UNIVERSIDADE DE LISBOA

Faculdade de Medicina

YB-1 expression in breast cancer: modulation of receptor status and therapeutic implications

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Orientada por:

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Dissertação especialmente elaborada para obtenção do grau de Mestre em Oncobiologia

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RESUMO

O cancro da mama é o segundo cancro mais frequente a nível mundial, sendo o mais incidente entre as mulheres. Apesar dos importantes avanços terapêuticos, o cancro da mama metastático continua a estar associado à maior taxa de mortalidade por cancro entre as mulheres.

O cancro da mama é uma doença bastante heterogénea que engloba vários subtipos histológicos e moleculares que influenciam o comportamento, a agressividade tumoral, e consequentemente, o prognóstico clínico. A classificação de cancro da mama com base na expressão molecular do receptor de estrogénio (RE), do receptor de progesterona (RP), do receptor 2 do factor de crescimento (HER2) e do índice proliferativo (Ki-67) tem particular interesse clínico e implicações terapêuticas. Do ponto de vista clínico, existem três subtipos de cancro da mama com maior relevância: o subtipo luminal com dois subgrupos: Luminal A (RE+ e/ou RP+, HER2-, Ki67 baixo) e Luminal B (RE+ e/ou RP+, HER2+ ou Ki67 elevado); o subtipo com sobreexpressão de HER2 ou HER2+ (RE-, PR-, HER2+); e o subtipo triplo negativo (RE-, RP- HER2-). O cancro de mama luminal é o mais comum, correspondendo a cerca de 60% de todos os casos, sendo também o menos agressivo. Uma vez que é caracterizado pela expressão de receptores hormonais e o seu crescimento é hormono-dependente, os doentes com cancro da mama luminal beneficiam de tratamento com hormono-terapia. As opções terapêuticas são várias e incluem o tamoxifeno e o fulvestrant que, por mecanismos diferentes, têm afinidade para o RE, impedindo a ligação do estrogénio e levando ao bloqueio da principal via de sinalização implicada na proliferação das células tumorais. No entanto, cerca de 30% dos casos de cancro de mama luminal torna-se resistente à terapia e os doentes vêm a desenvolver cancro metastático. O cancro metastático é a maior causa de morte por cancro e é considerado o maior desafio quer para os médicos e doentes, quer para a investigação. Assim, é muito importante descobrir novos mecanismos de resistência à terapia, bem como biomarcadores de prognóstico e/ou preditivos de resposta às diversas opções terapêuticas.

A YB-1 é uma proteína multifuncional, que pertence à familia de proteinas *Y-Box Binding Proteins* e que está envolvida em vários processos celulares importantes para o crescimento e o desenvolvimento celular. Geralmente localizada no citoplasma da célula, uma vez activada por fosforilação a YB-1 é translocada para o núcleo onde actua como factor de transcrição e promove a expressão de genes associados ao crescimento, ao ciclo celular e à quimio-resistência. Vários estudos demonstraram o papel oncogénico da YB-1, que foi já associada a todos os *hallmarks* do cancro. Em relação ao cancro da mama, especificamente, a expressão elevada de YB-1 está associada ao subtipo molecular triplo negativo e a um pior prognóstico dos doentes.

Nesta dissertação propusemos estudar a proteína YB-1 em cancro da mama luminal. Neste tipo de cancro, foi já demonstrado *in vitro* que a expressão aumentada de YB-1 está associada à perda da expressão de RE e resistência à hormono-terapia, e ao aumento da transcrição de HER2. Desta forma, colocámos a hipótese de que a expressão elevada de YB-1 em tumores primários de cancro da mama RE+ pode não só estar associada a um mau prognóstico, mas também à resistência à hormono-terapia e à perda de RE nas metástases. Para abordar esta hipótese, avaliámos retrospectivamente a associação entre YB-1 e p-YB-1 e a sobrevida livre de doença e sobrevida global, usando uma coorte de 80 tumores primários e 51 metástases emparelhadas. Avaliámos ainda uma possível associação com alterações no RE, RP e HER2 nas metástases.

Os níveis de proteína foram detectados por imunohistoquímica e as amostras foram avaliadas por dois médicos patologistas independentes. Os resultados mostram que níveis elevados de YB-1 ou a localização nuclear de p-YB-1 estão associados à ausência de RE (p=0,0383 e p=0,0306, respectivamente). Em relação aos *outcomes* clínicos, a elevada expressão de YB-1 nos tumores, bem como a localização nuclear de YB-1 ou p-YB-1, mostraram estar associadas a menor sobrevivência global (p=0,0437; p=0,0221 e p=0,0163, respectivamente). Embora não tenha sido clara uma associação entre a expressão de YB-1 e as alterações moleculares que se observaram entre os tumores primários e as metástases, 45,8% das metástases com *status* molecular

diferente do tumor primário, eram positivas para YB-1 nuclear, em comparação com 37% sem YB-1 nuclear.

Globalmente, a nossa análise de amostras de cancro da mama humano corrobora a importância da YB-1 como biomarcador de mau prognóstico e mostra que esta proteína está implicada na negatividade de RE. Além disso, conseguimos mostrar pela primeira vez que a hormono-terapia adjuvante pode estar implicada na seleção de clones com expressão elevada de YB-1 e que isto pode estar associado com o pior prognóstico de doentes com tumores com elevada expressão de YB-1.

Assim, também questionámos se a resistência adquirida à hormono-terapia em cancro da mama RE+ é acompanhada por uma alteração na expressão de YB-1; e sendo assim, se isso afecta a sensibilidade a outras terapias alvo. Para responder a essas questões, seleccionámos *in vitro* linhas celulares com resistência adquirida a diferentes terapias e avaliámos: a expressão de YB-1, RE, RP, HER2 e Ciclina D1; e a sensibilidade à hormono-terapia, terapia anti-HER2, e inibidores de mTOR e CDK4/6, actualmente usados como agentes únicos ou em combinação, para tratar cancro da mama luminal.

Os nossos resultados mostram que a sobreexpressão de YB-1 em células de cancro da mama luminal, MCF-7, leva à diminuição da expressão de receptores hormonais (RE e RP), e aumenta ligeiramente a expressão de HER2. Em consequência, estas células tornam-se menos sensíveis à hormono-terapia (tamoxifeno e fulvestrano) e mais sensíveis à terapia anti-HER2 (lapatinib). De seguida, e para testar se a expressão de YB-1 estaria associada à resistência adquirida, mantivemos quatro linhas celulares de cancro da mama luminal expostas a baixas concentrações de fármacos diferentes por um periodo de cinco meses e avaliámos os níveis de expressão de YB-1, bem como os níveis de expressão dos receptores hormonais RE, RP e HER2. Os nossos resultados mostram que as células MCF-7 resistentes aos fármacos têm uma diminuição na expressão dos receptores hormonais e Ciclina D1, embora não tenham sido detectadas diferenças nos níveis de expressão de YB-1. Em concordância com estes resultados, a linha celular derivada de MCF-7 e resistente ao tamoxifeno apresentou uma menor taxa proliferativa e com menor dependência do estradiol,

quando comparada com a linha parental não tratada. No entanto, as linhas celulares derivadas de T47D resistentes ao tamoxifeno, fulvestrano e lapatinib mostram ter um aumento de *YBX1, ESR1 e ERBB2*, embora nem sempre se traduza num aumento ao nível da proteína.

Adicionalmente, avaliámos a resposta das linhas celulares derivadas de MCF-7 e T47D resistentes à hormono-terapia, a três terapias usadas na clínica para cancro da mama luminal metastático: lapatinib (anti-HER2), everolimus (inibidor do mTOR) e palbociclib (inibidor da CDK4/6). Curiosamente, os clones resistentes à hormono-terapia não se tornam mais resistentes às terapias alternativas, havendo até uma tendência para serem mais sensíveis, em especial a linha MCF-7 resistente ao Fulvestrano.

Em conclusão, os nossos resultados demonstram que a proteína YB-1 está associada a um pior prognóstico em cancro da mama e que a sua expressão se correlaciona negativamente com os níveis de RE. Assim, esta proteína poderá ser um biomarcador de resposta à terapia em cancro da mama luminal, uma vez que a terapia *standard* é direccionada para o RE.

Palavras-chave: Cancro da mama luminal; Y-Box binding protein 1 (YB-1); resistência à terapia; Biomarcadores preditivos e/ou prognósticos.

ABSTRACT

Breast cancer (BC) is the most common cancer among women worldwide, and Luminal BC is the most frequent and less aggressive subtype of BC, that affects about 60% of BC new cases. Luminal BC is characterized by the expression of hormone receptors, estrogen receptor (ER) and/or progesterone receptor (PR), and it could be divided in two subgroups depending of HER2 overexpression. Therefore, Luminal BC benefits from hormone-therapy (HT), as tamoxifen and fulvestrant, that blocks the ER-pathway and control the cell growth and development. However, there is a percentage of tumours that became resistant to therapy and develop metastases. Metastatic BC is the biggest challenge, and the major responsible for BC-related deaths.

