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Y-box binding protein 1 (YB-1) relevance in estrogen receptor-positive (ER+) breast cancer

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Dissertação

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RESUMO

O cancro da mama é uma doença heterogénea determinada por várias características clínicas e patológicas, incluindo parâmetros histológicos e marcadores moleculares como o recetor de estrogénio (RE), recetor de progesterona (RP) e o recetor 2 do fator de crescimento epidérmico humano (HER2). A classificação de cancro da mama em três principais grupos com interesse clínico reflete essencialmente a classificação molecular: subtipo Luminal, Luminal A (RE+ e/ou RP+, HER2-, Ki67 baixo) e Luminal B (RE+ e/ou RP+, HER2+ ou Ki67 elevado); subtipo com sobre-expressão de HER2 (RE-, PR-, HER2+); e subtipo triplo negativo (RE-, RP- HER2-).

Os tumores da mama positivos para o RE são os mais frequentes (cerca de 80%) e são os que apresentam melhor prognóstico. No entanto, cerca de 30% irão progredir com metastização à distância, resultando num aumento da taxa de mortalidade. O cancro da mama RE+ é dependente de estrogénios que ativam o RE, um factor de transcrição importante. Neste contexto, o gene *YBX1*, que codifica a proteína YB-1 (Y-box binding protein 1), foi recentemente identificado como parte de um preditor de mau prognóstico em cancro da mama dependente de RE.

A proteína YB-1 parece estar envolvida em todos os *hallmarks* do cancro, sendo um indicador de mau prognóstico, recidiva, invasão e resistência a fármacos. Em cancro da mama, a proteína YB-1 encontra-se normalmente sobreexpressa, e tem sido associada com a progressão da doença e com a ausência de RE e RP.

Devido à sua capacidade de ligação aos ácidos nucleicos, a proteína YB-1 é um regulador importante de transcrição e tradução de vários genes relacionados com o cancro, como *ERBB2*, *cyclin A/B1*, *E2F*, *MDR1*, *MYC*, *PIK3CA*, e também um componente principal das partículas de ribonucleoproteínas mensageiras, contribuindo assim para a sua estabilização e regulação de tradução. O papel funcional da proteína YB-1 parece ser dependente da sua localização celular, que é estreitamente regulada. Em condições fisiológicas normais a proteína é maioritariamente citoplasmática, embora possa também ser encontrada no núcleo. A translocação nuclear surge, geralmente, em resposta a um *stress* ou estímulo, e pode ocorrer por clivagem proteolítica ou fosforilação da proteína. A proteína YB-1 é fosforilada no resíduo de serina 102 localizada no *cold shock domain*, pela cinase p90 ribossomal S6 e pela cinase Akt serina/treonina.

Recentemente, utilizando um modelo animal de xenotransplantes ortotópicos de cancro da mama RE+ vs. RE-, com modulação dos níveis de estradiol, o nosso grupo mostrou que o gene *YBX1* se encontra sobrexpresso em tumores RE+ que cresceram na presença de 17β-Estradiol (E2), comparativamente a tumores RE+ que cresceram na ausência de E2. Assim, o objetivo deste projeto foi explorar a relevância biológica e clínica da regulação de YB-1 mediada pelo RE em cancro da mama. A nossa hipótese é que as terapias dirigidas ao

RE possam afectar o gene *YBX1*, levando a uma diminuição da sua expressão e que a cessação da hormonoterapia possa desencadear um aumento da sua expressão e consequentemente da proliferação tumoral. Para testar a nossa hipótese usámos uma abordagem translacional, baseada em modelos celulares de cancro da mama e na análise de grupos clínicos relevantes de pacientes com cancro da mama.

O estudo do efeito do E2 na expressão do gene *YBX1* na linha celular de cancro da mama positiva para RE MCF-7, mostrou que a adição de E2 ao meio de cultura per se não é suficiente para alterar os níveis de expressão de *YBX1*. De facto, a análise de um painel de 124 genes regulados pelo RE revelou que a sua regulação *in vitro* difere substancialmente do que foi observado *in vivo*, o que indica que existirão outros fatores e/ou mecanismos envolvidos, quer sejam intrínsecos da célula ou derivados do hospedeiro, que poderão afetar a relação entre o RE e *YBX1*. O efeito do E2 nos níveis de YB-1 foi também avaliado ao nível da proteína por Western Blot e imunofluorescência, utilizando anticorpos específicos contra YB-1 total e a forma fosforilada no resíduo de serina 102 (p-YB-1). O estímulo com E2 induziu um aumento da p-YB-1 e da sua localização nuclear. Este efeito mostrou ser dependente do RE, uma vez que foi inibido pelo tamoxifeno e não foi observado na linha celular RE negativa MDA-MB-231. Assim, embora não tenha sido observado um efeito da ativação da via do RE pelo E2 na expressão do gene *YBX1*, foi detetado um aumento na fosforilação da proteína YB-1, o que sugere que no modelo *in vitro*, a expressão e atividade de YB-1 pode ser modulada pela via RE ao nível pós-transducional.

No contexto clínico, o nosso principal objetivo consistiu em estabelecer uma possível correlação entre a expressão de YB-1 e o cancro da mama RE+. Deste modo, analisámos pela primeira vez a expressão de p-YB-1 em amostras cancro da mama, num conjunto de 60 amostras de tumor primário e 32 metástases emparelhadas. Observou-se uma associação entre níveis elevados de p-YB-1 e tumores RE e RP negativos (*P*=0,006 e *P*=0,037, respetivamente). Relativamente aos *outcomes* clínicos, a expressão de YB-1 e p-YB-1 correlacionou-se com uma diminuição da sobrevivência livre de recidiva (*P*=0.0442, HR 0.5514 95%CI 0.3088-0.9846 e *P*=0.0108, HR 0.058 95%CI 0.1230-0.7606 respetivamente), mas não com a sobrevivência global (*P*=0.2473, HR 0.6097 95%CI 0.3704-1.292 e *P*=0.0687, HR 0.4789 95%CI 0.2168-1.058). Desta forma, YB-1 e p-YB-1 são importantes biomarcadores de pior prognóstico, nomeadamente para risco de recidiva, especialmente em pacientes com tumores RE-, RP-. Em amostras de metástases, observou-se uma correlação positiva entre a expressão elevada de p-YB-1 e metástases RP negativas (*P*=0,030), sendo que a marcação de YB-1 não mostrou associação significativa com nenhum dos parâmeros clínicos. Verificámos ainda não existirem níveis diferentes de expressão de ambos os marcadores nos tumores primários e metástases, refletindo uma alteração durante a progressão tumoral (Teste McNemar: *P*=0.7728 e *P*=0.0771, respetivamente; Paired t-test: *P*=0.5754 e *P*=0.1883,

respetivamente). Este estudo consiste n a primeira análise da expressão de YB-1 e p-YB-1 em tumores primários da mama e metástases emparelhadas.

Visto que a deteção da proteína YB-1 em tecidos tumorais é um marcador de mau prognóstico, procurámos de seguida avaliar se a deteção de YB-1 no soro de doentes com cancro da mama poderia ser igualmente significante. A deteção de níveis séricos de proteínas é uma técnica minimamente invasiva e de fácil aplicação. Para esta análise foi utilizado um grupo de amostras de soro disponível de doentes com cancro da mama e metástases ósseas. Foi detetada a presença de YB-1 no soro de 22 doentes, correlacionada com a presença de metástases extra-ósseas (*P*=0.044). A análise multivariada mostrou que os doentes com YB-1 no soro apresentaram uma progressão da doença óssea mais rápida (HR 3.29, 95% CI 1.13 – 9.60, *P*=0.029), no entanto sem diferenças ao nível da sobrevivência global (HR 2.04, 95% CI 0.86 – 4.87, *P*=0.108). Este estudo corresponde à primeira análise dos níveis séricos de YB-1 em doentes com cancro da mama.

Em suma, os resultados obtidos neste projeto mostram que não só a proteína YB-1, mas também p-YB-1 e YB-1 secretada têm valor de prognóstico em doentes com cancro da mama, reforçando a sua utilidade clínica como fator de prognóstico e possível alvo terapêutico. Este estudo mostrou ainda a regulação *in vitro* e *in vivo* de *YBX1* não é exclusivamente dependente da presença de E2. No geral, este estudo gerou dados significativos e colocou questões importantes que serão abordadas em projetos futuros.

Palavras-chave:
Cancro da mama; Recetor de estrogénio; Hormonoterapia; YB-1; Biomarcador de prognóstico

ABSTRACT

Estrogen receptor-positive (ER+) tumors are the most frequent breast cancers (BC), and have the better prognosis. Nevertheless, about 30% of patients with ER+ BC will develop distant metastases, with increased mortality rates. ER+ BC is dependent on estrogens that activate ER, an important transcription factor. In this context *YBX1*, which encodes for Y-box binding protein 1 (YB-1), was recently identified as part of an ER-dependent poor prognosis predictor for ER+ BC. YB-1 is an oncoprotein overexpressed in BC, where it has been associated with disease progression and ER/PR negativity. Recently, using an orthotopic mouse model of ER+ vs. ER- BC, our group showed that *YBX1* is overexpressed in ER+ tumors growing under the presence of 17β-Estradiol (E2).

Therefore, this project aimed to establish a biological and clinical link between ER and YB-1 expression in BC. Using BC cell lines, we showed that E2 per se did not altered YB-1 expression, at the mRNA or protein level, but induced its phosphorylation. These results need further investigation to address the mechanism behind YB-1/ER connection we observe in the *in vivo* model. Next, we explored the prognostic value of p-YB-1 expression and secreted YB-1 in the clinical setting. We demonstrated that p-YB-1 is a biomarker of decreased distant metastases-free survival and overall survival, and that secreted YB-1 correlates with faster bone disease progression in patients with BC and bone metastases.

In conclusion, the results obtained in this project demonstrate that not only YB-1 but also p-YB-1 and secreted YB-1 have prognostic value in BC patients, reinforcing its clinical utility as prognostic factor and putative target. We also showed that the *in vitro* and *in vivo* regulation of *YBX1* is not exclusively dependent on the presence of E2. Overall, this work generated significant data and raised important questions to be addressed in future projects.

Keywords:
Breast Cancer; Estrogen Receptor; Hormone therapy; YB-1; Prognostic biomarker.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microliter
µM	Micro molar
18S	18S ribosomal RNA
AI	Aromatase Inhibitor
AKT	Serine/threonine kinase
B2M	Beta 2 Microglobulin
BC	Breast Cancer
BM	Bone Metastases
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
ChIP-seq	Chromatin Immunoprecipitation sequencing
CI	Confidence Interval
CIP	Calf Intestinal Alkaline Phosphatase
CRS	Cytoplasmatic Retention Site
CSD	Cold Shock Domain
csFBS	Charcoal-Stripped Fetal Bovine Serum
Ct	Threshold Cycle
CTD	C-terminal domain
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle Medium
DMFS	Distant Metastasis Free-Survival
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
E2	17β-Estradiol
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
ERE	Estrogen Response Element
FBS	Fetal Bovine Serum
FFPE	Formalin-Fixed Paraffin-Embedded
FOXA1	Forkhead box protein A1

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
h	hours
HER2	Human Epidermal Growth Factor Receptor 2
HKG	Housekeeping Genes
HR	Hazard-ratio
HRP	Horseradish Peroxidase
HT	Hormone Therapy
IARC	International Agency for Research on Cancer
IF	Immunofluorescence
IHC	Immunohistochemistry
kDa	KiloDalton
KO	Knockout
Luc	Luciferase
MAPK	Mitogen-Activated Protein Kinase
MCF-7	Michigan Cancer Foundation-7 (Breast Cancer Cell Line)
MDA-MB-231	M.D. Anderson – Metastatic Breast 231 (Breast Cancer Cell Line)
MHC II	Major Histocompatibility Complex Class II
min	Minutes
mL	Milliliter
MMPs	Matrix Metalloproteinases
mRNA	messenger Ribonucleic Acid
mRNP	Messenger Ribonucleoprotein Particles
NLS	Nuclear Localization Signal
nM	Nanometer
NSG	NOD scid gamma
ºC	Degree Celsius
OR	Odds ratio
OS	Overall Survival
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0,05% Triton X-100
PCR	Polymerase Chain Reaction
Pen	Penicillin
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PR	Progesterone Receptor

RNA	Ribonucleic Acid
RPKMs	Reads Per Kilobase of transcript per Million mapped reads
RPL13A	Ribosomal Protein L13a
RT	Room Temperature
RT-qPCR	Reverse Transcription-semi quantitative Real Time PCR
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SERD	Selective Estrogen Receptor Down Regulator
SERM	Selective Estrogen Receptor Modulator
SREs	Skeletal-related events
Strep	Streptomycin
TAM	Tamoxifen
TNBC	Triple Negative Breast Cancer
UV	Ultraviolet
V	Volts
WHO	World Health Organization
YB-1	Y-box Binding Protein 1

1. INTRODUCTION

Breast Cancer (BC) is the second most common cancer, after lung cancer. Among women, BC is the most frequent type of cancer, with 1,671 million new cases diagnosed worldwide in 2012, and an overall incidence of 11,9%[1]. According to World Health Organization (WHO) and the International Agency for Research on Cancer (IARC), in 2012, 521,907 women died from BC, representing the fifth highest mortality rate among all cancers[2]. Based on GLOBOCAN prediction for 2020, 1,979,022 new cases will be diagnosed and 622,676 women will die from this disease worldwide[34]. In Portugal, we observe an identical scenario, with 6,088 new cases diagnosed in 2012, corresponding to the third most common cancer in the general population, and the most frequent in the female population[5]. Also in 2012, 1,570 women died from BC in Portugal (16% mortality rate)[6].

1.1 Breast cancer subtypes

BC is a heterogeneous disease characterized by various clinical and pathological parameters, including histological evidences and molecular markers such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2)[7]. The clinically relevant classification of BC recapitulates the molecular classification in three major subtypes: Luminal BC, including Luminal A (ER+ and/or PR+, HER2-, low Ki67) and Luminal B (ER+ and/or PR+, HER2+ or high Ki67); HER2 amplified BC (ER-PR-HER2+); and TNBC BC (ER-/PR-/HER2-)[8–10].

Luminal BC is the most common subtype, corresponding to approximately 60% of all BC. It also has the better prognosis, exhibiting low tumor grade, low tumor growth, and lower risk of relapse at an early stage[11]. However, and despite major improvements in clinical management and outcomes, almost 30% of ER+ patients will develop metastatic disease predominantly in bone and soft tissue during the following 15 years[12]. Bone recurrence represents 65% of all ER+ metastatic BC. When tumor cells spread to the bone, their interaction with the bone microenvironment will favor bone osteolysis, leading to the onset of skeletal related events (SRE), increasing morbidity and mortality.

1.2 Estrogen Receptors (ERs)

Estrogen receptors (ERs) are ligand-induced transcription factors that control cell differentiation and proliferation[13,14]. There are two major estrogens receptors, ER alpha (ERα) and ER beta (ERβ), encoded by *ESR1* and *ESR2* respectively[15,16]. ERβ seems to act as a negative modulator of ERα and its expression is minor in tumor cells. ERα is the one with major relevance in clinical treatment of BC and from now on will be referred to as ER.

ER is overexpressed in 60% of all BC and is mainly activated by 17β-Estradiol (E2), the main systemic steroid ovarian hormone (review in[17]). ER pathway is involved in main

cellular functions, such as proliferation, differentiation, invasion, apoptosis and angiogenesis, promoting tumor growth. ER exerts its main function in the nucleus. When E2 binds to ER, it promotes its dimerization and translocation to the nucleus, where it will recruit co-factors and modulate gene expression. The specificity of the transcriptional response depends on the complex ER/co-factors that binds to estrogen response elements (EREs) in the promoter region of the target genes. Binding to DNA can also occur indirectly through interaction with another transcription factors, such as FOXA1.

Studies of ER-related genes have been mostly restricted to model systems[10]. Recently, using a cohort of BC patients with different clinical outcomes, ChIP-seq mapping of ERα chromatin binding events allowed the identification of predictive signatures of endocrine resistance and prognostic of clinical outcome[18]. In fact, ER-binding events in different genomic regions clustered into differential groups that correlate with survival. Genes clustered in good prognosis group contained genomic regions mainly with EREs, whereas the poor prognosis set contained EREs as much as FOXA1-binding motifs. In this context, we are currently addressing the relation between *YBX1*, part of the poor prognosis predictor and the ER pathway.

