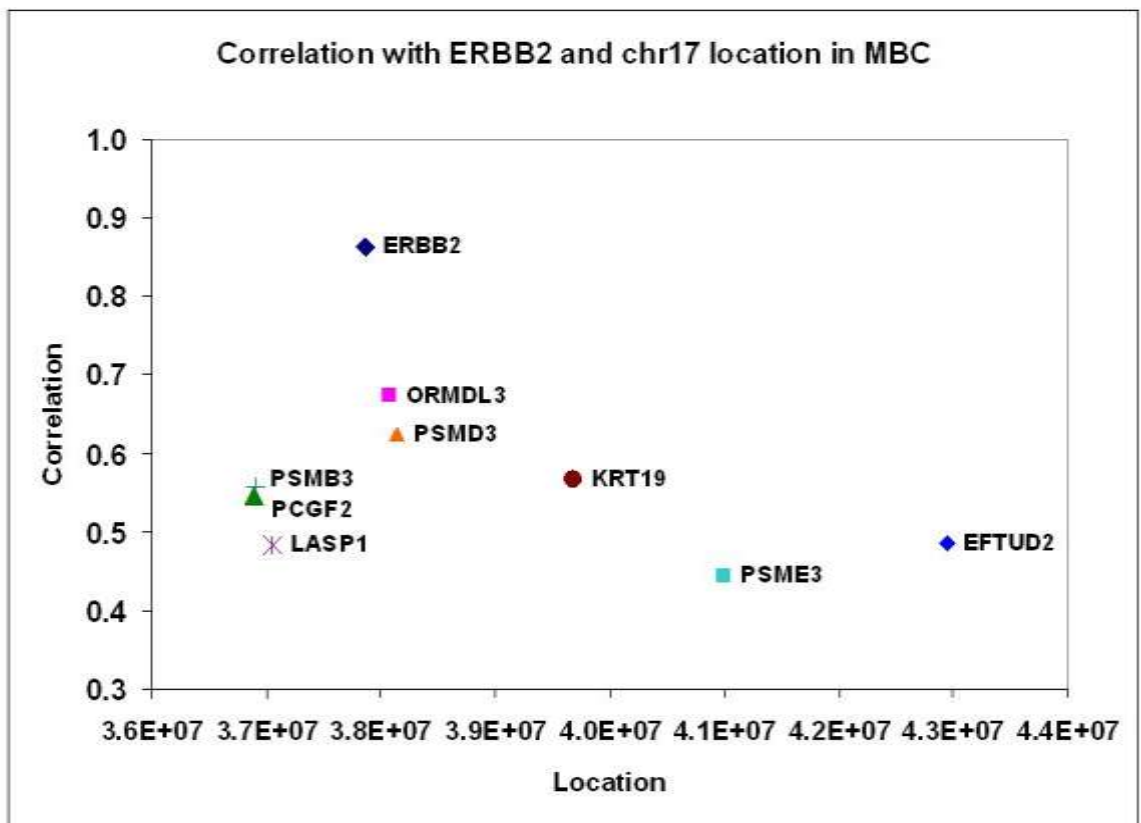
↓ = all DE genes are down-regulated

↑ ↓ = DE genes are both up- and down- regulated

**Tab. 3 – Number of clones significantly correlated with receptors in MBC and FBC**

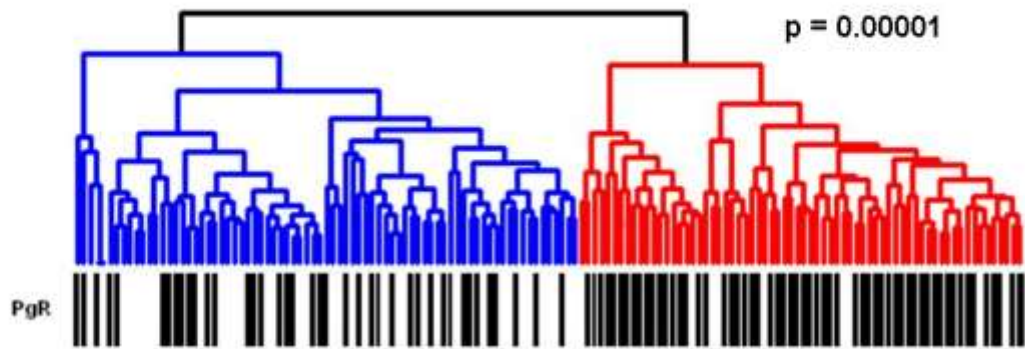
CORRELATED CLONES			
GENE	MBC	FBC	Common
ERBB2	243	2288	45
PGR	159	582	26
AR	441	86	7



**A****B**

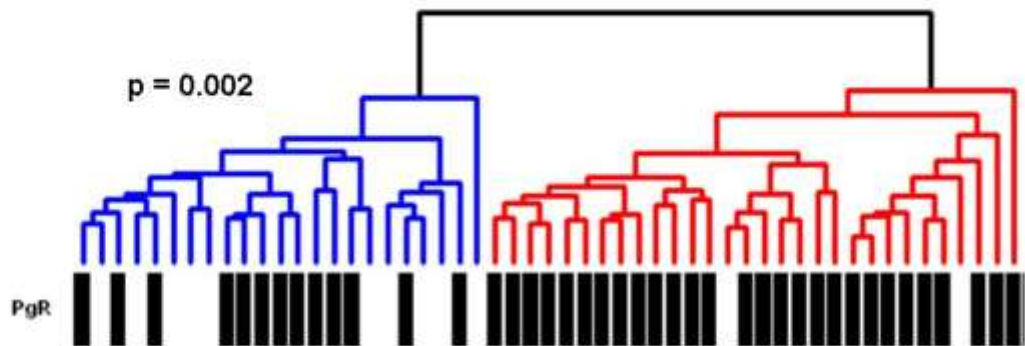
**A**

FBC (Chanrion et al)



**B**

FBC (our dataset)



**C**

MBC (our dataset)