YB-1 is a multifunctional protein that is involved in a variety of cellular processes related with growth and development. Normally, it is localized on cytoplasm and upon activation by phosphorylation, YB-1 translocates to the nucleus. As a transcription factor, YB-1 promotes the transcription of genes related with cell cycle, growth and drug resistance. Therefore, YB-1 has been associated with all hallmarks of cancer in diverse types of cancers, including BC. In this project we aimed to associate YB-1 with prognosis and resistance to HT in luminal BC, using a clinical and *in vitro* approach. Overall, our analysis in a cohort of 80 primary BC tumours and 51 paired metastases corroborates the importance of YB-1 as a biomarker of poor prognosis and shows that it is associated with ER negativity. Moreover, we provide the first data that shows that adjuvant HT may be implicated in the selection of clones with elevated YB-1 expression, which may be associated with the poor outcome of YB-1^{High} patients.

Our *in vitro* results show that YB-1 overexpression decreases the expression of hormone receptors, and that cells become more resistant to HT and more sensitive to lapatinib (anti-HER2 therapy). Moreover, cells with acquired resistance to tamoxifen, fulvestrant or lapatinib show some alterations in gene transcription, but HT-resistant cells remain sensitive to alternative therapeutics such as lapatinib, everolimus or palbociclib.

All things considered, our results suggest that YB-1 could be a predictive biomarker of HT-resistance and tumour aggressiveness, and that it deserves further studies.

Keywords: Luminal Breast Cancer; Y-Box Binding Protein 1 (YB-1); Therapeutic Resistance; Prognostic Biomarker

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
ºC	Degree Celsius
µl	Microliter
µM	Micro Molar
18S	18S Ribosomal RNA
a.a.	amino acids
AKT	Serine/threonine Kinase
AI	Aromatase Inhibitor
BC	Breast Cancer
BT474	Breast-474 (Breast Cancer Cell Line)
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CI	Confidence Interval
CO_2	Carbone Dioxide
csFBS	Charcoal-Stripped Foetal Bovine Serum
CSD	Cold Shock Domain
CT	Cycle Threshold
CTD	C-terminal Domain
DAB	3,3-Diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
Dnase I	Deoxyribonuclease I
E2	17β-Estradiol
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-Mesenchymal Transition
ER	Estrogen Receptor
FBS	Foetal Bovine Serum
FFPE	Formalin-Fixed Paraffin-Embedded
FOXA1	Forkhead Box Protein A1
FULV	Fulvestrant
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
h	Hours
H-score	Histoscore
HER2	Human Epidermal Growth Factor Receptor 2
HKGs	House Keeping Genes
HR	Hazard-Ratio
HRP	Horseradish Peroxidase
HT	Hormone Therapy
IARC	International Agency for Research on Cancer
IGF	Insulin-like Growth Factor

IHC	Immunohistochemistry
KDa	Kilo Dalton
LAP	Lapatinib
Luc	Luciferase
MCF-7	Michigan Cancer Foundation-7 (Breast Cancer Cell Line)
MDA-MB-231	M.D. Anderson – Metastatic Breast 231 (Breast Cancer Cell Line)
MDA-MB-361	M.D. Anderson – Metastatic Breast 361 (Breast Cancer Cell Line)
MHCI	Major Histocompatibility Complex Class I
MHCII	Major Histocompatibility Complex Class II
min	Minutes
mL	Millilitre
MMPs	Matrix Metalloproteinases
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
nM	Nanomolar
OE	Overexpression
OR	Odds Ratio
OS	Overall Survival
PABP	Poly-A Binding Protein
PBS	Phosphate-Buffered Saline
PBS-T	Phosphate-Buffered Saline with 0,05% Triton X-100
PCR	Polymerase Chain Reaction
Pen	Penicillin
PI3K	Phosphoinositide 3-Kinase
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin Homolog
RFS	Relapse-Free Survival
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT	Room Temperature
RT-qPCR	Reverse Transcription - Semi Quantitative Real Time PCR
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
SERDs	Selective Estrogen Down Regulators
SERMs	Selective Estrogen Receptor Modulators
Strep	Streptomycin
sYB-1	Secreted Y-box Binding Protein 1
T47D	Tumor-47 Ductal (Breast Cancer Cell Line)
TAM	Tamoxifen
TNBC	Triple Negative Breast Cancer
UTR	Untranslated Region
WHO	World Health Organization
YB-1	Y-box Binding Protein 1

1. INTRODUCTION

1.1. Breast Cancer Epidemiology

Breast Cancer (BC) is the second most commonly diagnosed cancer worldwide. According to the International Agency for Research on Cancer (IARC), BC accounts for about 12% of all new cancer cases, closely after lung cancer. In women, BC is by far the most common cancer and the leading cause of cancer death worldwide. IACR estimates that in 2018, BC will correspond to 25% of all new cancer cases (2,15 million new BC cases), causing 15% of all cancer-related deaths (630,000 deaths from BC).[1] These numbers reflect that despite great advances in BC early detection and treatment, BC incidence and mortality is still very high.

In Portugal, although the incidence of BC is lower than in the rest of Europe, it is still the most frequent female cancer. The World Health Organization (WHO) data shows that in 2018 there were 6,974 women diagnosed with BC in Portugal and 1,748 deaths, corresponding to 12% of all cancer deaths. (GLOBOCAN 2018, https://www.uicc.org/new-global-cancer-data-globocan-2018; accessed in November 2018)

1.2. Breast Cancer Molecular Subtypes

BC is a heterogeneous disease, comprising different histological and molecular subtypes and treatment sensitivity profiles. Therefore, prognosis is also heterogeneous and depends on the tumour characteristics.[2]

BC is classified based on histological evidences, the molecular status of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki-67 expression. There are three major subtypes of BC with clinical interest.[2,3] 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 Triple Negative BC (TNBC; ER-, PR-, HER2-). Luminal BC is the most common (60% of all cases) and less aggressive type of BC, while TNBC is associated

with worst prognosis.[4,5] The molecular status of a tumour is extremely relevant for clinical management, since the presence of ER, PR and HER2 predict response to targeted-therapies.

1.2.1. Luminal BC and ER signaling

Luminal BC is characterized by the expression of Hormone Receptors, mainly ER, and its growth is dependent of an ER-pathway, which is constitutively activated.[6] Hormone therapy is the first line therapeutic option for Luminal BC and shows higher efficacy for tumours with elevated levels of ER expression.[7] Although tumour growth is more indolent in Luminal BC (especially in Luminal A BC), and these tumours are associated with good response rates, resistance to therapy often occurs, and approximately 30% of patients will relapse.[4] Among ER+ BC, the overexpression of HER2 (HER2+) can also influence the prognosis and response to therapy, and ER+/HER2+ BC has been shown to be associated with higher relapse rates when compared with ER+/HER2- BC.[3]

ERs are cytoplasmic receptors which, upon activation by estrogen (17β-estradiol), are translocated to the nucleus and act as transcription factors.[8,9] ER downstream genes mostly include genes involved in the proliferation and differentiation.

There are two ERs, namely ERα and ERβ, encoded by *ESR1* and *ESR2* genes, respectively.[6] Both ERα and ERβ have a major affinity for estrogen. Despite having independent transcription activity as homodimers, they can also bind to each other and act as a heterodimer.[9] Both ERs are expressed in hormone-dependent cells, however ERα is mostly related with the growth of mammary epithelial cells, while ERβ has a major effect in the differentiation of the mammary gland.[10,11] In ER+ BC is ERα which is overexpressed, increasing the tumour growth, and is considered an oncogenic marker.[12] On the contrary, ERβ expression is normally downregulated in tumour tissues and there are no clear evidences of positive effects by targeting this protein.[10] The impact of ERβ in estrogen-dependent cancer cells is still unclear, but it has been

suggested that it could act as an inhibitory modulator of ERα activity.[13] From herein, ER+ BC will mean BC expressing ERα.