Despite its major role in genomic activity, ER pathway is also important in membrane and intracellular signaling pathways. At this level, ER regulation occurs by interaction with membrane tyrosine kinase receptors, like epidermal growth factor receptor (EGFR) and HER2[19,20]. Downstream activation of PI3K/AKT and Ras/MAPK signaling pathways can contribute to endocrine therapy resistance[21,22].

Clinical evidences raise the hypothesis that ER regulated genes could also have a major role in the development of site specific metastases, like the predominant bone metastases (BM) in ER+ BC, but the molecular mechanisms are still unknown.

1.2.1 ER pathway as a therapeutic target

Presently, hormone therapy (HT) is the most effective option in BC treatment by blocking ER pathway and therefore its activity in tumor development[23]. HT for BC includes selective estrogen receptor modulators (SERMs), selective estrogen receptor down regulators (SERDs), and aromatase inhibitors (AIs). SERMS, such as Tamoxifen (TAM), antagonize estrogen effects on target specific genes[17]. Nevertheless, although most ER+ BC patients are eligible for endocrine therapy, even in advanced disease stage. the clinical indication will depend on tumor aggressiveness, risk of relapse and expression levels of molecular receptors. In general, Luminal A BC shows the best response to HT[24].

Despite HT effectiveness some patients with primary tumors and the majority of patients with distant metastases will develop resistance to the treatment (reviewed in [23]). However, the molecular mechanisms of acquired resistance are not completely understood.

Tumor resistance may be due to loss of ER or loss of its activity; crosstalk between ER and growth factors; and alteration in one of the downstream targets could affect ER pathway and mediate resistance by providing another proliferation or survival stimuli. Understanding the molecular mechanisms behind resistance to HT could allow the identification of new biomarkers, contributing to improve personalized treatment for BC patients.

1.3 Y-box binding protein 1 (YB-1)

YB-1 (Y-box binding protein 1), encoded by *YBX1* gene (1p34), is a multifunctional protein member of the cold-shock protein superfamily[25]. YB-1 is constitutively expressed in tissues, but at different levels according to development stage and type of tissue (reviewed in [26]). YB-1 plays a major role in several cellular functions, like proliferation, apoptosis, cell cycle control, drug resistance, epithelial-mesenchymal transition (EMT) and stress response.

YB-1 was initially identified as a transcriptional regulator that binds to Y-box in the promoter region of MHC II genes[27] and to enhancers of the *EGFR* and *ERBB2* genes[28]. YB-1 protein has 324 amino acids, 36kDa of molecular mass, and an electrophoretic mobility of approximately 50kDa. YB-1 has three major domains: a N-terminal domain; a cold shock domain (CSD), with a phosphorylation site at Serine 102 residue (Ser102); and a C-terminal domain (CTD), which has a cytoplasmic retention site (CRS), a nuclear localization signal (NLS), and a cleavage site.

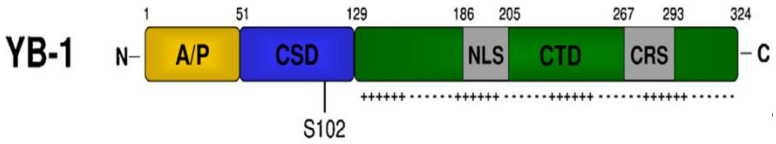


Figure 1 Representative diagram of YB-1 protein structure. N-terminal domain contains alanine and proline residues (A/P domain); CSD, cold-shock domain; Ser 102, phosphorylation site at serine 102; NLS, nuclear localization signal; CTD, C-terminal domain; CRS, cytoplasmic retention site. Adapted from C.Kosnopfel et al., *European Journal of Cell Biology*, 2014

Due to its ability to bind to nucleic acids, YB-1 is an important regulator of transcription and translation of several genes, including *ERBB2*[28], *cyclin A/B1*[29], *E2F*[30], *MDR1*[31], *MYC*[32], *PIK3CA*[33], and is also a major component of messenger ribonucleoprotein particles (mRNPs)[34], contributing to its stabilization and acting as regulator of its own translation. Therefore, the functional role of YB-1 seems to depend on its cellular localization which is highly regulated. In normal conditions YB-1 is mainly cytoplasmic, mostly located in the perinuclear region, although it can also be found in the nucleus[35]. Generally, YB-1 translocation to the nucleus occurs in response to stress, namely UV irradiation, oxidative stress and drugs, and it may be promoted by cytokines and growth factors. In addition, YB-1 translocation to the nucleus can occur by proteolytic cleavage of the CRS[36], or upon phosphorylation. YB-1 is

phosphorylated at Serine 102, located at the CSD, by the p90 ribosomal S6 kinase[37] and the serine/threonine kinase Akt (RSK1/2)[38].

1.3.1 YB-1 as an oncoprotein

YB-1 has been implicated in all hallmarks of cancer[39] (reviewed in [40]). Elevated YB-1 levels have been correlated with cancer progression and poor prognosis in several types of cancers, such as breast[41–43], prostate[44], gastric[45], non-small cell lung cancer[46], osteosarcoma[47], melanoma[48] and multiple myeloma[49]. An association between YB-1 levels and an increase of proliferation and/or inhibition of apoptosis has been shown *in vitro* in different tumor cell lines[50] and seems to be dependent of YB-1 translocation to the nucleus[38].

In melanoma, both nuclear localization and phosphorylation of YB-1 have been correlated with tumor progression using a human tissue array[48]. In MCF-7 BC cells, a point mutation in Ser102 residue decreased its nuclear localization, and affected EGFR and HER2 regulation, showing a major role of phospho-YB-1^{Ser102} (p-YB-1) in the regulation of these genes.[51].YB-1 also contributes to EMT in BC cells, by increasing levels of metalloproteases (MMPs) which play an important role in invasion and metastization[52,53]. Importantly, YB-1 also plays a role in mitosis and has been considered a centrosomal protein, with functions in chromosomal instability and influence in actin filaments[54].

YB-1 is commonly expressed in BC and a marker of poor prognosis, EMT, relapse and drug resistance[31,42,52,53]. And as previously mentioned, *YBX1* was recently identified as being part of the ER-related predictor of poor prognosis in BC patients[18]. In BC, YB-1 nuclear localization has been associated with the expression of *MDR1* and P-glycoprotein, which contribute to the development of a multi drug resistance phenotype [31]. More recently, it was shown in a transgenic mice model that overexpression of YB-1 in mammary epithelial cells contributes to the development of different histological types of BC, another strong evidence of YB-1 role as an oncoprotein[54].

1.3.2 Detection of YB-1 in human breast cancer

Among BC subtypes, YB-1 is more expressed in basal-like tumors, and is correlated with an aggressive tumor phenotype[42]. In Luminal BC YB-1 expression is inversely correlated with ER and PR levels [30]. In accordance, it has been shown that YB-1 knock-down in MCF-7 cells leads to an increase in ER expression [55].

In a large cohort of 4,049 BC patients, high YB-1 expression, assessed by immunohistochemistry (IHC), was related to lower overall survival (OS) and higher risk of relapse across all BC subtypes, with a superior predictive value in comparison with ER and HER2[42]. In this study it was also shown a positive correlation between the nuclear localization of YB-1 and HER2 positivity, and a negative correlation with ER expression. Recently, a meta-

analysis that included 8 studies that assessed the prognostic role of YB-1 by IHC, has shown that YB-1 correlates with OS[43].

Despite significant clinical data, the relation between ER and YB-1 regulation has not been investigated.

1.3.3 Secreted YB-1

In addition to the intracellular role of YB-1, this protein has also been reported to be secreted and act as an extracellular mitogen, in a monocytic model of inflammation [56]. In BC, addition of YB-1 to MCF-7 cells *in vitro*, has also shown to promote proliferation [57]. Secreted YB-1 was also identified in plasma of patients with different types of malignancies, including BC[58]. Therefore, secreted YB-1 may be a strong biomarker of malignancy[59].

Taking into account the major role of YB-1 in oncogenesis, and the finding that YB-1 is present in the serum of cancer patients, including BC, further studies need to be performed in order to analyze the importance and predictive value of secreted YB-1.

2. OBJECTIVES

Previously in our lab, we investigated how the expression of ER-regulated genes, identified as part of a poor prognosis predictor[18], varied *in vivo* according to the BC subtype and 17β-Estradiol (E2) availability (unpublished data). MCF-7$^{GFP+Luc+}$ and MDA-MB-231$^{GFP+Luc+}$ human BC cell lines (Luminal A and TNBC, respectively) were orthotopically engrafted in the mammary fat pad of NSG mice, in the absence of endogenous E2 (ovariectomized mice) and in presence or absence of an exogenous source of E2 (E2 or placebo sub-cutaneous slow release pellets, respectively). Data from gene expression profiling showed that 32 genes were upregulated in ER+ tumors supplemented with E2, being *YBX1* the most up-regulated gene (23,98 fold). As expected, in MDA-MB-231 (ER-) tumors, gene expression did not change with E2 supplementation.

Due to the potential oncogenic role of *YBX1*, we decided to explore the biological and clinical significance of ER-related *YBX1* up-regulation in BC. We hypothesized that ER-targeted therapies could affect *YBX1*, down-regulating its expression, and that HT cessation could trigger an increase in *YBX1* and, consequently, in tumor cell proliferation. To address our hypothesis, we use a translational approach, based in cellular models of BC and analysis of clinically relevant cohorts of patients with BC.

In the scope of this specific project, the specific aims were:

1) to address *in vitro* the effect of ER pathway activation over YB-1 expression, namely:
    a. to evaluate the impact of E2 stimuli over the expression of *YBX1*;
    b. to assess the effect of ER pathway activation by E2 in YB-1 expression and localization;
2) to address a possible correlation between YB-1 expression and ER pathway in the clinical setting, namely:
    a. to analyze the prognostic value of YB-1 and p-YB-1 in patients with advanced BC and its correlation with clinicopathologic characteristics;
    b. to evaluate the potential of secreted YB-1 as biomarker of BC and its correlation with clinicopathologic characteristics.

3. MATERIALS AND METHODS

3.1 Cell culture and cellular assays

The BC cell lines MCF-7$^{GFP+Luc+}$ and MDA-MB-231$^{GFP+Luc+}$ were gently provided by Sérgio Dias Lab, IMM. Both cell lines were routinely propagated in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% (v/v) fetal bovine serum (FBS, Gibco), 1% (v/v) Penicillin/Streptomycin (Pen/Strep, 10,000 U/mL Penicillin, 10,000 µg/mL Streptomycin, Gibco). The medium of MCF-7$^{GFP+Luc+}$ cell line was supplemented with 0,01mg/mL Insulin (Gibco). Cells were maintained at 37ºC with 5% CO_2 in a humidified atmosphere, and medium was replaced every 2 or 3 days. For experiments with 17β-Estradiol (E2, Sigma-Aldrich), cells were seeded in 6-well plates, at a density of 4x10^{5}cells/well, in DMEM supplemented with 10% charcoal-stripped fetal bovine serum (csFBS, Gibco) or 0,1% FBS. After 24h, medium was replaced with fresh medium supplemented with 1 or 10nM E2, with or without 10µM Tamoxifen (TAM, Sigma-Aldrich).

3.2 RNA isolation, cDNA synthesis and RT-qPCR

Cells were treated as described above. At different time-points, medium was removed and cells were washed with 1xPBS. Total RNA was extracted with NZY Total RNA Isolation kit (NZY Tech), according to the manufacturer's instructions. Incubation with DNase I was extended to 30min to remove any genomic DNA contamination. Total RNA was quantified in NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and 1µg of total RNA was used to synthesize complementary DNA (cDNA).

For cDNA synthesis, Oligo(dT)18 primer and NZY M-MuLV First-Strand cDNA Synthesis Kit (NZY Tech) were used according to manufacturer's instructions.

Interest genes were amplified by real time semi quantitative PCR (qPCR) using ViiA™ Real-Time PCR System (Applied Biosystems). Reactions were performed using 2x TaqMan Gene Expression Master Mix (Applied Biosystems), 900 nM TaqMan® Gene Expression Assay (Applied Biosystems), or 2x iTaq™ Universal SYBER® Green Supermix (Biorad), 10µM specific primers (Invitrogen) and 5% cDNA (v/v) in a final volume of 20 µl in Micro Amp Optical 384-well Plates (Applied Biosystems). Reactions were run in triplicate, and non-template and RNA template controls were included. The TaqMan® Gene Expression Assays used were: *18S* (Hs99999901_s1), *GAPDH* (Hs02758991_g1), *RPL13A* (Hs00744303_s1), *YBX1* (Hs03044127_g1) and *ESR1* (Hs0017486_m1). Gene array assay was performed with a Custom Inventoried TaqMan® Array Plate, Format 96 + Endogenous Controls (Standard) (Applied Biosystems). *c-myc* specific primer (Invitrogen) sequence: exon 1 (190) forward 5'-GCC GCA TCC ACG AAA CTT T-3' reverse 5'-TCC TTG CTC GGG TGT TGT AAG-3'.

Cycling conditions were the following: holding at 50ºC for 2 min, initial denaturation at 95ºC for 10min, followed by 40 cycles of 95ºC for 15 second and 60ºC for 1min. Relative mRNA expression levels were normalized to *RPL13A*, *GAPDH* and *B2M* and calculated using the $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$ method.

3.3 Western Blot

Cells were treated as described above. At different time-points cells were transferred to 1.5 mL tubes and centrifuged for 5min, at 1,000 x g and 4ºC. The pellet was re-suspended and lyzed in RIPA Lysis buffer (Santa Cruz), containing 1:100 (v/v) phosphatase inhibitor cocktail (Sigma), 1:100 protease inhibitor cocktail, 1:100 PMSF and 1:100 sodium orthovanadate (all from Santa Cruz). After cooling on ice for 30min, the extracts were sonicated for approximately 1 min in Soniprep 150 (MSE) and centrifuged at 10,000 x g for 20min at 4ºC. Supernatants were transferred to a new tube and quantified using Pierce™ BCA Protein Assay Kit (Thermo Scientific), according to manufacturer's instructions. Absorbance was measured at 562nm and protein concentration was determined by standard curve of known albumin concentrations. 5x SDS-PAGE Sample Loading Buffer (NZY Tech) was added to each extract, proteins were denatured for 5 min at 95ºC and stored at -20ºC.

Proteins were loaded into 10% SDS-PAGE gels (10µg per lane), and separated by denaturant electrophoresis at 120V. The Precision Plus Protein Standard (Bio Rad) was used as a ladder. Proteins were electro blotted onto nitrocellulose membranes (iBlot 2 NC Regular Stacks, Thermo Fisher Scientific), using Invitrogen Dry Erase mini-gel drying system (Invitrogen) according to manufacturer's instructions. Membranes were blocked for 1h in 5% Bovine Serum Albumin (BSA, Santa Cruz) or 5% non-fat dry milk, in PBST (1xPBS with 0,05% Tween, Sigma-Aldrich). Membranes were incubated with the following primary antibodies: rabbit anti-human YB1 (D299) (1:1,000, Cell Signaling) and rabbit anti-human p-YB1 (Ser102) (1:500, Cell Signaling), diluted in 5%BSA in PBST, overnight at 4ºC; mouse anti-human β-Actin (1: 25,000, Abcam), diluted in 5% non-fat milk in PBST, for 1h at room temperature (RT). Membranes were rinsed 3x 10 min in PBST, and incubated with the respective secondary horseradish peroxidase (HRP) antibody for 2h at RT: donkey polyclonal anti-rabbit (1:4000, Santa Cruz) or donkey anti-mouse (1: 2,000, Santa Cruz), diluted in 5% non-fat milk in PBST. After washing membranes, proteins were detected using Amersham ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences) or the SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific), according to manufacturer's instructions. Signal was detected on radiographic film (Fujifilm), using Curix. Membranes were reused after stripping with Stripping Reagent (Dako), according to manufacturer's instructions.

3.4 Immunofluorescence

MCF-7$^{GFP+Luc+}$ cells (4x10^{5}cells/well) were seeded in 6-well plates over glass cover slips, in 10% csFBS medium. Medium was replaced with fresh medium supplemented with 10nM E2 ± 10μM Tamoxifen, and cells were incubated for 2 or 24h. Following incubation, medium was removed, cells were rinsed twice with 1xPBS, fixed with 3,7% paraformaldehyde (PFA) for 10 min at RT, and permeabilized with 0,5% Triton X-100 for 10 min at RT. Cells were incubated with primary antibodies, rabbit anti-human YB1 or rabbit anti-human phospho YB1 (Ser102), diluted 1:100 in 1% PBS, 0,3% Triton X-100, 1% BSA, overnight at 37ºC, and then incubated with secondary antibody, AffiniPure Goat anti-rabbit Cy3 (1:200, Jackson Immunoresearch), for 1h at 37ºC. Fixation was performed with 2% Formaldehyde for 10min at 4ºC, cover slips were mounted in Vectashield Mount. Medium (Baptista Marques) with DAPI (1:1,000, Sigma-Aldrich) and stored at 4ºC.

Images were acquired with a Zeiss LSM 7 Live Confocal Laser Line-Scanning Microscope (Carl Zeiss MicroImaging) and analyzed in ImageJ Software (version 1.50b, National Institutes of Health, USA).

3.5 Immunohistochemistry

Protocol optimization was performed in an anonymized human BC formalin-fixed paraffin-embedded (FFPE) sample from Histology and Comparative Pathology Laboratory, IMM. The same sample was used as positive control of all experiments. Expression of YB-1 and p-YB-1 was evaluated by IHC in a cohort of FFPE samples from human primary breast tumors (n=60) and paired distant metastases (n=32), stored at the Pathology Archive of Hospital de Santa Maria-CHLN.

Deparaffinization and antigen retrieval was performed in PT Link Pre-Treatment Module for Tissue Specimens (Dako), using Antigen Retrieval pH6 solution (Dako), at 94ºC for 20min. Activity of endogenous peroxidase was blocked with Blocked Endogenous Peroxidase Solution (Dako) for 10 min at RT, and total protein was blocked by incubation with Protein Block Solution (Dako), for 20min at RT. Incubation with primary antibodies was performed overnight at 4ºC with the following antibodies: rabbit anti-human YB1 (D299) (Cell Signaling), diluted 1:50 in Antibody Diluent (Dako) and rabbit anti-human p-YB1 (Ser102) (Cell Signaling), diluted 1:500 in Protein Block Solution. The visualization system Dako REALTM EnVisionTM Detection System, peroxidase/DAB+, rabbit/mouse (Dako) was used according to manufacturer's instructions, with 2 min of incubation with DAB. Slides were counterstained with hematoxylin, dehydrated and diaphanized in running water, alcohols at 70%, 96% and 100% (30seconds each) and xylene for 10 min.

Sections were mounted with Quick-D mounting medium (Klinipath) and visualized in a bright field microscope (Leica DM2500).

Negative controls included the omission of primary antibodies and a phosphatase treated tissue as specific negative control for p-YB-1. In this case, slides were incubated with 0,3U/µl Calf Intestinal Alkaline Phosphatase (CIP, New England BioLabs, inc.), for 2h at 37ºC in a Hybridizer (Dako) after blockage of endogenous peroxidase. Samples were analyzed by a Medical Pathologist. Staining intensity was classified from 0 to 3: (0) absence of staining, (1) weak, (2) moderated and (3) strong staining. Samples were scored according to the percentage of cells with each intensity and randomized to high or low YB-1 and p-YB-1 using different types of cut off values.: the median; the value obtained using cut-off finder software[60,61], taking into account the percentage of cells with cytoplasmic staining of intensity equal or superior to 2 and overall survival (OS) as endpoint; the presence of cells with cytoplasmic staining of intensity 3 or 2 with nuclear staining; positive and negative nuclear staining; the presence of cells with cytoplasmic staining graded with score 3. Final dichotomization between high and low levels of YB-1 and p-YB-1 was performed using cut-off finder software and OS as endpoint.

3.6 YB-1 quantification in serum samples

Serum samples for the quantification of YB-1 were obtained from a prospective collection of serum from patients with BC and BM, followed at the Oncology Division of Hospital de Santa Maria – CHLN. All patients signed an informed consent and the use of these samples to analyze biomarkers was previously approved by the Ethics Committee of Hospital de Santa Maria – CHLN. Baseline cohort (n=44) correspond to samples collected at the time of BM diagnosis. YB-1 was quantified using Human YBX1/YB1 Sandwich ELISA kit (LSBio, LifeSpan BioSciences, Inc.), according to manufacturer's instructions. Absorbance was measured in InfiniteM200 Plate Reader (Tecan) at 450nm and YB-1 concentration was calculated based on standard curve.

3.7 Statistical analysis

Statistical analyses were performed with the software GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) or Stata 13.1 software (StataCorp LP).

In vitro assays were performed in triplicate and values were expressed as the mean. Differences between groups were analyzed by two-tailed unpaired t-test.

Demographic and clinicopathological characteristics of patients are described using frequencies for categorical variables and central tendency, dispersion and range for continuous variables. Univariate association of these characteristics and YB-1 levels was performed using two-tailed unpaired t-test, Fisher's exact test and Chi-squared test when appropriate. The concordance between YB-1 and p-YB-1 in primary tumors and paired

metastasis was analyzed with McNemar's test[62]. Percentage survival and cumulative DMFS plots were performed using Kaplan–Meier. Univariate differences between survival rates were tested for significance using the log-rank test, while multivariate analysis for survival was tested using Cox proportional hazards models. All patients with missing data in relevant variables were excluded from the multivariate analysis. Significance was defined by a *P*-value<0, 05.

4. RESULTS AND DISCUSSION

4.1 Effect of E2 over *YBX1* expression in BC cells *in vitro*

4.1.1 E2 stimuli does not affect *YBX1* expression in MCF-7 BC cells

To study the impact of E2 on *YBX1* expression we started by assessing *YBX1* expression in MCF-7 cells, at different time points after exposure to E2. Since *YBX1* expression was quantified by RT-qPCR we first assessed the effect of E2 on housekeeping genes (HKGs), used as endogenous controls for data normalization.

HKGs used for RT-qPCR normalization must have stable expression independently of factors added to the experiment. *GAPDH* is commonly used as an HKG. However, it has been shown that *GAPDH* expression changes in ER+ BC cells exposed to E2 and TAM, and *RPL13A* was found to be the best internal reference gene in these conditions [63,64].

We analyzed the fold change of *GAPDH*, *18S* and *RPL13A* by RT-qPCR, in MCF-7 cells seeded in medium supplemented with 10%FBS, 0.1%FBS or 10% cs-FBS, and exposed to 1, 10 or 100nM E2 for 3-6h. We observed that both *GAPDH* and *18S* had unstable expression levels, under E2 stimulus, independently of the culture medium.

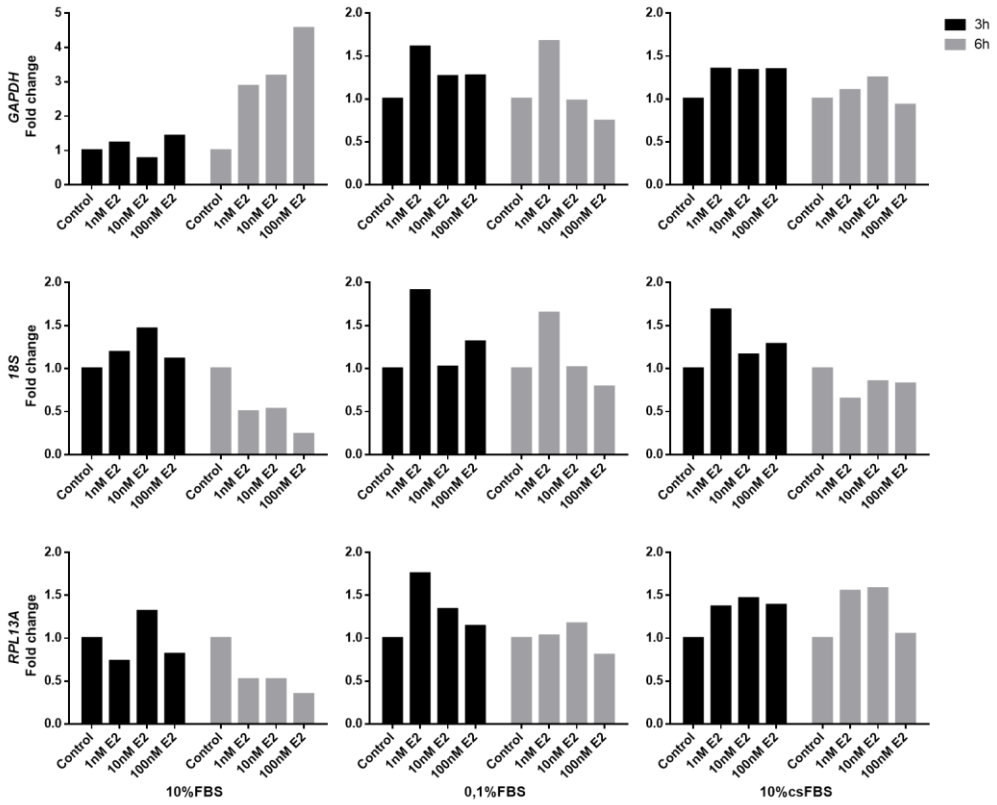


Figure 2 Expression pattern of housekeeping genes (HKGs) under 17β-Estradiol (E2) stimulus in the estrogen receptor positive (ER+) MCF-7 cell line. Cells were incubated in complete medium with 10% FBS, 0,1% FBS or 10% cs-FBS, and treated with different concentrations of E2. Total RNA was extracted 3 or 6h post treatment. *GAPDH, 18S* and RPL13A fold changes were compared to untreated cells using comparative Ct method ($2^{-\Delta Ct}$). Results representative of three independent experiments.

RPL13A expression was more stable, especially in 10% csFBS medium (Figure 2). Stability of these three reference genes was also confirmed using BestKeeper Gene software[65] (Figure S1 and Table S1).

As a result, we were able to demonstrate that *RPL13A* is an adequate HKG to be used as an endogenous control in our experiments.

To test the effect of E2 on *YBX1* expression, cells were cultured in medium supplemented with cs-FBS to ensure the absolute absence of steroids prior to stimulus. TAM was used to block ER and the effect of E2. MDA-MB-231 cells used to demonstrate the dependence of ER of a possible effect of E2 over *YBX1* expression. However, our results show that addition of E2 to culture medium does not affect *YBX1* expression in MCF-7 cells (Figure 3A). Results were consistent between three independent experiments, and similar at different alternative time points (Figure S2). Basal *YBX1* expression in MCF-7 and MDA-MB-231 cells and in different human tissues (Figure S3) was also consistent with values reported at the EMBL-EBI Expression Atlas[66,67] and GTEX Analysis Release V6[68], respectively (Figure S4). Therefore, the selected *YBX1* assay was not contributing to the lack of observed effect of E2 over *YBX1* expression.

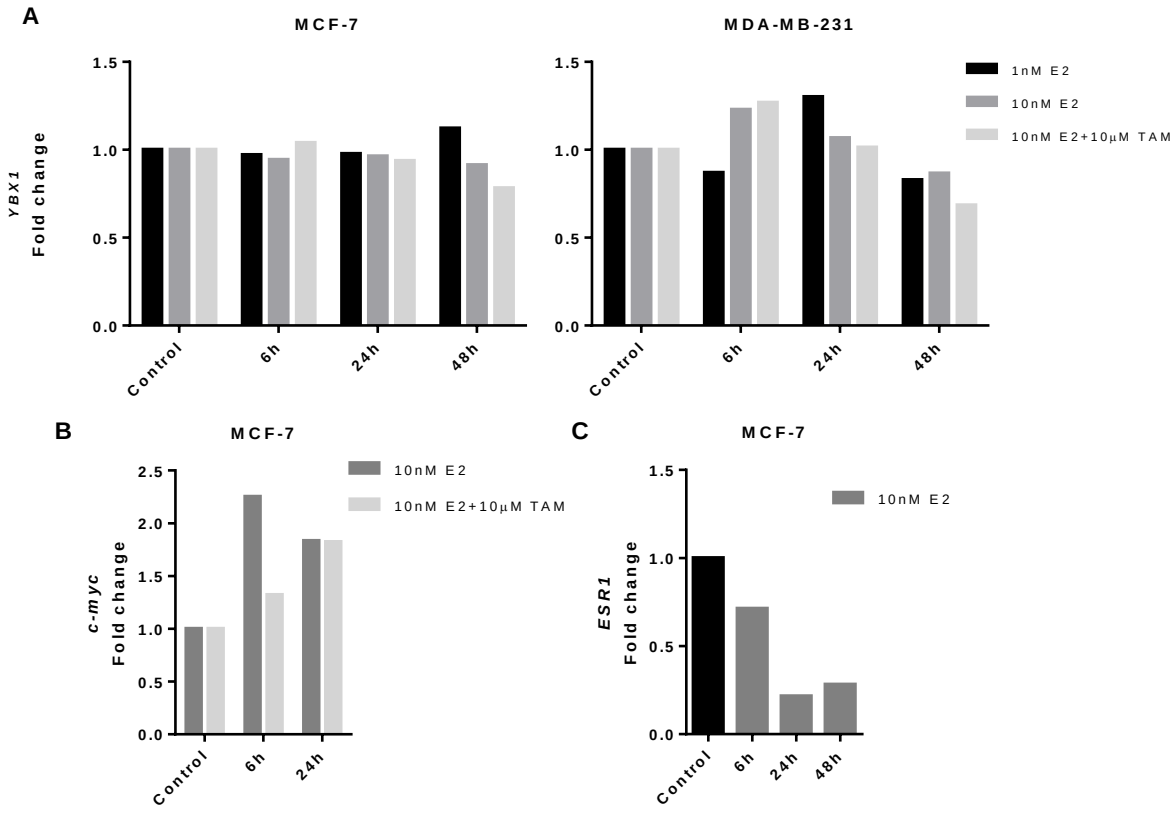


Figure 3 *YBX1* expression under 17β-Estradiol (E2) in BC cell lines. Expression of *YBX1* (A), *c-myc* (B) and *ESR1*(C) was analyzed by RT-qPCR in MCF-7 or MDA-MB-231 BC cells, after incubation with different concentrations of E2±Tamoxifen (TAM). Gene fold change was determined using the $2^{-\Delta\Delta Ct}$ method and *RPL13A* was used as housekeeping gene. Results representative of three independent experiments.

As confirmatory assays, next we assessed ER activation by E2 in our experimental conditions, by testing the expression of *c-myc* and *ESR1*. *c-myc* was previously shown to be up-regulated in similar conditions[69], and *ESR1* is known to be down-regulated by E2 in order to control cells sensitivity to the ligand[70–72]. We also observed that E2 induced an up-regulation of *c-myc* at 6h, an effect inhibited by TAM (Figure 3B). Also, *ESR1* expression was decreased as expected (Figure 3C).

A direct association between ER and *YBX1* expression was only reported *in vitro* using antiestrogens to reduce *YBX1* mRNA levels[73]. Using MCF-7 cells it was shown that treatment with TAM for 120h reduced *YBX1* expression by 20%. Moreover, overexpression of *YBX1* resulted in down-regulation of ER. However, the effect of E2 was not addressed.

The lack of effect of ER pathway activation on *YBX1* expression *in vitro* is not concordant with our previous *in vivo* data, where we observed an up-regulation in *YBX1* expression in MCF-7 orthotopic xenografts growing in mice supplemented with E2. This indicates that additional factors and/or mechanisms, either cell-intrinsic or host-derived may control the regulation between ER and *YBX1*. Importantly, there are obvious differences in the microenvironment of cells grown in culture and in engrafted tumors, and E2 levels may also be different. Moreover, it is known that ER binding affinity not always correlate linearly with E2-induced transcriptional regulation[74].

Among other factors, this can be due to cellular amounts and roles for other transcription factors and co-activators. Moreover, genes' upregulation by E2-exposure is time dependent[69] Importantly, the analysis of gene expression under E2 stimuli, comparing *in vitro* cell cultures and mouse xenografts, is not totally concordant, although suggesting a low level of comparison[74,75].

Therefore, to address the differences between *in vivo* and *in vitro* effect of E2 over MCF-7 cells, we next analyzed the complete gene expression array, previously used with MCF-7 xenografts, using mRNA extracted from MCF-7 cells growing *in vitro*. In this case gene expression was normalized to *B2M* mRNA, which had the lowest standard deviation between samples in a panel of four HKGs (*18S*, *GAPDH*, *βActin*, *B2M*), and fold change was calculated relative to untreated cells. We confirmed that *YBX1* expression was not affected by E2.