1.2.1.1. Hormone therapy and acquired resistance

The current standard of care treatments for BC include not only surgery, radiotherapy and chemotherapy, but also, targeted therapies like hormone therapy (HT) and a wide variety of biological therapies. Therapeutic regimens often include a combination of different therapies, depending on staging and tumour characteristics, including the molecular status.[14]

In the case of Luminal BC there is benefit from the use of HT, which includes aromatase inhibitors (AIs), selective estrogen receptor modulators (SERMs) and selective estrogen downregulators (SERDs). AIs, like anastrozole or letrozole, block the enzymatic activity of aromatase, decreasing the production of estrogen in the adipose tissue.[15] Since AIs do not inhibit the synthesis of ovarian estrogen, they are used to treat postmenopausal women. In the premenopausal setting, SERMs or SERDs are used to block the binding of estrogen to ER. SERMs, like tamoxifen, bind to ER and impair estrogen binding to the receptor, decreasing its activity in gene transcription.[16] SERDs, like fulvestrant, also have a high affinity to ER, but additionally induce ER degradation.[17] In both cases, the ER-pathway is inhibited, decreasing proliferation and inducing apoptosis.[9]

However, tumours often manage to escape therapy, either adjuvant leading to relapse, or metastatic leading to progression and death. Advanced (or metastatic) cancer correspond to tumour cells which were able to leave the primary tumour, spread into distant organs and grow from micro to macro metastases, and represents one of the biggest challenges for clinicians and researchers. As mentioned before, nearly 30% of ER+ BC patients will relapse on the course of their disease, mainly in bone, but also in lung, liver and brain. Relapse may occur within months or even years after diagnosis of primary tumour.

Known mechanisms of resistance to HT include the loss of ER accompanied by the upregulation of Epidermal Growth Factor Receptor (EGFR)[18], Insulin-like Growth Factor (IGF)[19] and HER2[20] proteins, or downstream activation of Phosphoinositide 3-

Kinase (PI3K) - Serine/threonine Kinase (AKT) - Mammalian Target of Rapamycin (mTOR) pathway.[21] Therefore, inhibitors of mTOR, like everolimus, have been used to control the proliferation, cell growth and tumour progression. Combining everolimus with HT has demonstrated benefit for metastatic BC patients, blocking the alternative pathway and restoring the sensitivity to HT.[22,23]

In the case of metastatic Luminal BC with HER2 amplification, some studies have shown that the combination of an AI, like letrozole or anastrozole, with anti-HER2 therapy, like lapatinib, versus AI alone is beneficial.[24.25] Moreover, also in ER+/HER2- tumours, the combination of an AI with anti-HER2 therapy has shown to be beneficial, since upregulation of HER2 has been described as one of the resistance mechanism adopted by tumour cells in response to AIs.[26] Additionally, the anti-HER2 trastuzumab has shown to reverse the resistance to AIs, upon HER2 increase and ER decrease in ER+/HER2- cells and xenografts.[27]

Likewise, the expression of cyclins are also frequently altered in cancer, particularly the cyclin A, B and D upregulation are observed in relapsed BC.[28,29] Cyclins are regulators of cell cycle progression, activated upon binding to cyclin-dependent serine/threonine protein kinases (CDKs), whose expression is dependent on cell cycle phase. CDK4 and CDK6 bind to D-type cyclins, and cyclin D/CDK4/6 complexes mediate the transition between G0/G1 and S phase of the cell cycle.[30] Moreover, cyclin D1 overexpression, downstream of ERα, is associated with higher proliferative rate and a stem cell phenotype.[31] In this context, cell cycle inhibitors or downregulators have been studied in order to control the cell cycle and growth responsible for tumour resistance. Currently, there are three cyclin-dependent kinase 4/6 (CDK4/6) inhibitors: ribociclib, palbociclib and abemaciclib, which have emerged as a substantial advance for patients with ER+/HER2- BC.[32] A randomised phase II trial of the CDK4/6 inhibitor palbociclib plus letrozole versus letrozole, and a phase III of palbociclib plus fulvestrant versus fulvestrant, showed significantly increased progression-free survival (PFS) when compared with HT alone in first-line and second-line treatment for advanced ER+/HER2- BC.[33]

Still, multiple and sequential resistance mechanisms are life-threatening, as patients often progress after first and subsequent lines of treatment. Therefore, it is

extremely important to identify new mechanisms of resistance as well as predictive biomarkers. In the scope of this project we focused on the prognostic and predictive value of Y-Box binding protein 1 (YB-1).

1.3. Y-Box Binding Protein 1

YB-1 is a multifunctional protein, encoded by *YBX1* gene, that belongs to a large family of DNA/RNA-binding proteins, which have an evolutionary ancient cold shock domain (CSD). Since YB-1 is involved in several cellular processes like proliferation, development and differentiation, this protein is ubiquitously expressed in somatic cells, predominantly in early-stages of development.[34]

YB-1 has 324 aminoacids and a molecular weight of 36KDa. Its structure contains three major domains: The N-terminal domain, rich in alanine and proline residues; the CSD, with a phosphorylation site at Serine 102; and a C-terminal domain (CTD), which has a nuclear localization signal (NLS), a cleavage site and a cytoplasmic retention site (CRS) (Figure 1). Therefore, YB-1 is able to bind to other proteins and nucleic acids.[34,35]

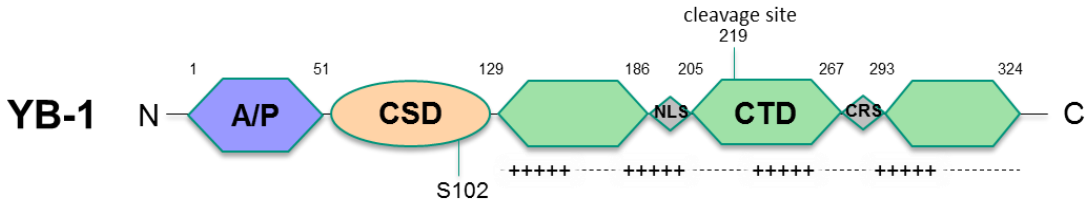

Figure 1. Representative scheme of YB-1 protein structure. YB-1 protein has three major domains: (**A/P)** N-Terminal Domain rich in alanine and proline residues; **(CSD) Cold-shock domain** that includes a phosphorylation site at Serine 102; **(CTD) C-Terminal Domain** that includes the nuclear localization site (NLS), cleavage site (a.a. 219) and cytoplasmic retention site (CRS). Adapted from [34].

Inside the cell, YB-1 can be found both in the cytoplasm and nucleus. Unphosphorylated YB-1 is maintained on the cytoplasm, where it plays a key role in balancing mRNA translation and stability in a dose-dependent manner, since at a low cytoplasmic YB-1/mRNA ratio the translation of mRNA and protein synthesis are activated.[35] There is also a nucleocytoplasmic shuttling of YB-1 bound to transcribed

mRNAs, regulating translation at the cytoplasm. Phosphorylation of cytoplasmic YB-1 leads to its translocation to the nucleus, where it binds to DNA and regulates transcription.

Although YB-1 has an ancient CSD and was first characterized as binding to the Y-box domain on genes' promoters[34], it is now known that it recognizes and bind to other motifs in DNA. Some studies have shown that YB-1 has higher affinity to the single stranded motifs GGGG, CACC and CATC than to the Y-box domain. Upon binding to DNA YB-1 recruits other proteins forming a transcription regulatory complex.[34]

YB-1 regulates transcription of several genes related with cell cycle, growth, drug resistance and stress response, acting as an activator or repressor. For example, nuclear YB-1 positively regulates the transcription of *EGFR*, *ERBB2*, *MDR1*, *PI3KCA*, *CCNA2* and *CCNB1*.[5,36] On the other hand, YB-1 inhibits the transcription of *ACTB, MHCI*, *MHCII*, *p53* and *VEGF*. Moreover, it has been shown that YB-1 is able to negatively regulate its own transcription. The promotor region of *YBX1* has a regulatory sequence which is recognized by Poly-A Binding Protein (PABP). However, YB-1 can also bind to this regulatory sequence, impairing the PABP's affinity and supressing its own mRNA translation.[37,38,39]

Additionally, YB-1 can be secreted by a non-canonical secretion mechanism[34]. It has been reported that secreted YB-1 (sYB-1) may participate on cell-cell signalling, normally in stress and inflammatory conditions, inducing cell cycle arrest.[40] Interestingly, sYB-1 is found in patients' serum from different tumours and different grades of malignancies, including BC.[41] In our previous work we have found an association between sYB-1 and the presence of extra-bone metastases and faster bone disease progression, in patients with BC and bone metastases.[42] Therefore, we hypothesize that the detection of sYB-1 in patients with BC and bone metastases may indicate a higher tumour burden, in bone and extra-bone locations, and is a biomarker of faster bone disease progression. Further studies will clarify the importance and role of sYB-1 in the cancer setting but the presence of sYB-1 in serum can be an important biomarker.

1.3.1. YB-1 as an Oncoprotein

Since YB-1 plays a key role in development and survival, the impact of YB-1 in oncogenesis has been the subject of several studies. Due to YB-1's role as transcription factor and its ability of binding to several proteins, it is not a surprise that YB-1 may affect and deregulate oncogenic signalling pathways. In fact, YB-1 has been shown to be correlated with all hallmarks of cancer, and an elevated YB-1 expression is associated with poor prognosis in a variety of human cancers including breast[43], prostate[44], non-small cell lung cancer[45,50], melanoma[46,47], osteosarcoma[48] and liver and gastric cancer.[49]

Increased YB-1 expression has been associated high proliferative rates in ovarian cancer and BC, by being a positive regulator of the PI3K/AKT pathway.[50,51] It was also shown that in melanoma cells MAPKs (PI3K/AKT) promote YB-1 phosphorylation, inducing its translocation to the nucleus, while NFkB pathway inhibits YB-1 activity. NFkB recognizes YB-1 and prevents its phosphorylation and translocation, mediating its expression and cell survival.[47] Furthermore, an association between YB-1 and cell cycle proteins also justifies the modulation of proliferation and apoptosis rates.[28] It has been shown that nuclear YB-1 accumulates during the transition from G1 to S phase of the cell cycle[36], activating *CCNA2*, *CCNB1* and *CCND1*[36,48,52], crucial for cell cycle progression. Moreover, it has been reported that in HER2-amplified tumours, YB-1 inhibits apoptosis of tumour cells[46], which may occur via mTOR/STAT3 pathway.[53,54]

Besides the effect of YB-1 on proliferation and apoptosis, which contributes to tumour aggressiveness, YB-1 was also identified as a driver for epithelial-to-mesenchymal transition (EMT) and drug resistance in many cancers such as BC[55,56], prostate cancer[44], and gastric cancer[49], through regulation of the transcription of EMT-associated genes, like the downregulation of *E-cadherin*, and up-regulation of *Snail*, *Slug* and *Vimentin*.[44,49,56]

Increased nuclear YB-1 has been reported as a key factor for drug resistance, by inducing the expression of *MDR-1*[5,57], and for invasiveness and metastases in BC.[58] Besides that, there is a strong association between YB-1 expression and cell growth in BC; and previous studies showed that differential proliferation rates are accompanied

by an increase of EGFR, HER2, p-ERK and c-Myc but a decrease of ERα, affecting the response to therapies.[59]

Thus, BC overexpressing YB-1 may have a higher capacity to develop and progress quickly, and in fact, the elevated expression of YB-1 in BC has been associated with highly aggressive phenotypes and decreased OS.[60] Also, it has been shown that YB-1 is more frequently elevated in basal-like tumours and HER2-amplified tumours.[59,61,62] Concordantly, it was reported a negative association between YB-1 levels and ER and PR expression, and positive association with HER2 in BC cells.[63]

All facts considered, when overexpressed, YB-1 modulates a variety of genes which are crucial to cancer development and aggressiveness, and YB-1 can be an important biomarker of poor prognosis in BC.

2. AIMS

As mentioned before, it was previously shown *in vitro* and in BC xenografts that YB-1 overexpression was responsible for ER down-regulation and HER2 up-regulation, contributing to anti-estrogen bypass and resistance to HT in BC cells. Therefore, we hypothesized that elevated YB-1 expression in primary ER+ breast tumours may not only be associated with poor prognosis but also with the loss of ER in metastases. To address this hypothesis, we retrospectively assessed the association between YB-1 and p-YB-1 and relapse-free survival (RFS) and overall survival (OS), using a cohort of primary tumours and paired metastasis; and evaluated a possible association with alterations in ER, PR and HER2 status in primary tumours and paired metastases.

Moreover, we also interrogated if the acquired resistance to HT in ER+/HER2- or HER2+ BC is related to YB-1 expression levels or accompanied by an alteration in YB-1 expression; and being so, if this affects the sensitivity to other targeted therapies. To address these questions, we selected *in vitro* cells with acquired resistance to different therapies, like HT, and evaluated: the expression of YB-1, ER, PR, HER2 and Cyclin D1; and the sensitivity to HT, anti-HER2 therapy, and mTOR and CDK4/6 inhibitors, currently used as single agents or in combination, to treat Luminal BC.

3. MATERIALS AND METHODS

3.1. Human Samples

3.1.1. Immunohistochemistry

Expression of YB-1 and p-YB-1 was evaluated by immunohistochemistry (IHC) in a retrospective cohort of 131 formalin-fixed paraffin-embedded (FFPE) samples from 80 human primary breast tumours and 51 paired metastases. In addition to a first sub-group of 94 samples previously evaluated[64], 36 new samples were provided by the Pathology Department from CUF Hospital, and all patients signed an Institutional informed consent.

IHC was performed as previously described.[64] Briefly, deparaffination and antigen retrieval were performed using the PT Link Pre-Treatment Module for Tissue Specimes (Dako), according to manufacturer's instructions, and 5µm tissue sections. Next, samples were incubated for 10 min at room temperature (RT) with Peroxidase Blocking Solution (Dako) to inhibit endogenous peroxidase activity, followed by an incubation with Protein Block Solution (Dako) for 20min at RT, and incubation with primary antibodies overnight at 4°C. Primary antibodies included: rabbit anti-human YB-1 (1:50, D299, Cell Signaling) and rabbit anti-human p-YB-1 (Ser102) (1:500, Cell Signaling); both diluted in Antibody Diluent (Dako). The visualization system Dako REAL EnVision 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, mounted with Quick-D mounting medium (Klinipath) and visualized in a bright field microscope (Leica DM2500).

Samples were blinded scored by two Medical Pathologists, and staining intensity was classified from 0 to 3: (0) absence of staining, (1) weak, (2) moderated and (3) strong staining. H-Score, which ranged from 0 to 300, was calculated based on the percentage of cells per intensity. Nuclear localization of YB-1 and p-YB-1 was classified as positive or negative.

For outcome analysis, the samples were dichotomized according to: 1) low or high expression of YB-1 or p-YB-1 using cut-off finder software and OS as endpoint; 2) positive or negative nuclear staining.

3.1.2. Statistical Analysis

Statistical analyses were performed using GraphPad Prism Software, version 7.0 (GraphPad Software, www.graphpad.com). For univariate analysis, RFS and OS were estimated using Kaplan-Meier curves and differences were determined using the log-rank test. Survival time was censored at death if patients were still alive at the last follow-up. Patients with missing data were excluded from the respective analysis. Two-tailed unpaired t-test, Fisher's exact test and Chi-squared test were used to determine the association between YB-1 or p-YB-1 expression and clinicopathological characteristics of patients. Unpaired t-test was used to determine the concordance between YB-1 and p-YB-1 expression in primary tumour and paired metastasis. Significance was defined by a p-value<0,05.

3.2. Cell Culture and Cellular Assays

3.2.1. Cell Lines

Human BC cell lines MDA-MB-231, MCF-7, and MDA-MB-361 were obtained from ATCC. T47D and BT474 BC cell lines were kindly provided by Dr. Phillippe Clézardin (INSERM). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 1% (v/v) Penicillin/Streptomycin (Pen/Strep, 10,000 U/mL Penicillin, 10,000 µg/mL Streptomycin, Gibco), and 10% (v/v) foetal bovine serum (FBS, Gibco), except for MDA-MB-361, where 20% (v/v) FBS was used. For MCF-7, T47D and BT474 the medium was supplemented with 0,01mg/mL Insulin (Gibco). Medium was replaced twice a week and cells were incubated at 37^{o}C with 5% CO_2 in a humidified atmosphere.

3.2.2. YB-1 Overexpression

MCF-7$^{YB\text{-}1\ OE}$ cell line was derived from MCF-7 cell line by transduction with YB-1 lentiviral activation particles (Santa Cruz). 4x10^4 cells were plated in 24-well plates, in 1 mL of complete medium, and 24 hours later, the medium was replaced by fresh medium containing 8µg/mL polybrene, and 30µl of lentiviral particles were added to each well. Cells were incubated for 24h and then, the medium was replaced by fresh complete culture medium. On the fifth day, infected cells were selected with an antibiotic cocktail containing 0,5µg/mL Puromycin, 5µg/mL Blasticidin and 200 µg/mL Hygromycin.

3.2.3. Cell Viability Assay

Cell viability was quantified using the Alamar Blue Assay. 1x10^4 cells per well were seeded in 96-well plates and incubated for 6 days in the presence of specific drugs. The medium was replaced every 2 days. For drug sensitivity assays, 1:10 (v/v) Alamar blue (Invitrogen) was added after seven days of drug exposure, and after 2h, the fluorescence was quantified (560nm excitation/590nm emission) on Microplate Reader TECAN Infinite M200. For proliferation assays, Alamar blue was added 24h after cell seeding, and fluorescence was measured daily. Each experiment was performed in triplicate, with four replicates per condition per experiment.

3.2.4. Selection of drug-resistant cell lines

To induce drug-resistance, cells were seeded in T25cm^2 flasks and incubated for 5 months in the presence of specific drug: 10nM Tamoxifen (Sigma-Aldrich); 10nM Fulvestrant (Sigma); 100nM Lapatinib (Stemcell); 100nM Everolimus (Stemcell); 10nM Palbociclib (Sigma). Medium was replaced twice a week. The sensitivity profile was assessed after 3 and 5 months of exposure using Alamar Blue Assay as described above.