Moreover, we observed that only seven of the 124 genes were overexpressed and one gene was down-regulated upon stimulation with E2, an effect totally or partially abrogated by TAM, confirming its ER-dependence (Table 1). Four genes had higher relative mRNA levels under E2 and TAM, although at borderline up-regulation levels. Full data can be found in Figure S5. Importantly, we only found three genes to be overexpressed *in vitro* and *in vivo* (*RRP12*, *ISG20* and *CUEDC1*), interestingly at very similar levels.

Table 1 Up and down-regulated ER related genes in MCF-7 cells treated with 17β-Estradiol (E2)±Tamoxifen (TAM).

	10nM E2	10nM E2+10μM TAM
XBP1	**3,35**	**1,50**
SFRS2	**1,77**	**1,02**
RRP12	**1,72**	**1,02**
KLRC3	**1,64**	**0,58**
ISG20	**1,63**	**1,55**
UCK2	**1,59**	**1,09**
CUEDC1	**1,58**	**1,03**
DHCR7	**1,22**	**1,56**
YBX1	**1,06**	**0,91**
NUDT4	**0,95**	**1,61**
RNF10	**0,86**	**1,64**
RND1	**0,79**	**1,61**
DUSP2	**0,37**	**1,40**

Notes: mRNA expression levels were measured by RT-qPCR and gene fold change was determined using the $2^{-\Delta\Delta Ct}$ method. B2M was used as a housekeeping gene, and fold difference was calculated relative to untreated cells. Up-regulated genes are represented in red and down-regulated genes in green.

As described in the literature, the best concordance found between *in vitro* cultures and tumor xenografts was of approximately 40% in around 22,000 transcripts analyzed[74]. In another study, comparison of *in vivo* E2-regulated genes with those regulated in identical cells *in vitro* after 6 and 24 h of E2 treatment demonstrate only 11% overlap[75]. In our study, we found no concordance with these two reported gene sets, as only the most up-regulated gene, *XBP1* was also reported to be up-regulated by E2, actually *in vitro* and *in vivo*[74]. The differences found between gene sets and studies can be due to not only to the experimental conditions and E2 uptake but also with the number of transcripts analyzed. In our study, we only include 124 genes, which could contribute to the lack of concordance.

It is clear that the ER-*YBX1* axis requires further studies to dissect the mechanism behind their co-regulation. In the scope of this work, we decided to address if despite we could not detect differences at the mRNA level, we could observe differences in YB-1 protein in MCF-7 cells exposed to E2.

4.1.2 E2 induces an increase in YB-1 phosphorylation

The effect of E2 in YB-1 protein expression and activation was assessed by Western blot and immunofluorescence, using specific antibodies against total YB-1 and YB-1 phosphorylated at residue Serine 102 (p-YB-1). For Western Blot, cell lysates were obtained from MCF-7 and MDA-MB-231 cells, cultured in 10% cs-FBS medium and treated with E2±TAM for different time points. We observed that YB-1 expression seems to remain unaltered after E2 stimulus in both cell lines (Figure 4). However, YB-1 phosphorylation increased with exposure to E2 in ER+ cells, with a maximum at 2h post stimulus. Twenty-four

hours after treatment, phosphorylated YB-1 returned to basal levels. The effect is ER-dependent, since the effect of E2 was abrogated by TAM and YB-1 phosphorylation was not observed in MDA-MB-231 cells exposed to E2.

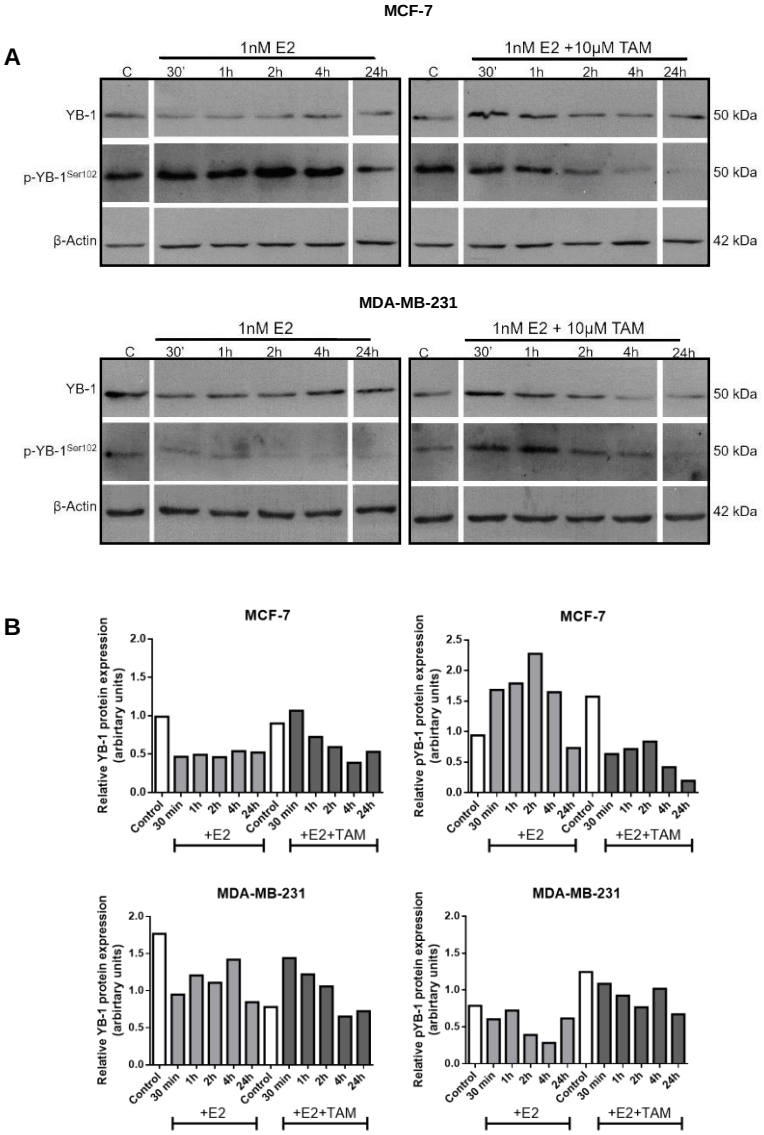


Figure 4 Effect of 17β-Estradiol (E2) in YB-1 protein expression and phosphorylation. (A) YB-1 and p-YB-1 protein levels were analyzed by Western Blot in MCF-7 and MDA-MB-231 BC cell lines exposed to 1nM E2±10μM Tamoxifen (TAM). β-Actin was used as loading control. (B) YB-1 protein levels were normalized by densitometry to β-Actin protein levels and p-YB-1 protein levels were normalized to total YB-1. Experiments were conducted in 10%csFBS medium.

YB-1 is phosphorylated at serine 102 (p-YB-1^{S102}) by Akt and RSK, leading to protein translocation from cytoplasm to the nucleus[37,38]. In fact, activated Akt has been found to be positively correlated with the protein expression of YB-1 in primary BC by screening tumor tissue microarrays[38]. In this study it was found that Akt binds to and phosphorylates the YB-1 cold shock domain at Ser102, and in an Ala102 mutant nuclear translocation of YB-1 was inhibited. In another study, it was found by *in vitro* kinase assay that RSK1 and RSK2 directly phosphorylate YB-1, being more effective activators of YB-1 than Akt[37]. Therefore, it will be

assessed if future experiments if the E2 effect on p-YB-1 we observe is Akt and/or RSK dependent.

To determine if YB-1 phosphorylation was correlated with its translocation to the nucleus, we next assessed protein expression and localization by Immunofluorescence Confocal Microscopy[76].Interestingly, we observed that when MCF-7 cells were exposed to E2 for 2h, YB-1 protein was mostly localized at the perinuclear region (white arrows), an effect abrogated by TAM and not visualized 24h post stimulus (Figure 5). E2 also increased p-YB-1, and induced its translocation to the nucleus.

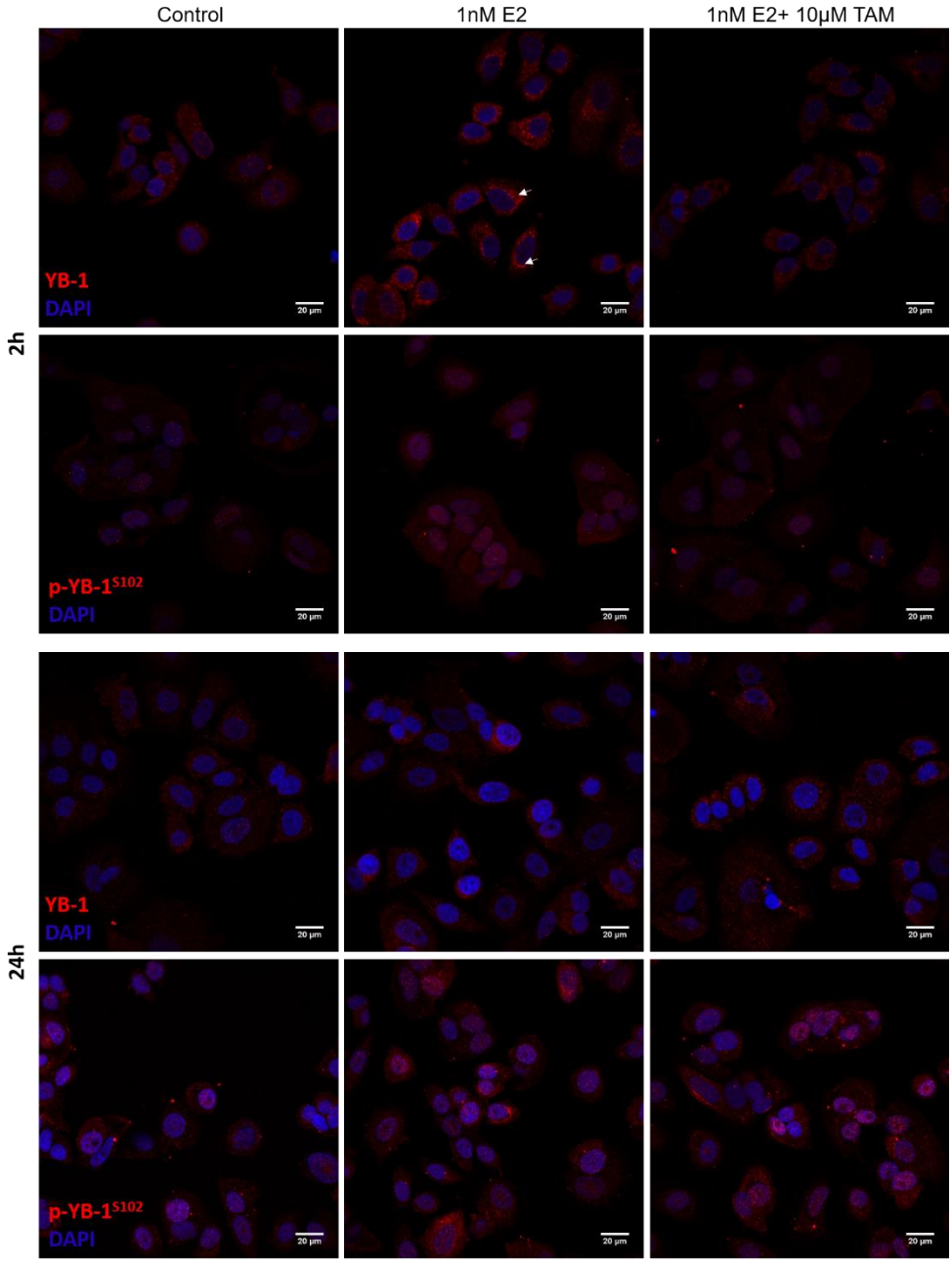


Figure 5 Localization and expression of YB-1 total and phosphorylated form under E2 stimuli. Protein expression and cellular localization were observed by immunofluorescence in MCF-7 cells exposed to 1nM E2±10μM Tamoxifen (TAM) for 2 and 24h. Nuclei were stained with DAPI. All experiments were performed in 10% cs-FBS medium.

Therefore, although we could not observe an effect of ER pathway activation by E2 on *YBX1* mRNA levels, we could detect an increase in YB-1 phosphorylation, suggesting that in an *in vitro* model, YB-1 expression and activity can be modulated by ER pathway at post-translational level.

Further studies are necessary to confirm the effect of ER pathway on YB-1 basal levels. Our results require further confirmation by Western blot in cytoplasmatic and nuclear cell extracts, and including the ER- MDA-MB-231 cell line as a negative control. The antibodies specificity will also be confirmed in YB-1 KO cells.

4.2 Prognostic value of YB-1 and p-YB-1 expression in primary tumors and paired metastases from BC patients

YB-1 has been described as a marker of poor prognosis across different types of cancers, including BC. However, results are not always concordant between cohorts, and YB-1 role as a biomarker is still under study[42,43].

Therefore, we proposed to assess the prognostic value of YB-1 in a cohort of patients with advanced BC, and to address a possible correlation with ER status. Moreover, based on the role of p-YB-1 in cellular processes[37,38,51], and on our results described above, it is also of great relevance to determine p-YB-1 role as a biomarker. As far as we know, there was no conducted study that assess p-YB-1 expression and its correlation with clinicopathological characteristics in human breast tumors. In this retrospective analysis we included a cohort of 60 patients with advanced BC, including 32 cases with paired samples of distant metastases.

First we optimized an immunostaining method based on the use of specific anti-YB-1 and anti-p-YB-1 antibodies, using the absence of primary antibody and treatment with phosphatase as the respective negative controls (Figure S6). Next, all slides were evaluated by a medical pathologist and staining intensities were graded from 0 to 3 (Figure S7). Representative images of IHC staining of YB-1 and p-YB-1 are shown in Figure 6. In both markers, staining was predominantly cytoplasmic, but could also be found in the nucleus. In most part of the cases, nuclear detection coincided with a strongest cytoplasmic staining. We observed the presence of both markers in normal breast tissue, although p-YB-1 staining was a rare event. This phenomena had been described in a previous study, where YB-1 staining was found in normal tissue adjacent to tumor cells[41]. The majority of tumor cells across all slides had a cytoplasmic score equal or superior to 2 for YB-1 immunostaining, while this score for p-YB-1 was found in a lower number of cases (25/60).

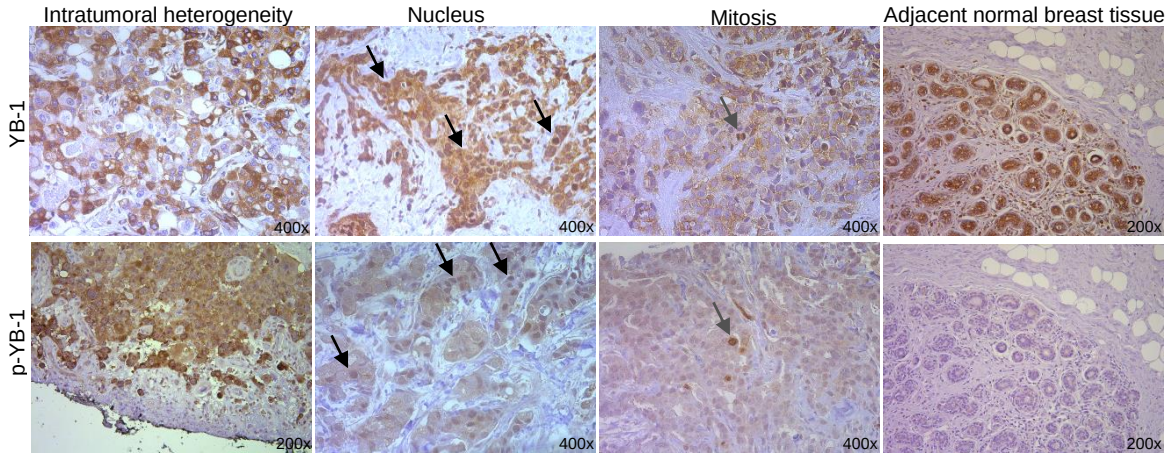


Figure 6 Histologic findings and expression of YB-1 and p-YB-1 in human breast cancer and adjacent normal tissue. Immunohistochemical staining with antibodies against YB-1 and p-YB-1 is heterogeneous within the tumor; both markers are present in both nucleus (black arrows) and cytoplasm of BC cells; strong intensity staining was observed with both antibodies in the nucleus of mitotic cells (grey arrows). YB-1 and p-YB-1 staining is observed in normal breast tissue.