3.2.5. E-Screen Assay

Sensitivity to estradiol was quantified using the E-Screen assay.[66] Briefly, cells were plated into 24-well plates, at a density of $2x10^4$ cells/mL and incubated for 24h hours in standard complete medium. The medium was replaced with phenol-red DMEM (Gibco) with 5% csFBS (Gibco) and 1% pen/strep, and cells were incubated in the absence or presence of 1nM or 10nM 17β-Estradiol (E2, Sigma-Aldrich). After five days, cell viability was quantified using the Alamar Blue Assay as described above, and the percentage of viable cells was normalized for control cells growing in a standard complete medium. This experiment was performed once with four replicates per condition.

3.3. RNA isolation, cDNA synthesis and RT-qPCR

Total RNA was extracted from under confluent cells growing in T25cm^2 flasks, with the NZY Total RNA Isolation kit (NZYTech), according to the manufacturer's instructions, and was quantified in a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Complementary DNA (cDNA) was synthesized from 1µg of total RNA, using Oligo(dT)18 primer and the NZY M-MuLV First-Strand cDNA Synthesis Kit (NZYTech), according to manufacturer's instructions.

Interest genes were amplified by semi-quantitative real time PCR (qPCR) in a ViiA7 Real-Time PCR System (Applied Biosystems), using 5% cDNA and: TaqMan Gene Expression Assays (Table 1) and the 2x TaqMan Gene Expression Master Mix (Applied Biosystems); or 10µM of specific primers (Table 2; Invitrogen) and 2x Universal SYBR Green Supermix (Biorad). Target gene expression was normalized against the housekeeping genes *GAPDH* or *18S*, and the data was analysed using the $2^{-\Delta\Delta Ct}$ method. RT-qPCR reactions were performed in triplicate and repeated for three different cDNAs synthesized from the same RNA sample.

Table 1. TaqMan Gene Expression Assays used in qPCR.

	Reference
YBX1	Hs00358903_g1
ESR1	Hs0017486_m1
ERBB2	Hs01001580_m1
PGR	Hs01556702_m1
GAPDH	Hs02758991_g1

Table 2. Specific primers used in qPCR.

	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
18S	GCCCTATCAACTTTCGATGGTAGT	CCGGAATCGAACCCTGATT
CCND1	ATGTCGTGGCCTCTAAGATGA	CAGGTTCCACTTGAGTTC

3.4. Western blotting

Proteins were extracted from under confluent cells growing in T25cm^2 flasks. Cells were trypsinized and harvested by centrifugation at 450g and 4^{o}C for 5 min. Cell pellets were lysed in 100µl of RIPA buffer (SIGMA), incubated for 10 min on ice, and centrifuged at 16,000g and 4^{o}C for 10 min. The supernatants were collected, and proteins quantified using Bradford Reagent (VWR Life Science) according to manufacturer's instructions. Protein extracts were diluted in 2X Sample Loading Buffer (NZYTech) with 1:100 protease inhibitors cocktail (Cell Signaling), denatured for 10 min at 95^{o}C and stored at -20^{o}C.

Equal amounts of protein (5µg per lane) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Thermo Fisher Scientific), using an iBlot 2 system (Invitrogen) according to manufacturer's instructions.

Membranes were incubated for 1h in 5% BSA (Santa Cruz) or 5% non-fat milk in PBS-T (1X PBS with 0,05% Tween, Sigma-Aldrich), and then, incubated with the primary antibodies. Membranes were washed with PBS-T (3X 10 min) and incubated 1h with secondary antibodies conjugated with horse radish peroxidase (HRP). Antibodies used, and incubation details are listed in Table 3. The washing step was repeated with PBS-T (3X 10 min) and proteins were detected using the HRP chemiluminescent substrate reagent kit (Invitrogen) on Amersham Imager 680 equipment, according to manufacturer's instructions.

Table 3. Antibodies used in Western blotting

Primary Antibody:	**Dilution**	**Diluent**	**Incubation period, Incubation temperature**
Rabbit anti-human YB-1 (D299, Cell Signaling)	1:1000		
Rabbit anti-human p-YB-1 (Ser102) (Cell Signaling)	1:500	5%BSA in PBS-T	Overnight, 4^{o}C
Rabbit anti-human ER (Cell Signaling)	1:1000		
Rabbit anti-human HER2 (Cell Signaling)	1:1000		
Rabbit anti-human CyclinD1 (Cell Signaling)	1: 1000		
Mouse-anti-human β-Actin (Cell Signaling)	1: 25000	5% non-fat milk in PBS-T	2 hours, RT
Secondary Antibody:			
Goat anti-rabbit IgG (HRP Conjugate) (Bio Rad)	1:5000	5% non-fat milk in PBS-T	1 hour, RT
Goat anti-mouse IgG (HRP Conjugate) (Bio Rad)			

3.5. Statistical Analysis

Statistical analysis and graphs were performed using GraphPad Prism Software, version 7.0 (GraphPad Software, www.graphpad.com) and error bars represent the standard error of the means (SEM). One-way ANOVA or unpaired t-test were used as appropriate. The levels of statistical significance were set at *p<0,05 **p<0,01 and ***p<0,001.

4. RESULTS

4.1. Prognostic value of YB-1 and p-YB-1 expression in primary BC

The exact applicability of YB-1 as a biomarker in BC is still unclear, but different studies have shown an association between elevated YB-1 expression in primary breast tumours and poor prognosis, as well as with ER/PR negativity. Moreover, to our knowledge, despite the importance of YB-1 phosphorylation in several cellular processes, the association between p-YB-1 and clinicopathologic characteristics was not addressed so far. Therefore, we proposed to determine the association between YB-1 and p-YB-1 expression and cellular localization in primary and metastatic breast tumours and clinicopathologic characteristics. For that purpose, we conducted a retrospective study, in a cohort of 80 primary BC tissue specimens. The clinicopathological characteristics of this cohort are presented in Supplementary Table 1. The median OS and RFS in this cohort was 4,93 and 2,74 years, respectively.

The expression of YB-1 and p-YB-1 was assessed by IHC in all 80 primary BC tissue specimens, and independently evaluated by two medical pathologists. The staining intensity was scored between 0 and 3, being (0) negative, (1) weak, (2) moderated and (3) strong staining (Figure 2.A). Two samples were considered not evaluable and excluded from further YB-1 analysis. Next, H-Score was calculated based on the percentage of cells per staining intensity and the samples were dichotomized between high or low YB-1 and p-YB-1 expression (Figure 2.B), using cut-off finder software and OS as endpoint, to determine the more significant cut-off. The cut-off values are shown in Table 4. In this study, 25,6% (20/78) of patients were classified as YB-1 high and 13,8% (11/80) as p-YB-1 high.

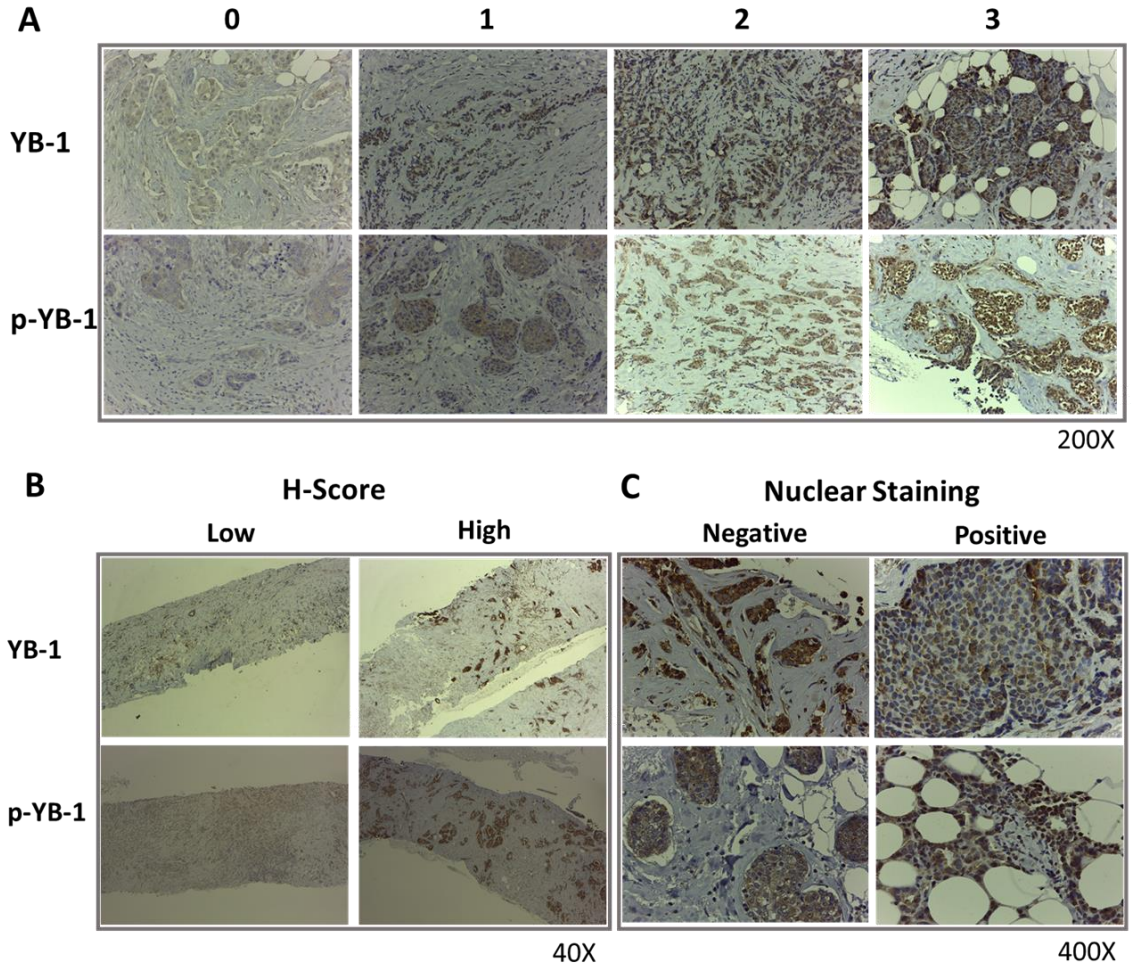

Figure 2. Representative images of YB-1 and p-YB-1 immunostaining in primary BC tissues. **(A)** The protein expression was scored into four levels of intensity (0-3); **(B)** Examples of low and high H-Score upon dichotomization; and **(C)** Examples of negative or positive nuclear staining.

Table 4. Cut-off values used for patients' dichotomization according to low or high YB-1 or p-YB-1 expression. P-value was calculated using log-rank test. HR – Hazard Ratio; CI – Confidence Interval.

.

	YB-1	p-YB-1
Cut off value	285	190
HR (95%CI)	1,56 (0,95-2,57)	0,32 (0,12-0,88)
P-value	0,074	0,02

Next, we analysed the association between YB-1 and p-YB-1 expression and clinical and pathological characteristics, namely: age at diagnosis, menopausal status, TNM stage, tumour grade, tumour size (T), positive lymph nodes (N), receptors' molecular status (positive or negative HER2, ER and PR), and metastasis site (Table 5). YB-1 expression was negatively associated with ER status (p=0,0383). We also observed a trend for visceral-only relapse in patients with low YB-1 (p=0,0555).

Table 5. Association between YB-1 and p-YB-1 expression in primary breast tumours and clinicopathologic characteristics.

Characteristics	YB-1		*P*	p-YB-1		*p*
	Low	High		Low	High	
Nº of Patients	58	20		69	11	
Age at diagnosis (years)						
Median (range)	53 (24 – 83)	50 (29 – 77)		54 (24 - 83)	50 (39 – 82)	
Menopausal Status (n, %)						
Premenopausal	18(31,0)	9 (45,0)		24 (34,8)	5 (45,5)	
Postmenopausal	36 (62,1)	10 (50,0)	0,4201#	40(58,0)	6 (54,5)	0,7405#
Unknown	4 (6,9)	1 (5,0)		5 (7,2)	0 (-)	
TNM Stage						
I	5 (8,6)	0 (-)		5 (7,3)	0 (-)	
II	15 (25,9)	4 (20,0)		18 (26,1)	1 (9,1)	
III	15 (25,9)	6 (30,0)	0,7217#	21 (30,4)	1 (9,1)	0,0938#
IV	11 (18,9)	2 (10,0)		11 (15,9)	3 (27,3)	
Unknown	12 (20,7)	8 (40,0)		14 (20,3)	6 (54,5)	
T						
1	18 (31,0)	4 (20,0)		21 (30,4)	1 (9,0)	
2	18 (31,0)	8 (40,0)		22 (31,9)	5 (45,5)	
3	2 (3,5)	2 (10,0)	0,2548$	4 (5,8)	0 (-)	0,6675$
4	6 (10,4)	3 (15,0)		9 (13,0)	0 (-)	
Unknwon	14 (24,1)	3 (15,0)		13 (18,9)	5 (45,5)	
N						
0	15 (25,9)	5 (25,0)		16 (23,2)	4 (36,3)	
1	8 (13,8)	5 (25,0)		12(17,4)	1 (9,1)	
2	6 (10,3)	2 (10,0)	0,9136$	8 (11,6)	1 (9,1)	0,1238$
3	7 (12,1)	3 (10,0)		10 (14,5)	0 (-)	
Unknown	22 (37,9)	5 (25,0)		23 (33,3)	5 (45,5)	
Tumor Grade						
1	1 (1,7)	0 (-)		1 (1,4)	0 (-)	
2	27 (46,6)	10 (50,0)	0,9317$	32 (46,4)	6 (54,6)	0,9185$
3	19 (32,8)	6 (30,0)		22 (31,9)	4 (36,3)	
Unknown	11 (18,9)	4(20,0)		14 (20,3)	1 (9,1)	
HER2						
Negative	33 (56,9)	11 (55,0)		40 (58,0)	5 (45,5)	
Positive	18 (31,0)	6 (30,0)	0,9999#	22(31,9)	2 (18,2)	0,9999#
Unknown	7 (12,1)	3 (15,0)		7 (10,1)	4 (36,3)	
ER						
Negative	12 (20,7)	9 (45,0)		18 (26,1)	3 (27,3)	
Positive	44 (75,9)	10 (50,0)	**0,0383#**	47 (68,1)	8 (72,7)	0,9999#
Unknown	2 (3,4)	1 (5,0)		4 (5,8)	0 (-)	
PR						
Negative	31 (53,5)	12 (60,0)		35 (50,7)	8 (72,7)	
Positive	25 (43,1)	6 (30,0)	0,4273#	30 (43,5)	2 (18,2)	0,1740#
Unknown	2 (3,4)	2 (10,0)		4 (5,8)	1 (9,1)	
Metastasis site						
Bone only	11 (19,0)	7 (35,0)		15 (21,7)	3 (27,3)	
Visceral only	32 (55,2)	5 (25,0)	0,0555$	36 (52,2)	7 (63,6)	0,5057$
Bone & Visceral	14 (24,1)	8 (40,0)		17 (24,6)	1 (9,1)	
Unknown	1 (1,7)	0 (-)		1 (1,5)	0 (-)	

#Fisher's exact test; $Chi-Square test

Next, we evaluated the association between YB-1 and p-YB-1 expression and clinical outcomes, namely OS and RFS. Kaplan-Meyer analysis shows that patients with elevated expression of YB-1 (YB-1^{High}) have decreased OS (p=0,0437; HR=0,538; 95%CI 0,2525-1,145) (Figure 3.A). There was no association between p-YB-1 and OS (p=0,0916; HR=2,321; 95%CI 1,114-4,835) (Figure 3.B); or between YB-1 (p=0,4615; HR=0,822; 95%CI 0,473-1,429) and p-YB-1 expression (p=0,2963; HR=1,468; 95%CI 0,7741-2,785) and RFS (Figure 3.C and D, respectively).