Samples were scored according to the percentage of cells with different intensity staining and randomized to high or low YB-1 and p-YB-1 using different types of cut off values as described in Materials and Methods. We analyzed OS and distant metastasis-free survival (DMFS) and chose the value established using cut-off finder software to dichotomization of samples. This cut off value was selected for all subsequent analyses (Table 2 and Table S2). Values obtained only take into account the percentage of cells with cytoplasmic staining of intensity equal or superior to 2. Nuclear staining was excluded once it was not correlated with outcomes (Table S2). Translocation of YB-1 to the nucleus can occur by phosphorylation at residue Ser102, as described above, or by proteolytic cleavage of the CRS containing the CTD[36–38]. Since anti-YB-1 (D299) binds to a C-terminal epitope, this antibody would not detect nuclear YB-1 translocated upon proteolytic cleavage. In fact, in studies using D299 antibodies[42,51,77], nuclear staining was rarely observed, whereas in studies using antibodies against N-terminal epitopes, nuclear detection was between 13 and 33%[31,41,78]. Overall, only cytoplasmatic YB-1 has been reported to have prognostic value across several studies[41–43,51,79].

Table 2 Cut-off values obtained for randomized samples between low and high YB-1 or p-YB-1 using cut-off finder software and overall survival (OS) as endpoint.

	YB1		p-YB1	
	Primary tumors	Metastases	Primary tumors	Metastases
Cut off value	62,5	72,5	35	20
HR (95% CI)	1,49 (0,75-2,96)	2,38 (0,97-5,84)	1,85 (0,94-3,63)	3,01 (1,27-7,15)
P-value	0,25	**0,052**	0,069	**0,009**

Regarding primary breast tumors, 44/60 samples had high YB-1 expression (73%), and 14/60 high p-YB-1 expression (23%) (Table 3). In this cohort YB-1 did not correlate with clinicopathological characteristics. Conversely, high p-YB-1 was associated with negative ER and PR tumors (*P*=0,006 and *P*=0,037, respectively). There was also a trend for association

of low p-YB-1 with stage I-II tumors (*P*= 0,079). In fact, across different studies, YB-1 correlation with clinicopathological characteristics is not uniform. A recent meta-analysis of eight studies containing a total of 1,094 BC patients (398 YB-1 positive and 696 YB-1 negative) has reported a correlation of high cytoplasmatic YB-1 with ER negativity (OR = 0.604, 95% CI = 0.388-0.941, *P* = 0.026), HER2 positivity (OR = 3.841, 95% CI = 2.637-5.594, *P* = 0.000), and high tumorous T stage (OR = 2.169, 95% CI = 1.295-3.632, *P* = 0.003)[43].

Table 3 Association between YB-1 and p-YB-1 and clinicopathological characteristics of patients with BC

Characteristics	YB-1 (n)		*P*	p-YB-1 (n)		*P*
	High	Low		High	Low	
No. of Patients	44	16		14	46	
Age at diagnosis (years)						
Median	50,59 (44) 42,17-58,42	51,20 (16) 43,23-66,45	0,499*	55,37 (14) 45,18-61,77	49,67 (46) 42,30-59,23	0,652*
Menopausal Status %						
Premenopausal	30 (13)	37,5 (6)	0,528£	36 (5)	30 (14)	1,000£
Posmenopausal	61 (27)	50 (8)		57 (8)	59 (27)	
Unkonwn	9 (4)	12,5 (2)		7 (1)	11 (5)	
Histology %						
Ductal carcinoma	93 (41)	81 (13)	1,000£	86 (12)	91 (42)	0,547
Lobular carcinoma	7 (3)	-		7 (1)	4 (2)	
Unkonwn	-	19 (3)		7 (1)	4 (2)	
TNM Stage %						
I	7 (3)	12,5 (2)	0,766#	-	11 (5)	0,079#
II	34 (15)	31 (5)		21 (3)	37 (17)	
III	36 (16)	25 (4)		43 (6)	30 (14)	
IV	14 (6)	19 (3)		21 (3)	13 (6)	
Unkonwn	9 (4)	12,5 (2)		14 (2)	9 (4)	
T %						
1	34 (15)	19 (3)	0,487#	36 (5)	28 (13)	0,976#
2	34 (15)	44 (7)		29 (4)	39 (18)	
3	5 (2)	12,5 (2)		-	9 (4)	
4	16 (7)	12,5 (2)		21 (3)	13 (6)	
Unkonwn	11 (5)	12,5 (2)		14 (2)	11 (5)	
N %						
0	20 (9)	31 (5)	0,575#	7 (1)	28 (13)	0,335#
1	25 (11)	12,5 (2)		29 (4)	20 (9)	
2	14 (6)	6 (1)		7 (1)	13 (6)	
3	16 (7)	12,5 (2)		21 (3)	13 (6)	
Unkonwn	25 (11)	37,5 (6)		36 (5)	26 (12)	
M %						
0	80 (35)	81 (13)	0,700£	64 (9)	85 (39)	0,380£
1	14 (6)	19 (3)		21 (3)	13 (6)	
Unkonwn	7 (3)	-		14 (2)	2 (1)	
Tumor Grade %						
1	-	6 (1)	0,286#	-	2 (1)	0,338#
2	48 (21)	37,5 (6)		57 (8)	41 (19)	
3	32 (14)	19 (3)		29 (4)	28 (13)	
Unkonwn	20 (9)	37,5 (6)		14 (2)	28 (13)	
HER2 %						
Positive	32 (14)	37,5 (6)	1,000£	36 (5)	33 (15)	0,754£
Negative	61 (27)	62,5 (10)		57 (8)	63 (29)	
Unkonwn	7 (3)	-		7 (1)	4 (2)	
ER %						
Positive	70 (31)	81 (13)	0,519£	43 (6)	83 (38)	**0,006£**
Negative	30 (13)	19 (3)		57 (8)	17 (8)	
PR %						
Positive	43 (19)	56 (9)	0,397£	21 (3)	54 (25)	**0,037£**
Negative	57 (25)	44 (7)		79 (11)	46 (21)	

Statistical analysis for categorical variables were performed using *Student's *t* test; £Fisher's exact test; #Chi-square test; Abbreviations: HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; TNM system – evaluation of tumor progression: T-Primary Tumor, N-Regional lymph nodes, M-Distant metastasis

Concerning clinical outcomes, YB-1 expression in primary tumors was associated with decreased DMFS (*P*=0.0442, HR 0.5514 95%CI 0.3088-0.9846) but not with OS (*P*=0.2473, HR 0.6097 95%CI 0.3704-1.292) (Figure 7). p-YB-1 expression in primary tumors was also associated with lower DMFS (*P*=0.0108, HR 0.058 95%CI 0.1230-0.7606), showing also a trend for lower OS (*P*=0.0687, HR 0.4789 95%CI 0.2168-1.058) (Figure 7). Therefore, YB-1 and p-YB-1 could be biomarkers of poor prognosis, namely for risk of relapse, especially in patients with ER-/PR- tumors.

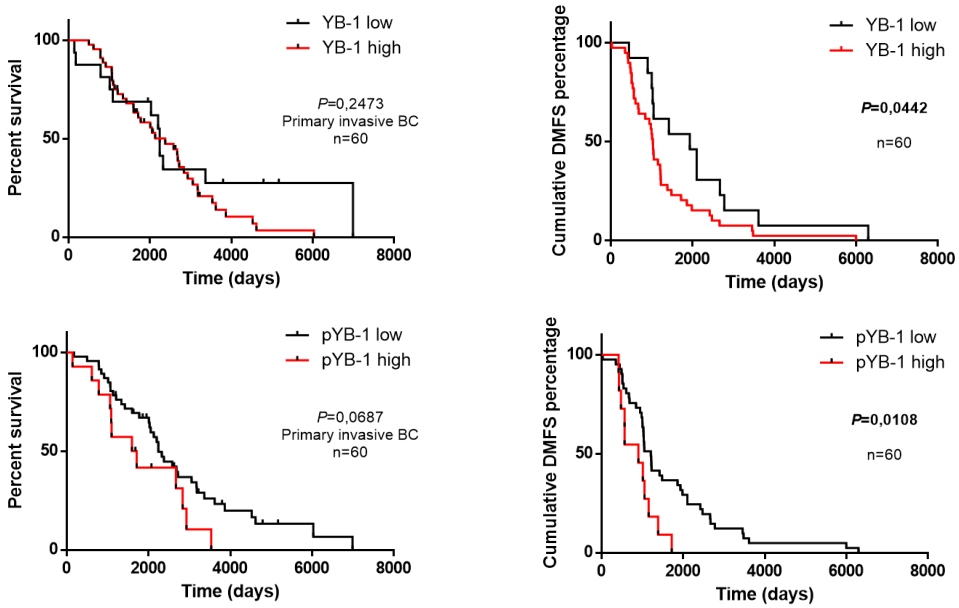


Figure 7 Kaplan-Meier overall survival (Percent survival) and distant metastasis-free survival (DMFS) curves according to YB-1 and p-YB-1(Ser102) in primary invasive BC patients (n=60). *P*-value was calculated using log-rank test.

Previous studies had already shown a correlation between YB-1 and OS and relapse[41–43,51,77,78]. Furthermore, Habibi and his colleagues revealed that YB-1 correlates with patient's outcome and disease recurrence in all BC subtypes[42]. However, YB-1 value as prognostic marker has been controversial, due to the disparity of results, especially regarding the association with clinicopathologic characteristics, as mentioned above. A recent analysis, pointed the small number of patients and a high dependence on the antibody used as causes for these disparities[80]. One important aspect will be the epitope availability, since the C-terminal portion of YB-1 is implicated in binding to mRNA or other proteins, and post-translational modifications may change YB-1 native conformation; epitope could be masked during fixation procedures[42,51,80]. However, it has been shown thar among the three commonly used epitopes (3-12, 23-53, 299-313) only epitope 23-53 may lead to a lack of specificity, and 3-12 will probably be the best epitope[80]. However, there are not available commercial antibodies raised against this epitope.

On the other hand, p-YB-1 was never described as a prognostic marker in BC. Detection of p-YB-1 in solid tumors was only reported in patients with ovarian cancer[81]. In this study, both p-YB-1 overall score and the presence of nuclear staining were significantly associated with poor overall survival (*P* = 0.02, HR = 1.86, 95% CI = 1.10 to 3.17 and *P* = 0.005, HR = 2.41, 95% CI = 1.34 to 4.33, respectively).

In our study we observe a significant correlation of p-YB-1 with ER-/PR- BC tumors, which has been already reported for YB-1[42,43,78,80]. Tumors deprived of hormone receptors include the more aggressive basal-like and HER2+ BC[7,10]. Further studies are needed to analyze the potential role of p-YB-1 as biomarker of poor prognosis in the different subtypes of BC. It should be noted that our cohort only includes patients with advance BC. For both markers, the significance in OS could be improved by increasing our cohort and adding a group of BC patients without metastatic disease.

We also assessed the relevance of both YB-1 and p-YB-1 in 32 paired metastases. Among metastases, 20/32 samples (62.5%) had high YB-1 expression and 10/31 high p-YB-1 expression (32.3%) (Table 4). YB-1 staining showed no association with receptor status at metastatic site, but high p-YB-1 was correlated with PR negativity in metastases (*P*=0,030). Our findings could be improved by increasing the number of samples and also by analyzing different sites of metastases individually.

Table 4 Association between YB-1 and p-YB-1 and clinical characteristics in paired metastasis of BC patients

Characteristics		YB-1 (n)		*P*	p-YB-1 (n)		*P*
		High	Low		High	Low	
No. of Patients		20	12		10	21	
Adjuvant Endocrine Therapy %							
	Yes	50 (10)	83 (10)	0,128[£]	40 (4)	76 (16)	0,116[£]
	No	45 (9)	17 (2)		50 (5)	24 (5)	
	Unknown	5 (1)			10 (1)		
Adjuvant Therapy %							
	Yes	90 (18)	92 (11)	1,000[£]	80 (8)	95 (20)	0,517[£]
	No	5 (1)	8 (1)		10 (1)	5 (1)	
	Unknown	5 (1)			10 (1)		
HER2 %							
	Positive	40 (8)	8 (1)	0,103[£]	40 (4)	19 (4)	0,381[£]
	Negative	60 (12)	92 (11)		60 (6)	81 (17)	
ER %							
	Positive	60 (12)	75 (9)	0,465[£]	60 (6)	71 (15)	0,685[£]
	Negative	40 (8)	25 (3)		40 (4)	29 (6)	
PR %							
	Positive	20 (4)	42 (5)	0,240[£]	-	43 (9)	**0,030[£]**
	Negative	80 (16)	58 (7)		100 (10)	57 (12)	

Statistical analysis for categorical variables were performed using [£]Fisher's exact test. Abbreviations: HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor;

In this setting, p-YB-1 was prognostic of decreased OS (*P*=0.009, HR 0.2394 95%CI 0.08186-0.7002) and patients with high YB-1 also show a trend for decreased OS (*P*=0.0656, HR 0.4672 95%CI 0.2070-1.05) (Figure 8).

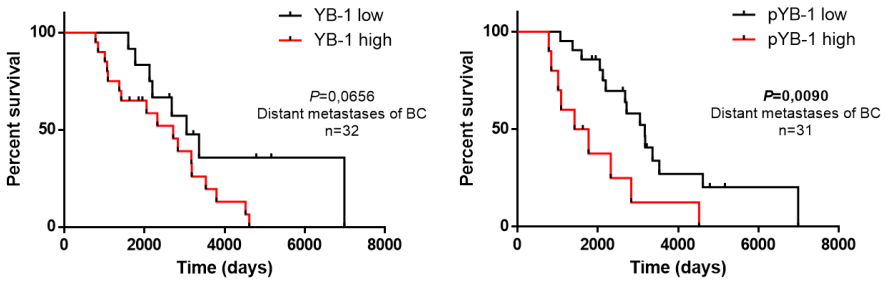


Figure 8 Kaplan-Meier overall survival (Percent survival) curves according to YB-1 and p-YB-1(Ser102) in paired metastases of BC patients (n=32). *P*-value was calculated using a log-rank test.

Next we assessed whether YB-1 and p-YB-1 expression differs between primary tumors and metastases, reflecting a change in expression associated to tumor progression. Statistical analysis and median comparison show that there is no association between YB-1 or p-YB-1 expression and the origin of the sample (McNemar's test: *P*=0.7728 and *P*=0.0771, respectively; Paired t-test: *P*=0.5754 and *P*=0.1883, respectively) (Figure 9). Despite p-YB-1 showed a trend to be higher at metastatic site, no significant result was obtained.

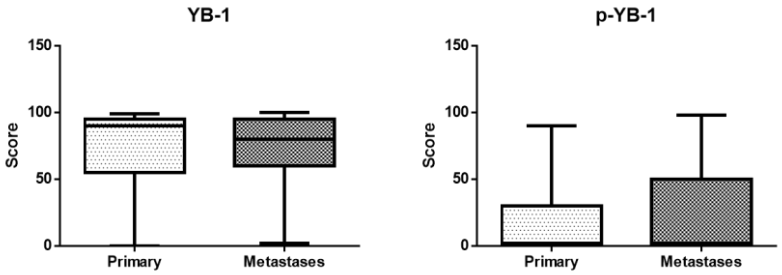


Figure 9 Blox graphs of YB-1 and p-YB-1 scores in primary and paired metastases samples

YB-1 expression was concordant between primary tumors and metastases in 19/31 cases (61,3%), while p-YB-1 was concordant in 22/30 cases (71,3%) (Figure 10).

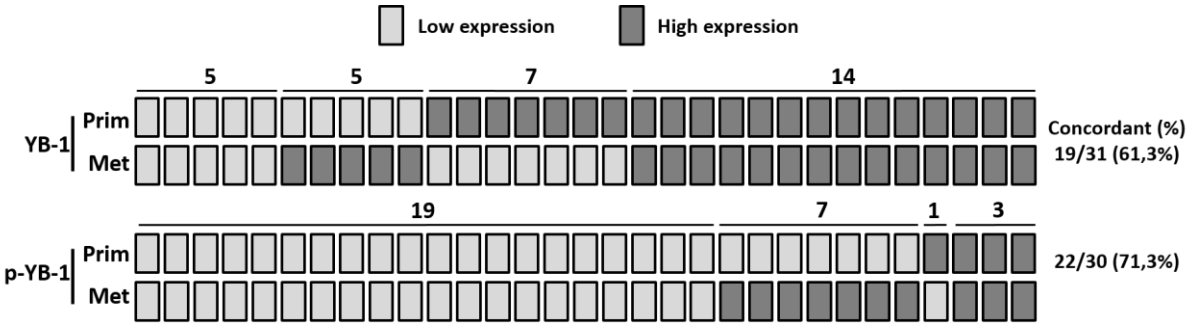


Figure 10 Concordance of YB-1 and p-YB-1 between primary tumors and paired metastases

Increased levels of p-YB-1 during tumor progression even not reaching statistically significance (*P*=0.0771), could have prognostic value for a more aggressive disease.