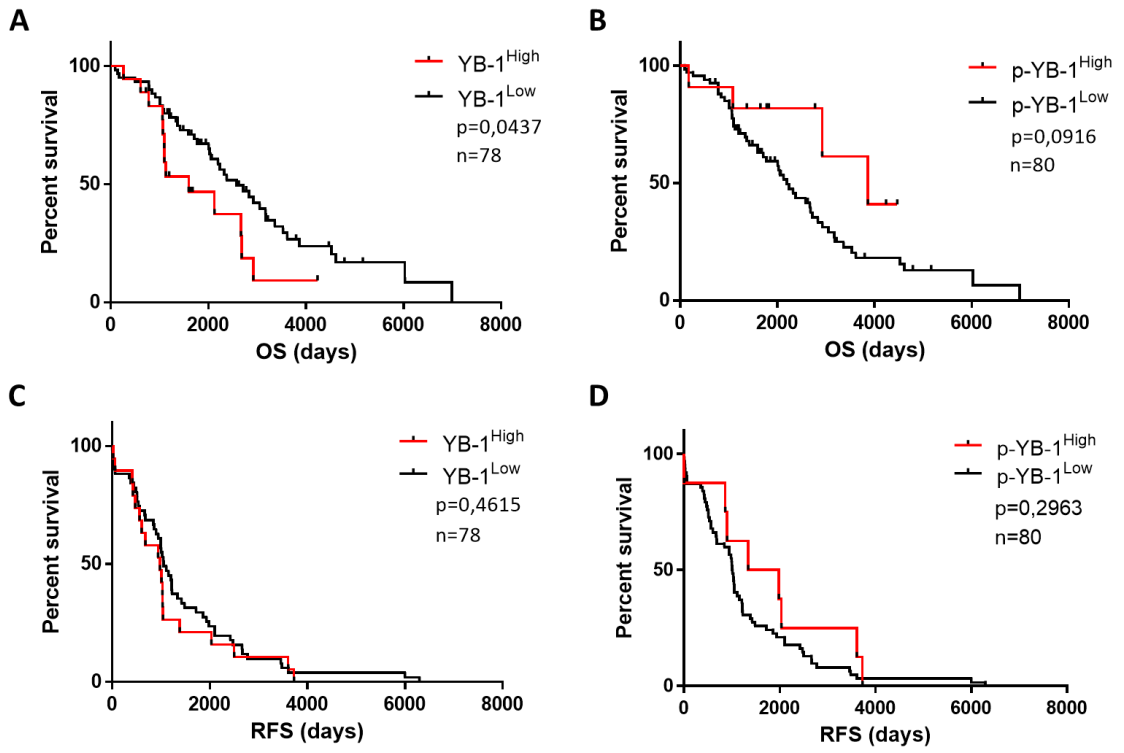

Figure 3. **High levels of YB-1 in BC tumours are associated with shorter patient survival.** Kaplan-Meier Overall Survival (OS) curves according to **(A) YB-1 expression** (HR=0,538; 95%CI 0,253-1,145) and **(B) p-YB-1 expression** (HR=2,321; 95%CI 1,114-4,835); and Relapse-free Survival (RFS) curves according to **(C) YB-1 expression** (HR=0,822; 95%CI 0,473-1,429) and **(D) p-YB-1 expression** (HR=1,468; 95%CI 0,774-2,785) in primary BC patients. P-Value and Hazard ratio (HR) was calculated using log-rank test and significance set at 0,05.

Since previous studies have shown that oncogenic function of YB-1 is associated with protein phosphorylation and nuclear translocation, we also evaluated the cellular localization of YB-1 and p-YB-1 (Figure 2.C) and analysed the association between nuclear YB-1 or p-YB-1 and the clinical and pathological characteristics referred above (Table 6). In this study, 29,5% of patients (24/78) had positive nuclear YB-1 and 36,3% (29/80) positive nuclear p-YB-1. Nuclear p-YB-1 was significantly associated with ER status (p=0,0306).

Table 6. Association between nuclear YB-1 and p-YB-1 expression in primary breast tumours and clinicopathologic characteristics.

Characteristics	Nuclear YB-1		*P*	Nuclear p-YB-1		*p*
	Positive	Negative		Positive	Negative	
Nº of Patients	24	54		29	51	
Age at diagnosis (years)						
Median (range)	50 (24 – 83)	54 (29 – 77)		54 (24 - 83)	50 (39 – 82)	
Menopausal Status (n, %)						
Premenopausal	11 (45,8)	16 (29,6)		10 (34,5)	19 (37,2)	
Postmenopausal	11 (45,8)	35 (64,8)	0,1865#	17 (58,6)	29 (56,9)	0,7405#
Unknown	2 (8,4)	3 (5,6)		2 (6,9)	3 (5,9)	
TNM Stage						
I	1 (4,2)	4 (7,4)		2 (6,9)	3 (5,9)	
II	4 (16,7)	15 (27,8)		6 (20,7)	13 (25,5)	
III	9 (37,4)	12 (22,2)	0,0924#	5 (17,2)	17 (33,3)	0,2341#
IV	6 (25,0)	7 (13,0)		8 (27,6)	6 (11,8)	
Unknown	4 (16,7)	16 (29,6)		8 (27,6)	12 (23,5)	
T						
1	6 (25,0)	16 (29,6)		5 (17,2)	17 (33,3)	
2	7 (29,1)	19 (35,2)		10 (34,5)	17 (33,3)	
3	1 (4,2)	3 (5,6)	0,4035$	0 (-)	4 (7,8)	0,4086$
4	4 (16,7)	5 (9,3)		4 (13,8)	5 (9,8)	
Unknwon	6 (25,0)	11 (20,3)		10 (34,5)	8 (15,8)	
N						
0	4 (16,7)	16 (29,6)		8 (27,6)	12 (23,5)	
1	5 (20,8)	8 (14,8)		6 (20,7)	7 (13,7)	
2	3 (12,5)	5 (9,3)	0,6549$	2 (6,9)	7 (13,7)	0,4618$
3	3 (12,5)	7 (13,0)		2 (6,9)	8 (15,8)	
Unknown	9 (37,5)	18 (33,3)		11 (37,9)	17 (33,3)	
Tumor Grade						
1	0 (-)	1 (1,8)		1 (3,5)	0 (-)	
2	8 (33,3)	29 (53,7)	0,0902$	13 (44,8)	25 (49,0)	0,9087$
3	10 (41,7)	15 (27,8)		10 (34,5)	16 (31,4)	
Unknown	6 (25,0)	9 (16,7)		5 (17,2)	10 (19,6)	
HER2						
Negative	12 (50,0)	32 (59,2)		16 (55,2)	29 (56,9)	
Positive	7 (29,2)	17 (31,5)	0,9999#	7 (24,1)	17 (33,3)	0,7891#
Unknown	5 (20,8)	5 (9,3)		6 (20,7)	5 (9,8)	
ER						
Negative	9 (37,5)	12 (22,2)		12 (41,4)	9 (17,6)	
Positive	14 (58,3)	40 (74,1)	0,1726#	15 (51,7)	39 (76,5)	**0,0306#**
Unknown	1 (4,2)	2 (3,7)		2 (6,9)	3 (5,9)	
PR						
Negative	12 (50,0)	31 (57,4)		14 (48,3)	29 (56,9)	
Positive	10 (41,7)	21 (38,8)	0,7979#	12 (41,4)	20 (39,2)	0,8067#
Unknown	2 (8,3)	2 (3,7)		3 (10,3)	2 (3,9)	
Metastasis site						
Bone only	4 (16,7)	14 (25,9)		4 (13,8)	14 (27,5)	
Visceral only	15 (62,5)	27 (50,0)	0,5768$	20 (69,0)	23 (45,1)	0,1338$
Bone & Visceral	5 (20,8)	12 (22,2)		5 (17,2)	13 (25,5)	
Unknown	0 (-)	1 (1,9)		0 (-)	1 (1,9)	

#Fisher's exact test; $Chi-Square test

We then evaluated the association between nuclear YB-1 and p-YB-1 and clinical outcomes. Kaplan-Meyer analysis shows that although not significant, OS curves tend to segregate patients, being the ones with positive nuclear YB-1 and p-YB-1 the ones with lower OS (YB-1: p=0,1754; HR=0,675; 95% CI 0,360-1,266; p-YB-1: p=0,7525; HR= 0,917; 95% CI 0,524-1,605) (Figure 4.A and B). This trend is more evident for patients who died within 5 years of follow-up. Since the median OS in this cohort was 4,93 years, we also performed a 5-year survival analysis, which shows that in fact nuclear YB-1 and p-YB-1 are markers of shortest OS (YB-1: p=0,0221 HR=0,432; 95% CI 0,182-1,022; p-YB-1: p=0,0163; HR=0,422; 95% CI 0,1923-0,926) (Figure 4.C and D). Concerning RFS, no differences were observed (YB-1: p=0,2594; HR=0,759; 95% CI 0,450-1,281; p-YB-1: p=0,8934; HR=0,971; 95% CI 0,617-1,529) (Figure 4.E and F).