The present study presents, to our knowledge, the first analysis of YB-1 and p-YB-1 expression in primary breast tumors and paired distant metastases. Moreover, as OS was significantly correlated with p-YB-1 at metastases, this biomarker could be considered in treatment adjustments as a marker of aggressiveness.

4.3 Prognostic value of seric YB-1 in BC patients with bone metastatic disease

Since YB-1 in tumor tissues has been shown to be a biomarker of poor prognosis, including decreased OS, attempts have been made to assess if YB-1 detection in the serum of patients could represent an equally significant, but of easier application, prognostic method[58].

Recently, it was identified a fragment of YB-1 in human serum[58] and its presence was associated with the diagnosis of different malignancies, including BC[59]. This study included a small cohort of BC patients and only seven out of ten were positive to seric YB-1.

Therefore, we aimed to assess the prognostic value of serum YB-1 in patients with BC. In this retrospective cohort study, we included 44 patients with BM from BC, to which peripheral blood was collected at the time of BM diagnosis. YB-1 was detected in the serum of 22 patients (50%), and correlated with the presence of extra-bone metastases (*P*=0.044), but not with other relevant clinicopathological characteristics (Table 5).

A non-significant trend towards ER-negativity, radiographically mixed BM lesions, and HT was also found. Since we anticipated that HT could decrease YB-1 levels in BC cells during the treatment, one possible explanation will be that BC cells are not the major contributors to secreted YB-1 and that secreted YB-1 may not be under ER-regulation.

Table 5 Association between YB-1 seric levels and clinical and pathological characteristics of patients with BM from BC

Characteristics	YB-1 (n)		*P*
	Positive	Negative	
No. of Patients	22	22	
Age at diagnosis (years)			
Median	48,4	55,9	0,425
Range	40,9-62,1	44,5-65,6	
Age (years) (%)			
<35	4,6 (1)	0 (0)	0,252
35-49	50 (11)	31,8 (7)	
50-69	27,2 (6)	54,6 (12)	
≥70	18,2 (4)	13,6 (3)	
Histology (%)			
Ductal carcinoma	77,4 (17)	77,4 (17)	1
Lobular carcinoma	4,5 (1)	9,1 (2)	
Other	4,5 (1)	9,1 (2)	
Unkonwn	13,6 (3)	4,4 (1)	
Hormone Receptor Status (%)			
ER+ or PR+	86,4 (19)	72,7 (16)	0,281
ER- and PR-	13,6 (3)	31,8 (7)	
Unkonwn	4,5 (1)	0 (0)	
HER2 (%)			
Positive	18,2 (4)	13,6 (3)	0,691
Negative	59,1 (13)	68,2 (15)	
Unkonwn	22,7 (5)	18,2 (4)	
Metastatic at diagnosis (%)			
Yes	18,2 (4)	18,2 (4)	1
No	77,3 (17)	68,2 (15)	
Unkonwn	4,5 (1)	13,6 (3)	
Age at diagnosis of bone involvement			
Median	59,1	61,3	0,378
P25-P75	46,8-75,9	52,8-67,0	
Unkonwn	0 (0)	9,1 (2)	
Radiographic pattern of bone lesions (%)			
Lytic	68,2 (15)	40,9 (9)	0,064
Blastic	18,2 (4)	9,1 (2)	
Mixed	9,1 (2)	36,4 (8)	
Unkonwn	4,5 (1)	13,6 (3)	
Metastatic disease outside bone			
Yes	77,3 (17)	45,5 (10)	**0,044**
No	22,7 (5)	50,0 (11)	
Unkonwn	0 (0)	4,5 (1)	
Receiving HT at time of YB-1 measurement (%)			
Yes	50,0 (11)	18,2 (4)	0,066
No	22,7 (5)	45,4 (10)	
Unkonwn	27,3 (6)	36,4 (8)	

Multivariate analysis showed that positive serum YB-1 is a marker of faster bone disease progression (HR 3.29, 95% CI 1.13 – 9.60, *P*=0.029) (Figure 11B). No significant differences were observed concerning OS (HR 2.04, 95% CI 0.86 – 4.87, *P*=0.108) (Figure 11A), and time to development of skeletal-related events (HR 1.45, 95% CI 0.53 – 4.00, *P*=0.467), although patients with positive YB-1 in serum had decreased median time between events (Figure 11C).

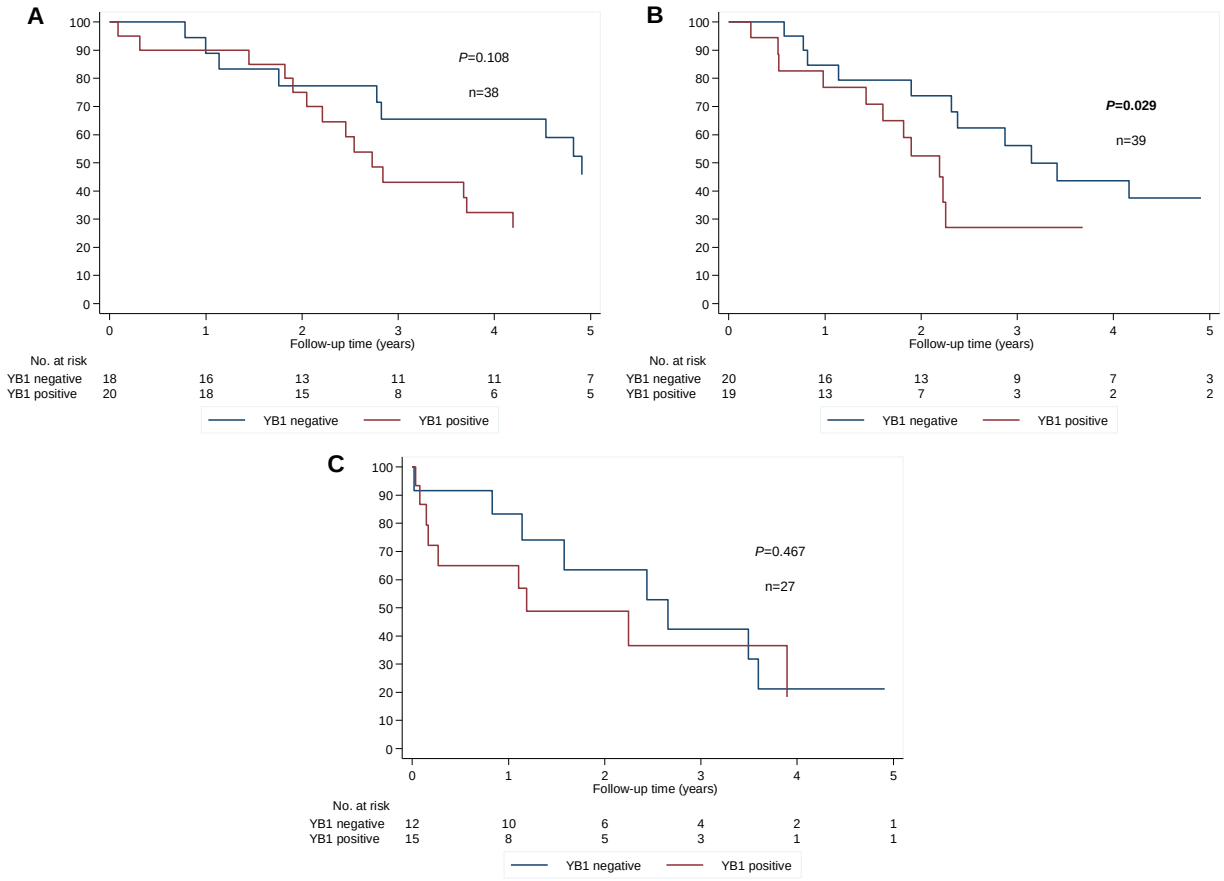


Figure 11 Kaplan-Meier Overall survival (A), Time to bone disease progression (B) and Skeletal Related Events (C) curves according to YB-1 secretion at baseline. Multivariate analysis controlling (A) for age at diagnosis, hormone receptor status, extra-bone metastases, (B) for age at diagnosis, hormone receptor status, extra-bone metastases and radiographic pattern, (C) for hormone receptor status and radiographic pattern of BM. *P*-value was calculated using log-rank test.

To the best of our knowledge, we present the first analysis of seric YB-1 in patients with BC. Our data suggests that the presence of YB-1 in serum of patients with BC and BM is a biomarker of a more aggressive disease, with faster bone disease progression. Interestingly it was recently found a direct interplay between YB-1 and IL-6 in regulating BC metastasis[53]. In this work overexpression of YB-1 in BC cell lines induced IL-6 production while stimulation with IL-6 increased YB-1 expression and YB-1 phosphorylation. Either approach was sufficient to induce EMT features, including increased cell migration and invasion. Very importantly, IL-6 is a known mediator of bone remodeling and plays a role in BM by inducing osteoclastogenesis (reviewed in [82]). Moreover, IL-6 has been found to be up-regulated in patients with BC. IL-6

also plays an important role in promoting metastases to organs other than the bone, by attracting circulating tumor cells to these organs, and we found a correlation with extra-bone metastases in patients with high seric YB-1.

In order to could elucidate why high YB-1 was found to be correlated with faster bone disease progression, we will explore in our cohort the association between seric YB-1 and IL-6, as it

5. CONCLUSION AND FUTURE PERSPECTIVES

YBX1 was recently described as an ER-regulated gene that is overexpressed in ER+ BC patients with poor clinic outcome[18]. Several studies have reported YB-1 as a marker of bad prognosis across all BC subtypes, being correlated with the absence of hormone receptors and linked to resistance to chemotherapy. Although several groups have shown that YB-1 may modulate ER in a negative way, the axis ER-YB-1 and its role in cancer is still unclear. This was the starting point for this project that aimed to establish a biological and clinical link between YB-1 and ER pathway.

In vitro, we observed that ER pathway activation by estrogens *per se* does not affect *YBX1* mRNA levels in an ER+BC cell line, but leads to an increase of YB-1 protein phosphorylation and nuclear translocation. We also observed that ER-regulation *in vitro* differs substantially from what we observed in the *in vivo* model. As previously mentioned these results require confirmation. So, taking into account that the localization and expression of YB-1 protein was not totally clear, it should be assessed in nuclear and cytoplasmic extracts. Importantly, mechanistic studies are needed to clarify how ER pathway mediates YB-1 phosphorylation and protein levels, and its relevance.

In the clinical setting our main goal was to address a possible correlation between YB-1 expression and ER+ BC. We analyzed for the first time p-YB-1 as a prognostic marker in BC. Notably, high expression of p-YB-1 in primary tumors was associated with decreased DMFS, especially in patients with ER-/PR- tumors. In metastases, high expression of p-YB-1 was associated with decreased OS in PR- paired samples. YB-1 was also correlated with decreased DMFS. Therefore, our findings contribute with new significant data to the putative use of p-YB-1 and YB-1 as biomarkers of prognosis in BC. This study requires further validation in larger and independent cohorts, including BC patients without metastases and more ER+BC patients.

We also conducted the first analysis with prognostic power of secreted YB-1 in the serum of BC patients. The seropositivity of YB-1 was correlated with faster bone disease progression and with the presence of extra-bone metastases. These findings suggest that evaluation of YB-1 levels in the serum is a useful minimally invasive technique to monitor the progression of bone metastatic disease in BC patients. Prospectively, to evaluate the prognostic value of YB-1 and a possible correlation with disease progression and OS, we plan to include a cohort of patients with only primary BC. We will also explore the axis YB-1/IL-6/BM in a mechanistic perspective. Moreover, the cellular source of seric YB-1 in cancer patients is still unknown. As far as we know, *in vitro* studies only identified secretion of YB-1 in granulocytes, lymphocytes and monocytes cells in response to inflammation[56,83]. Further *in vitro* studies will be performed to assess the secretion of YB-1 by BC cells. Globally, the results generated in this project are extremely relevant for the research of YB-1 in BC.

6. REFERENCES

1. GLOBOCAN 2012 world incidence. at <http://globocan.iarc.fr/old/pie_pop.asp?selection=224900&title=World&sex=0&type=0&window=1&join=1&submit=%C2%A0Execute%C2%A0>

2. GLOBOCAN 2012 world mortality. at <http://globocan.iarc.fr/old/pie_pop.asp?selection=224900&title=World&sex=0&type=1&window=1&join=1&submit=%C2%A0Execute%C2%A0>

3. GLOBOCAN. at <http://globocan.iarc.fr/old/burden.asp?selection_pop=224900&Text-p=World&selection_cancer=3152&Text-c=Breast&pYear=8&type=0&window=1&submit=%C2%A0Execute%C2%A0>

4. GLOBOCAN. at <http://globocan.iarc.fr/old/burden.asp?selection_pop=224900&Text-p=World&selection_cancer=3152&Text-c=Breast&pYear=8&type=1&window=1&submit=%C2%A0Execute%C2%A0>

5. GLOBOCAN 2012 portugal incidence. at <http://globocan.iarc.fr/old/pie_pop.asp?selection=158620&title=Portugal&sex=0&type=0&window=1&join=1&submit=%C2%A0Execute%C2%A0>

6. GLOBOCAN 2012 portugal mortality. at <http://globocan.iarc.fr/old/pie_pop.asp?selection=158620&title=Portugal&sex=0&type=1&window=1&join=1&submit=%C2%A0Execute%C2%A0>

7. Schnitt, S. J. Classification and prognosis of invasive breast cancer: from morphology to molecular taxonomy. *Mod. Pathol.* **23 Suppl 2,** S60–S64 (2010).

8. Cheang, M. C. U. *et al.* Ki67 Index , HER2 Status , and Prognosis of Patients With Luminal B Breast Cancer. **101,** (2009).

9. Cheang, M. C. U. *et al.* Basal-Like Breast Cancer Defined by Five Biomarkers Has Superior Prognostic Value thanT riple-Negative Phenotype. **14,** 1368–1377 (2008).

10. Perou, C. M. *et al.* Molecular portraits of human breast tumours. *Nature* **406,** 747–752 (2000).

11. Clark, G. M., Osborne, C. K. & McGuire, W. L. Correlations between estrogen receptor, progesterone receptor, and patient characteristics in human breast cancer. *J. Clin. Oncol.* **2,** 1102–9 (1984).

12. Kennecke, H. *et al.* Metastatic behavior of breast cancer subtypes. *J. Clin. Oncol.* **28,** 3271–3277 (2010).

13. Breast, H., Clarke, B. & Potten, S. Human Breast'. 4987–4991 (1997).

14. Rachner, T. D., Schoppet, M., Niebergall, U. & Hofbauer, L. C. 17β-Estradiol inhibits osteoprotegerin production by the estrogen receptor-α-positive human breast cancer cell line MCF-7. *Biochem. Biophys. Res. Commun.* **368,** 736–741 (2008).

15. Kuiper, G. G. J. M., Enmark, E. V. A., Pelto-huikkot, M., Nilssont, S. & Ii, J. G. and ovary Cloning of a novel estrogen receptor expressed in rat prostate. **93,** 5925–5930 (1996).

16. Mosselman, S., Polman, J. & Dijkema, R. ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett* **392,** 49–53 (1996).

17. Williams, C. & Lin, C.-Y. Oestrogen receptors in breast cancer: basic mechanisms and clinical implications. *Ecancermedicalscience* **7,** 370 (2013).

18. Ross-Innes, C. S. *et al.* Differential oestrogen receptor binding is associated with clinical

outcome in breast cancer. *Nature* 1–16 (2012). doi:10.1038/nature10730

19. Yarden, R. I., Wilson, M. A. & Chrysogelos, S. A. Estrogen suppression of EGFR expression in breast cancer cells: A possible mechanism to modulate growth. *J. Cell. Biochem.* **81,** 232–246 (2001).

20. Newman, S. P., Bates, N. P., Vernimmen, D., Parker, M. G. & Hurst, H. C. Cofactor competition between the ligand-bound oestrogen receptor and an intron 1 enhancer leads to oestrogen repression of ERBB2 expression in breast cancer. *Oncogene* **19,** 490–7 (2000).

21. Guo, S. & Sonenshein, G. E. Forkhead box transcription factor FOXO3a regulates estrogen receptor alpha expression and is repressed by the Her-2/neu/phosphatidylinositol 3-kinase/Akt signaling pathway. *Mol. Cell. Biol.* **24,** 8681–90 (2004).

22. Cui, X. *et al.* Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: progesterone receptor as a potential indicator of growth factor activity in brea. *Mol. Endocrinol.* **17,** 575–88 (2003).

23. Osborne, C. K. & Schiff, R. Mechanisms of Endocrine Resistance in Breast Cancer. *Annu. Rev. Med.* **62,** 233–247 (2011).

24. Goldhirsch, A. *et al.* Strategies for subtypes-dealing with the diversity of breast cancer: Highlights of the St Gallen international expert consensus on the primary therapy of early breast cancer 2011. *Ann. Oncol.* **22,** 1736–1747 (2011).

25. Wolffe, A. P., Tafuri, S., Ranjan, M. & Familari, M. The Y-box factors: a family of nucleic acid binding proteins conserved from Escherichia coli to man. *New Biol.* **4,** 290–8 (1992).

26. Lyabin, D. N., Eliseeva, I. a. & Ovchinnikov, L. P. YB-1 protein: Functions and regulation. *Wiley Interdiscip. Rev. RNA* **5,** 95–110 (2014).

27. Didier, D. K., Schiffenbauer, J., Woulfe, S. L., Zacheis, M. & Schwartz, B. D. Characterization of the cDNA encoding a protein binding to the major histocompatibility complex class II Y box. *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7322–6 (1988).

28. Sakura, H., Maekawa, T., Imamoto, F., Yasuda, K. & Ishii, S. Two human genes isolated by a novel method encode DNA-binding proteins containing a common region of homology. *Gene* **73,** 499–507 (1988).

29. Jurchott, K. *et al.* YB-1 as a cell cycle-regulated transcription factor facilitating cyclin A and cyclin B1 gene expression. *J. Biol. Chem.* **278,** 27988–96 (2003).

30. Lasham, A. *et al.* YB-1, the E2F pathway, and regulation of tumor cell growth. *J. Natl. Cancer Inst.* **104,** 133–146 (2012).

31. Bargou, R. C. *et al.* Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic MDR1 gene expression. *Nat. Med.* **3,** 447–50 (1997).

32. Bommert, K. S. *et al.* The feed-forward loop between YB-1 and MYC is essential for multiple myeloma cell survival. *Leukemia* **27,** 441–50 (2013).

33. Astanehe, A. *et al.* The transcriptional induction of PIK3CA in tumor cells is dependent on the oncoprotein Y-box binding protein-1. *Oncogene* **28,** 2406–18 (2009).

34. MINICH, W. B., MAIDEBURA, I. P. & OVCHINNIKOV, L. P. Purification and characterization of the major 50-kDa repressor protein from cytoplasmic mRNP of rabbit

reticulocytes. *Eur. J. Biochem.* **212,** 633–638 (1993).

35. Koike, K. *et al.* Nuclear translocation of the Y-box binding protein by ultraviolet irradiation. *FEBS Lett.* **417,** 390–394 (1997).

36. Sorokin, A. V *et al.* Proteasome-mediated cleavage of the Y-box-binding protein 1 is linked to DNA-damage stress response. *EMBO J.* **24,** 3602–12 (2005).

37. Stratford, A. L. *et al.* Y-box binding protein-1 serine 102 is a downstream target of p90 ribosomal S6 kinase in basal-like breast cancer cells. *Breast Cancer Res.* **10,** R99 (2008).

38. Sutherland, B. W. *et al.* Akt phosphorylates the Y-box binding protein 1 at Ser102 located in the cold shock domain and affects the anchorage-independent growth of breast cancer cells. *Oncogene* **24,** 4281–92 (2005).

39. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144,** 646–74 (2011).

40. Lasham, A., Print, C. G., Woolley, A. G., Dunn, S. E. & Braithwaite, A. W. YB-1: oncoprotein, prognostic marker and therapeutic target? *Biochem. J.* **449,** 11–23 (2013).

41. Janz, M. *et al.* Y-box factor YB-1 predicts drug resistance and patient outcome in breast cancer independent of clinically relevant tumor biologic factors HER2, uPA and PAI-1. *Int. J. Cancer* **97,** 278–82 (2002).

42. Habibi, G. *et al.* Redefining prognostic factors for breast cancer: YB-1 is a stronger predictor of relapse and disease-specific survival than estrogen receptor or HER-2 across all tumor subtypes. *Breast Cancer Res.* **10,** R86 (2008).

43. Wang, X. *et al.* Prognostic role of YB-1 expression in breast cancer : a. **8,** 1780–1791 (2015).

44. Giménez-Bonafé, P. *et al.* YB-1 is upregulated during prostate cancer tumor progression and increases P-glycoprotein activity. *Prostate* **59,** 337–49 (2004).

45. Wu, Y. *et al.* Strong YB-1 expression is associated with liver metastasis progression and predicts shorter disease-free survival in advanced gastric cancer. *J. Surg. Oncol.* **105,** 724–30 (2012).

46. Shibahara, K., Sugio, K. & Osaki, T. Nuclear Expression of the Y-Box Binding Protein , YB-1 , as a Novel Marker of Disease Progression in Non-Small Cell Lung Cancer Nuclear Expression of the Y-Box Binding Protein , YB-1 , as a Novel Marker of Disease Progression in Non-Small Cell Lung Cancer. **7,** 3151–3155 (2001).

47. Expression of YB-i Protein Correlates with Expression in Human Osteosarcoma. *Clin. Cancer Res.* **4,** 2273–2277 (1998).

48. Sinnberg, T. *et al.* MAPK and PI3K/AKT mediated YB-1 activation promotes melanoma cell proliferation which is counteracted by an autoregulatory loop. *Exp. Dermatol.* **21,** 265–70 (2012).

49. Chatterjee, M. *et al.* The Y-box binding protein YB-1 is associated with progressive disease and mediates survival and drug resistance in multiple myeloma. *Blood* **111,** 3714–22 (2008).

50. Lasham, A. *et al.* The Y-box-binding protein, YB1, is a potential negative regulator of the p53 tumor suppressor. *J. Biol. Chem.* **278,** 35516–23 (2003).

51. Wu, J. Disruption of the Y-Box Binding Protein-1 Results in Suppression of the Epidermal Growth Factor Receptor and HER-2. *Cancer Res.* **66,** 4872–4879 (2006).

52. Lovett, D. H., Cheng, S., Cape, L., Pollock, A. S. & Mertens, P. R. YB-1 alters MT1-MMP trafficking and stimulates MCF-7 breast tumor invasion and metastasis. *Biochem. Biophys. Res. Commun.* **398,** 482–8 (2010).

53. Castellana, B., Aasen, T., Moreno-bueno, G. & Dunn, S. E. Interplay between YB-1 and IL-6 promotes the metastatic phenotype in breast cancer cells. (2015). doi:10.18632/oncotarget.5664

54. Bergmann, S. *et al.* YB-1 provokes breast cancer through the induction of chromosomal instability that emerges from mitotic failure and centrosome amplification. *Cancer Res.* **65,** 4078–4087 (2005).

55. Ito, T. *et al.* Alteration of Y-box binding protein-1 expression modifies the response to endocrine therapy in estrogen receptor-positive breast cancer. *Breast Cancer Res. Treat.* **133,** 145–59 (2012).

56. Frye, B. C. *et al.* Y-box protein-1 is actively secreted through a non-classical pathway and acts as an extracellular mitogen. *EMBO Rep.* **10,** 783–9 (2009).

57. Moiseeva, N. I. *et al.* Effects of Extracellular YB 1 Protein on Cultured Cells of Human Breast Cancer 1. **7,** 21–28 (2013).

58. Tacke, F. *et al.* Y-box protein-1/p18 fragment identifies malignancies in patients with chronic liver disease. *BMC Cancer* **11,** 185 (2011).

59. Tacke, F. *et al.* High prevalence of Y-box protein-1/p18 fragment in plasma of patients with malignancies of different origin. *BMC Cancer* **14,** 33 (2014).

60. Budczies, J. *et al.* Cutoff Finder: A Comprehensive and Straightforward Web Application Enabling Rapid Biomarker Cutoff Optimization. *PLoS One* **7,** 1–7 (2012).

61. Cutoff Finder: introduction. at <http://molpath.charite.de/cutoff/>

62. McNemar test calculator. at <http://graphpad.com/quickcalcs/mcNemar1/>

63. Pawlowski, V., Hornez, L., Peyrat, J. & Re, F. Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer. **36,** 1038–1042 (2000).

64. Shah, K. N. & Faridi, J. S. Estrogen, tamoxifen, and Akt modulate expression of putative housekeeping genes in breast cancer cells. *J. Steroid Biochem. Mol. Biol.* **125,** 219–225 (2011).

65. Pfaffl, M. W., Tichopad, A., Prgomet, C. & Neuvians, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **26,** 509–515 (2004).

66. Experiment < EMBL-EBI. at <http://www.ebi.ac.uk/gxa/experiments/E-MTAB-2770?_specific=on&queryFactorType=CELL_LINE&queryFactorValues&geneQuery=YBX1&exactMatch=true&serializedFilterFactors=DISEASE%3Abreast+invasive+carcinoma>

67. Experiment < EMBL-EBI. at <http://www.ebi.ac.uk/gxa/experiments/E-MTAB-2706?_specific=on&queryFactorType=CELL_LINE&queryFactorValues=MCF-7&geneQuery=YBX1&exactMatch=true&serializedFilterFactors=DISEASE:breast adenocarcinoma,ORGANISM_PART:breast>

68. GTEx Portal. at <http://www.gtexportal.org/home/gene/YBX1>

69. Klinge, C. M. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res.* **29,** 2905–2919 (2001).

70. Berkenstam, A., Glaumann, H., Martin, M., Gustafsson, J.-Å. & Norstedt, G. Hormonal Regulation of Estrogen Receptor Messenger Ribonucleic Acid in T47D CO and MCF-7 Breast Cancer Cells. *Mol. Endocrinol.* **3,** 22–28 (1989).

71. Ellison-zelski, S. J., Solodin, N. M. & Alarid, E. T. Repression of ESR1 through Actions of Estrogen Receptor Alpha and Sin3A at the Proximal Promoter □. **29,** 4949–4958 (2009).

72. Alley, B. E. R. T. W. O. M. Proteasome-dependent degradation of the human estrogen receptor. **96,** 1858–1862 (1999).

73. Popp, S. L. *et al.* Antiestrogens Suppress Effects of Transforming Growth Factor-β in Breast Cancer Cells via the Signaling Axis Estrogen Receptor-α and Y-box Binding Protein-1. **2480,** 2473–2480 (2013).

74. Creighton, C. *et al.* Genes regulated by estrogen in breast tumor cells in vitro are similarly regulated in vivo in tumor xenografts and human breast tumors. *Genome Biol* **7,** R28 (2006).

75. Harvell, D. M. E., Richer, J. K., Allred, D. C., Sartorius, C. A. & Horwitz, K. B. Estradiol regulates different genes in human breast tumor xenografts compared with the identical cells in culture. *Endocrinology* **147,** 700–713 (2006).

76. Miyashita, T. Confocal microscopy for intracellular co-localization of proteins. *Methods Mol. Biol.* **261,** 399–410 (2004).

77. Gluz, O. *et al.* Y-box-binding protein YB-1 identifies high-risk patients with primary breast cancer benefiting from rapidly cycled tandem high-dose adjuvant chemotherapy. *J. Clin. Oncol.* **27,** 6144–51 (2009).

78. Dahl, E. *et al.* Nuclear detection of Y-box protein-1 (YB-1) closely associates with progesterone receptor negativity and is a strong adverse survival factor in human breast cancer. *BMC Cancer* **9,** 410 (2009).

79. Mylona, E. *et al.* Y-box-binding protein 1 (YB1) in breast carcinomas: Relation to aggressive tumor phenotype and identification of patients at high risk for relapse. *Eur. J. Surg. Oncol.* **40,** 289–296 (2014).

80. Woolley, A. G. *et al.* Prognostic association of YB-1 expression in breast cancers: a matter of antibody. *PLoS One* **6,** e20603 (2011).

81. Kang, Y. *et al.* Role of focal adhesion kinase in regulating YB-1-Mediated paclitaxel resistance in ovarian cancer. *J. Natl. Cancer Inst.* **105,** 1485–1495 (2013).

82. Ara, T. & Declerck, Y. A. Interleukin-6 in bone metastasis and cancer progression. *Eur. J. Cancer* **46,** 1223–31 (2010).

83. Hanssen, L. *et al.* YB-1 is an early and central mediator of bacterial and sterile inflammation in vivo. *J. Immunol.* **191,** 2604–13 (2013).

7. APPENDICES

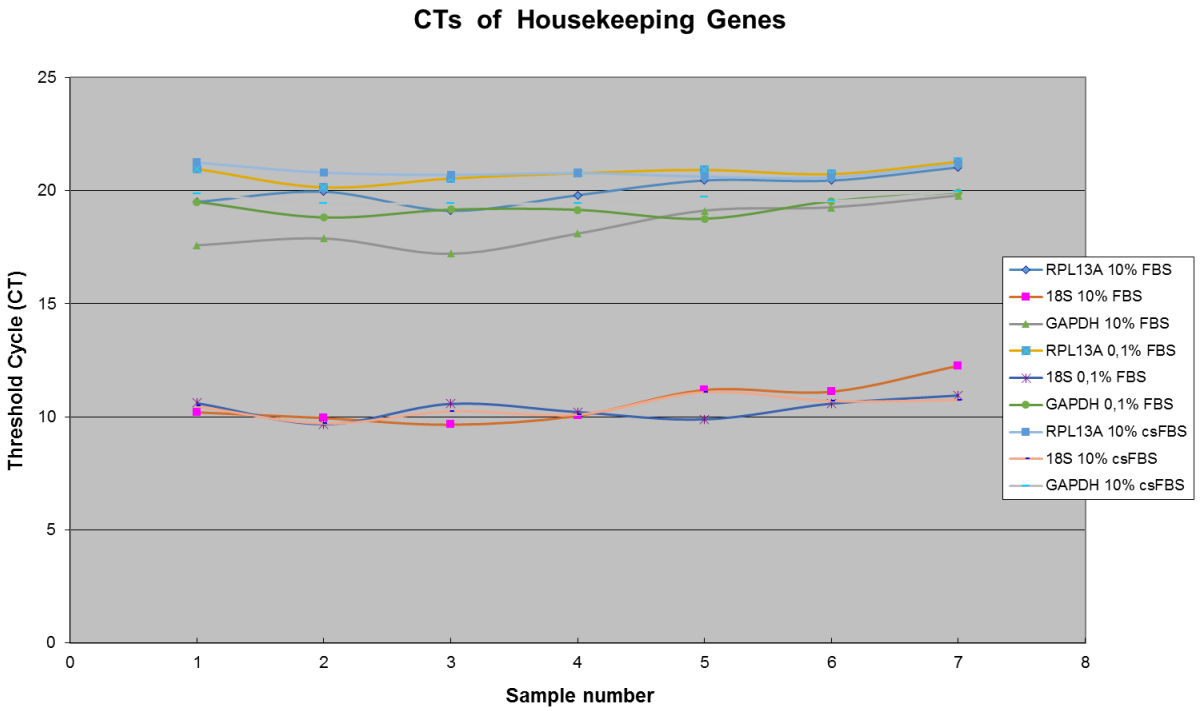


Figure S1 CT values of HKGs under E2 stimuli. Stability of HKGs' expression in cells cultured in different media and under stimuli with increased concentrations of E2.

Table S1 CT data of HKGs using BestKeeper software tool

	RPL13A **10% FBS**	***18S*** **10% FBS**	***GAPDH*** **10% FBS**	***RPL13A*** **0,1% FBS**	***18S*** **0,1% FBS**	***GAPDH*** **0,1% FBS**	***RPL13A*** **10% csFBS**	***18S*** **10% csFBS**	***GAPDH*** **10% csFBS**
n	7	7	7	7	7	7	7	7	7
geo Mean [CT]	20,03	10,60	18,39	20,76	10,35	19,25	20,84	10,43	19,62
ar Mean [CT]	20,04	10,63	18,41	20,76	10,36	19,26	20,84	10,44	19,62
min [CT]	19,11	9,65	17,21	20,14	9,67	18,75	20,59	9,71	19,42
max [CT]	21,03	12,26	19,77	21,27	10,95	19,92	21,25	11,09	19,96
std dev [± CT]	0,51	0,77	0,82	0,25	0,37	0,33	0,21	0,36	0,19
CV [% CT]	2,57	7,21	4,48	1,20	3,57	1,73	1,02	3,42	0,97
min [x-fold]	-1,90	-1,93	-2,27	-1,53	-1,60	-1,42	-1,19	-1,64	-1,15
max [x-fold]	1,99	3,17	2,60	1,43	1,52	1,59	1,33	1,58	1,26
std dev [± x-fold]	1,43	1,70	1,77	1,19	1,29	1,26	1,16	1,28	1,14

Notes: Descriptive statistics of three candidate HKGs based on their CT values. Abbreviations: n, number of samples; geo Mean, geometric mean of CT; ar Mean, arithmetic mean of CT; min and max, extreme values of CT; std dev, standard deviation of the CT; CV, coefficient of variance expressed as a percentage on the CT level; min [x-fold] and max [x-fold] extreme values of expression levels expressed as an absolute x-fold over- or under-regulation coefficient; std dev [± x-fold], standard deviation of the absolute regulation coefficients.

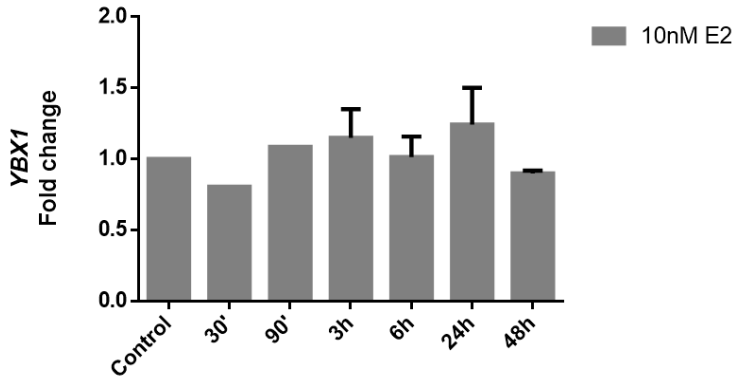


Figure S2 *YBX1* expression under E2 in MCF-7 cell line. Expression of *YBX1* was analyzed by RT-qPCR in MCF-7 breast cancer cell line, after incubation with 10nM E2. Gene fold change was determined using the $2^{-\Delta\Delta Ct}$ method and RPL13A was used as endogenous control. Results are represented as the mean of three independent experiments.

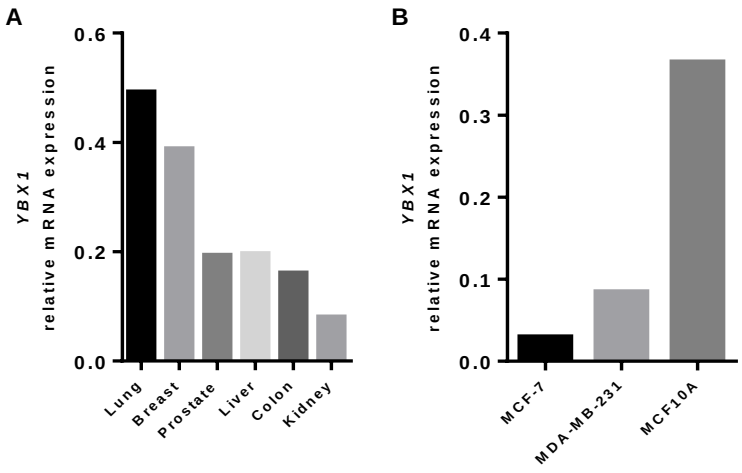
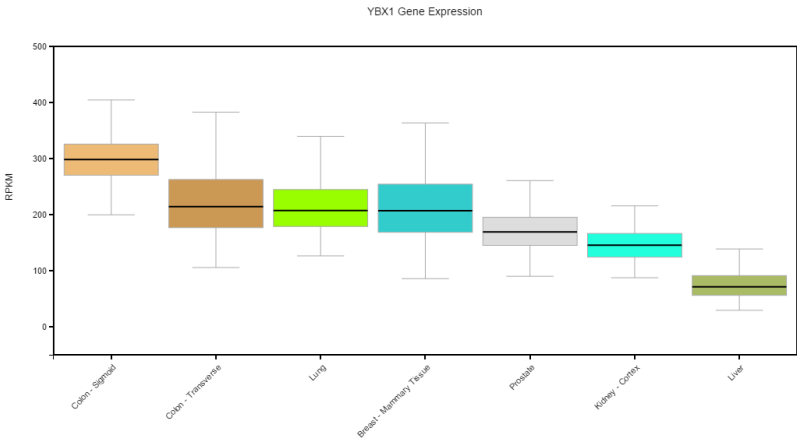


Figure S3 Evaluation of basal *YBX1* expression in human tissues and breast epithelial or tumor cell lines. *YBX1* mRNA was analyzed in human tissues (A) and breast epithelial (MCF10A) and tumoral (MCF-7 and MDA-MB-231) cell lines (B) by RT-qPCR. Relative expression levels were determined by comparative Ct method ($2^{-\Delta Ct}$). GAPDH was used as endogenous control gene.

A



B

Cell Line	Disease	Expression levels
MCF-7	Breast Adenocarcinoma	352
MDA-MB-231	Breast Invasive Carcinoma	508
MCF 10A	Breast fibrocystic disease	674

Figure S4 Reported basal *YBX1* expression levels in human tissues (A) and breast cell lines (B). Expression levels were quantified in Reads Per Kilobase of transcript per Million mapped reads (RPKMs). Data Source: GTEX Analysis Release V6 (A) and Expression Atlas, EMBL-EBI (B).

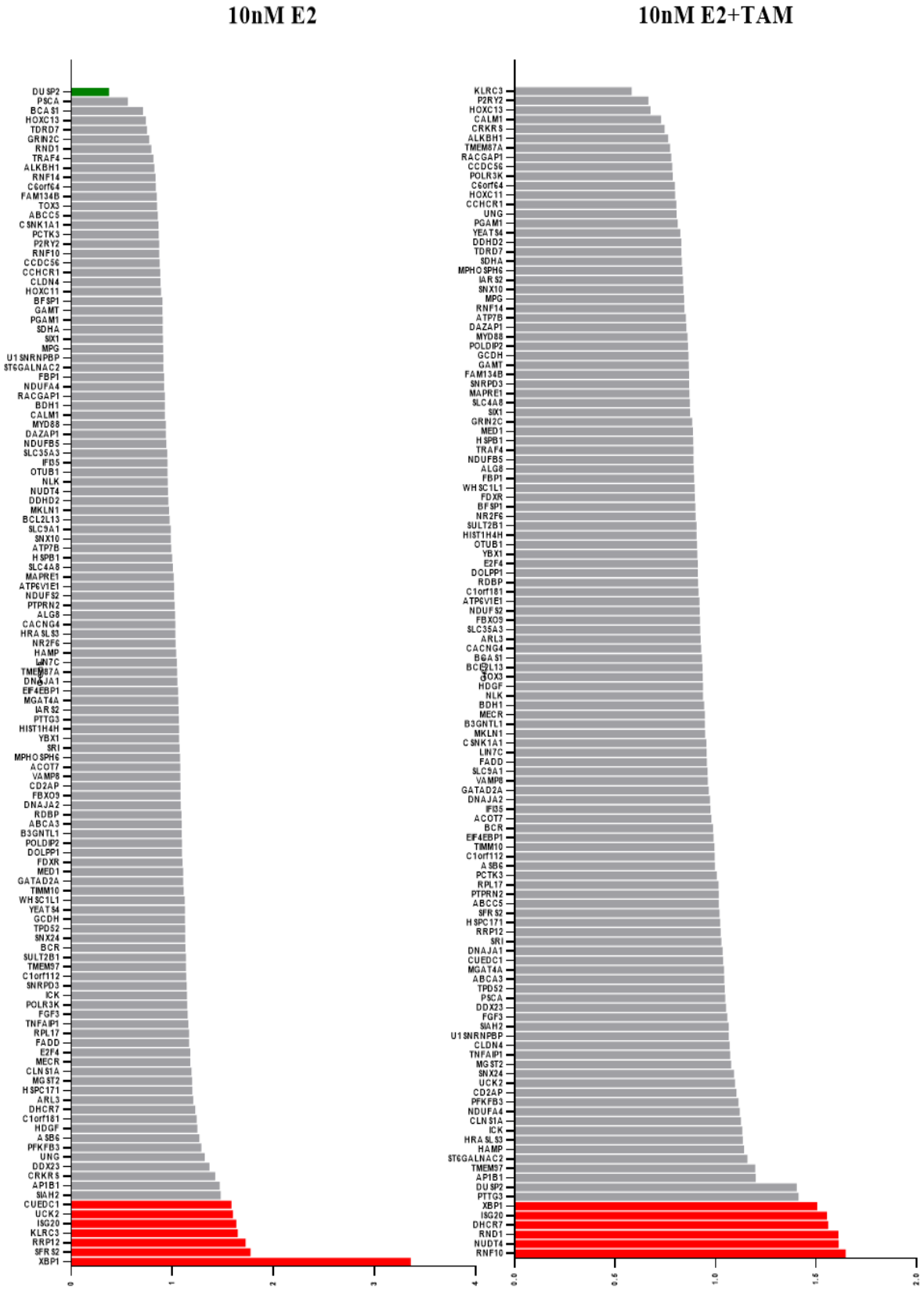


Figure S5 Up and down-regulated ER related genes in MCF-7 cells treated with 17β-Estradiol (E2)±Tamoxifen (TAM). mRNA expression levels were measured by RT-qPCR and gene fold change was determined using the $2^{-\Delta\Delta Ct}$ method. B2M was used as a housekeeping gene, and fold difference was calculated relative to untreated cells. Up-regulated genes are represented in red and down-regulated genes in green.

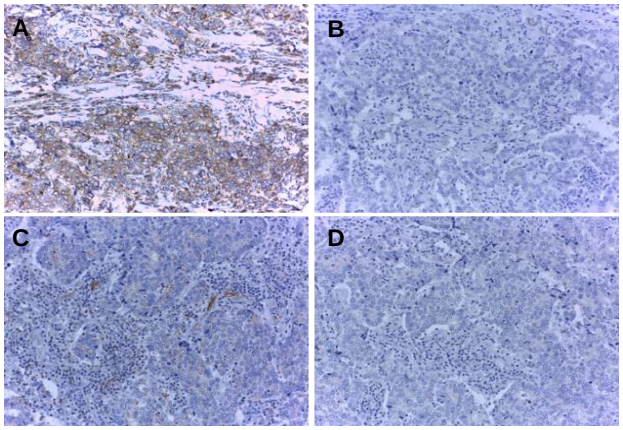


Figure S6 Optimization of detection of YB-1 and p-YB-1 by immunohistochemistry. FFPE tissue sections were incubated with primary antibodies against YB-1 (A) and p-YB-1 (C). Negative controls were performed by omission of the primary antibody for YB-1 (B) or by incubation with phosphatase (CIP) for p-YB-1 (D). Magnification x200.

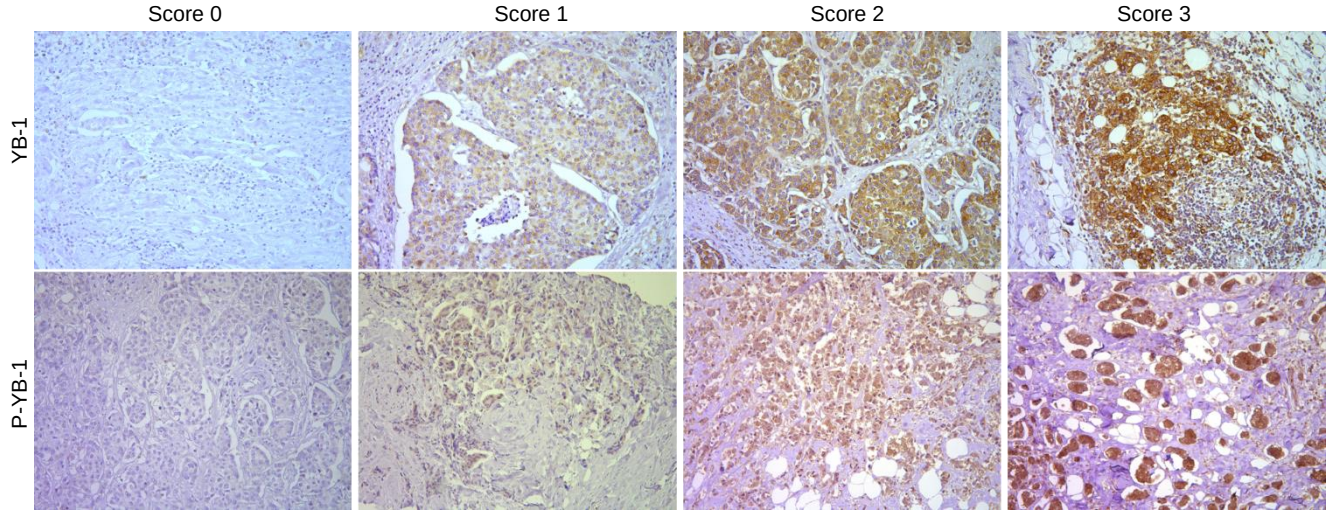


Figure S7 Immunohistochemical staining intensity scores for YB-1 and p-YB-1. (0) no staining, (1) weak, (2) moderated and (3) strong staining. Magnification x200

Table S2 Types of scores tested to analyze overall survival and disease-free survival for patients with primary breast cancer and/or metastatic disease.

		n	Type of cut off value	Outcome	HR	95% CI	*P*
YB-1	Primary tumors	59	Median	OS	1,31	0,74-2,33	0,350
			Cut off finder	OS	0,69	0,37-1,29	0,247
			C3+/-	OS	0,68	0,36-1,29	0,238
			N+/-	OS	1,20	0,67-2,17	0,544
			High/Low	OS	0,93	0,54-1,59	0,786
			Median	DMFS	1,19	0,68-2,06	0,543
			Cut off finder	DMFS	0,55	0,31-0,98	**0,044**
			C3+/-	DMFS	0,93	0,51-1,70	0,810
			N+/-	DMFS	1,19	0,66-2,14	0,564
			High/Low	DMFS	1,24	0,71-2,16	0,444
	Metastases	32	Median	OS	1,75	0,78-3,93	0,177
			Cut off finder	OS	0,47	0,21-1,05	0,066
			C3+/-	OS	1,72	0,75-3,95	0,199
			N+/-	OS	0,82	0,35-1,90	0,637
p-YB-1	Primary tumors	59	Median	OS	0,61	0,34-1,10	0,102
			Cut off finder	OS	0,48	0,22-1,06	0,069
			Pos/Neg	OS	0,77	0,42-1,42	0,402
			N+/-	OS	1,98	0,93-4,22	0,075
			Median	DMFS	0,57	0,31-1,02	0,060
			Cut off finder	DMFS	0,31	0,12-0,76	**0,011**
			N+/-	DMFS	1,28	0,62-2,64	0,502
			Pos/Neg	DMFS	0,79	0,44-1,42	0,431
	Metastases	32	Median	OS	0,50	0,22-1,14	0,100
			Cut off finder	OS	0,24	0,08-0,70	**0,009**
			Pos/Neg	OS	1,80	0,80-4,04	0,157
			N+/-	OS	0,89	0,31-2,52	0,822

Notes: Different score types were used in order to find the best cut off. *P*-values were calculated using a log-rank test. Abbreviations: HR, Hazard-ratio; C3+/-, positive/negative score 3 staining at cytoplasm; N+/-, positive/negative nuclear staining; High correspond to staining 3 or 2 with nuclear staining and low referred to staining 1 or 2 without nuclear staining. Pos/Neg apply to the presence or absence of immunostaining; OS, Overall survival; DMFS, Distant Metastases-Free Survival.

Table S3 Population characteristics of BC samples and paired metastasis

No. of patients	60
Age at diagnosis	
Median	50,74
Range	42,61-61,17
Posmenopausal women	58,3% (35)
Histology	
Invasive Ductal Carcinoma	90% (54)
Invasive Lobular Carcinoma	5% (3)
ER +	73,3% (44)
PR +	46,7% (28)
HER2 +	33,3% (20)
Adjuvant Endocrine Therapy	51,7% (31)
Median time to relapse	2,83 years
No. of paired metastases	32
Metastases samples	
Visceral	50% (16)
Bone	12,5% (4)
Soft tissue	21,9% (7)
Others	15,6% (5)