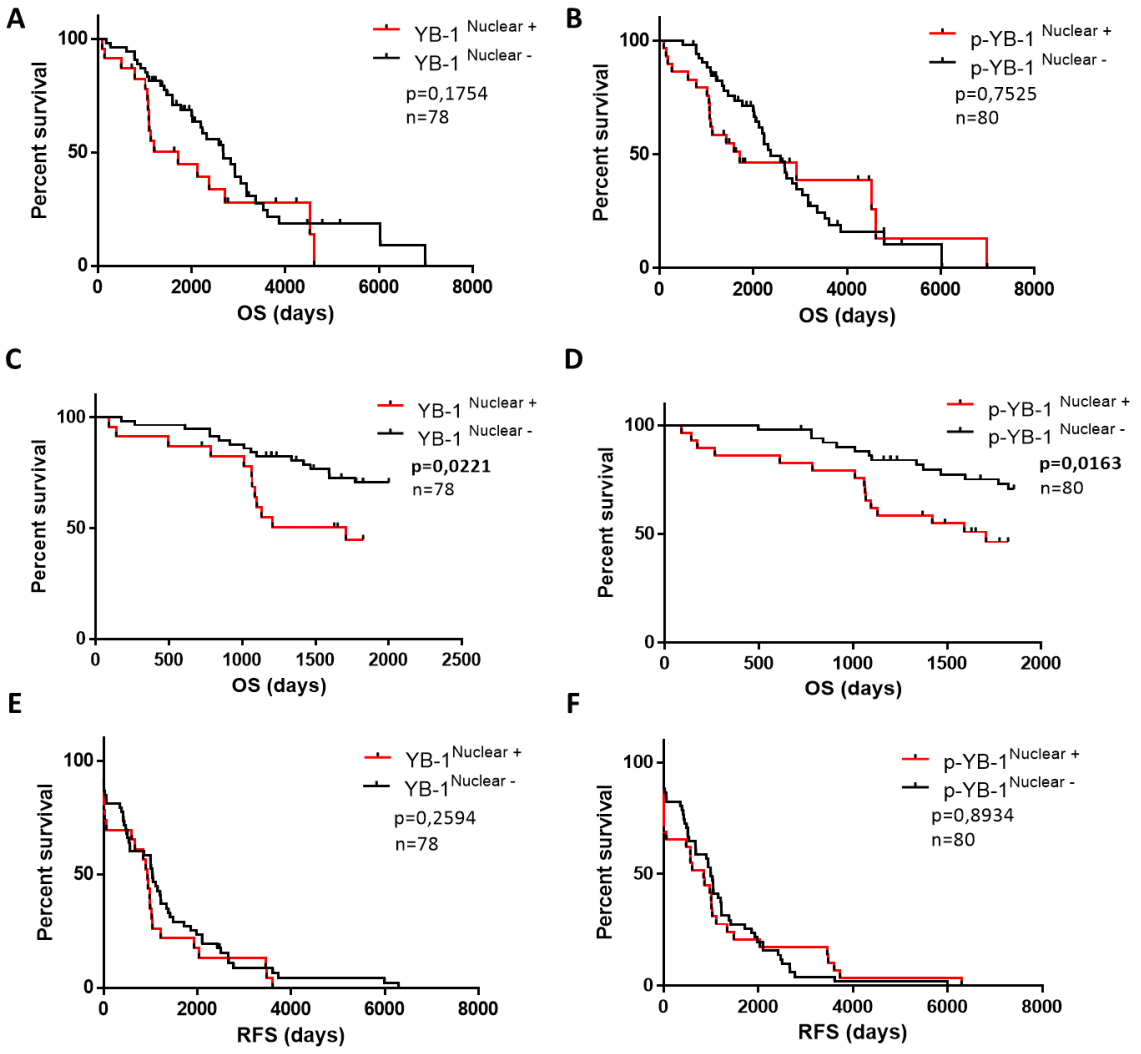

Figure 4. **Positive nuclear YB-1 or p-YB-1 is associated with poor 5-years survival.** Kaplan-Meier Overall Survival (OS) curves according to **(A)** YB-1 nuclear staining (HR=0,675; 95% CI 0,360-1,266), **(B)** p-YB-1

nuclear staining (HR= 0,917; 95% CI 0,524-1,605); Kaplan-Meier 5-years Overall Survival (OS) curves according to **(C)** YB-1 nuclear staining (HR=0,432; 95% CI 0,182-1,022), **(D)** p-YB-1 nuclear staining (HR=0,422; 95% CI 0,1923-0,926); and Kaplan-Meier Relapse-free Survival (RFS) curves according to **(E)** YB-1 nuclear staining (HR=0,759; 95% CI 0,450-1,281), **(F)** p-YB-1 nuclear staining (HR=0,971; 95% CI 0,617-1,529). P-Value and Hazard ratio (HR) were calculated using log-rank test and significance set at 0,05.

4.2. Prognostic value of YB-1 and p-YB-1 expression in metastatic BC

Since YB-1 expression is implicated in tumour aggressiveness and progression, we also analysed the expression of YB-1 and p-YB-1 in 51 paired metastases. Patients were dichotomized as described above and 31,4% (16/51) were classified as YB-1 high and 9,8% (5/51) as p-YB-1 high; without association with clinicopathologic characteristics (Table 7).

Table 7. Association between YB-1 and p-YB-1 expression in metastatic tissue and clinicopathologic characteristics.

Characteristics	**YB-1 (%)**		***p***	**p-YB-1 (%)**		***P***
	Low	**High**		**Low**	**High**	
Nº of Patients	35	16		46	5	
Age at diagnosis (years)						
Median (range)	53 (30 – 82)	63 (39 – 77)		54 (30 - 82)	66 (45 – 81)	
Adjuvant HT						
Yes	21 (60,0)	13 (81,2)		28 (60,9)	4 (80,0)	
No	12 (34,3)	3 (18,8)	0,3238[#]	14 (30,4)	1 (20,0)	>0,9999[#]
Unknown	2 (5,7)	0 (-)		4 (8,7)	0 (-)	
HER2						
Negative	23 (65,7)	10 (62,5)		31 (67,4)	2 (40,0)	
Positive	10 (28,6)	3 (18,75)	0,7290[#]	11 (23,9)	2 (40,0)	0,5654[#]
Unknown	2 (5,7)	3 (18,75)		4 (8,7)	1 (20,0)	
ER						
Negative	11 (31,4)	3 (18,8)		13 (28,3)	1 (20,0)	
Positive	21 (60,0)	11 (68,7)	0,4967[#]	29 (63,0)	3 (60,0)	>0,9999[#]
Unknown	3 (8,6)	2 (12,5)		4 (8,7)	1 (20,0)	
PR						
Negative	19 (54,3)	7 (43,7)		24 (52,2)	2 (40,0)	
Positive	14 (40,0)	6 (37,5)	0,9999[#]	18 (39,1)	2 (40,0)	>0,9999[#]
Unknown	2 (5,7)	3 (18,8)		4 (8,7)	1 (20,0)	
Metastasis site						
Bone only	7 (20,0)	3 (18,75)		9 (19,6)	2 (40,0)	
Visceral only	22 (62,9)	10 (62,5)	0,9876[#]	28 (60,8)	3 (60,0)	0,3961[#]
Bone & Visceral	6 (17,1)	3 (18,75)		9 (19,6)	0 (-)	

[#]Fisher's exact test.

Concerning the impact of metastatic YB-1 and p-YB-1 in survival after relapse, we observed a non-significant tendency toward decreased OS in patients with elevated YB-1 (p=0,2955; HR=0,661; 95%CI 0,277-1,577) (Figure 5.A); and no association between p-YB-1 and OS (p=0,1276; HR and 95% CI: undefined) (Figure 5. B). In this analysis, one patient with missing follow-up data was excluded.

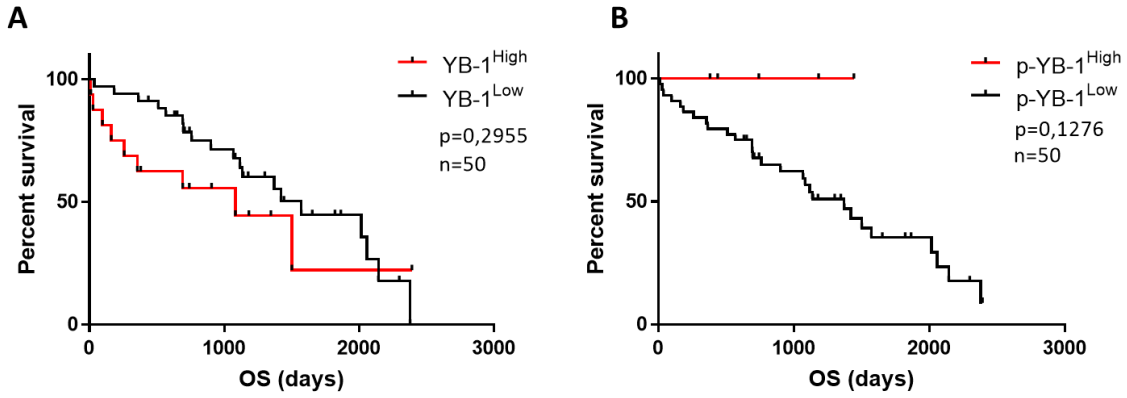

Figure 5. High levels of YB-1 in BC metastasis are not associated with shorter patient survival. Kaplan-Meier Overall Survival (OS) curves according to **(A) YB-1 expression** (HR=0,661; 95%CI 0,277-1,577) and **(B) p-YB-1 expression** (HR and 95%CI: undefined); P-Value and Hazard ratio (HR) was calculated using log-rank test and significance set at 0,05.

Additionally, we analysed the association of nuclear YB-1 and p-YB-1 expression in metastatic tissue, with clinical and pathological characteristics (Table 8). One sample in the case of nuclear YB-1 and two samples in the case of nuclear p-YB-1 were classified as “undefined” and excluded from analysis. In this cohort, 28% (14/50) of patients had positive nuclear YB-1 and 50% (24/48) positive nuclear p-YB-1. Positive nuclear YB-1 was associated with adjuvant HT (p=0,0039), and positive nuclear p-YB-1 with visceral-only recurrence (p=0,0350) (Table 8).

Table 8. Association between nuclear YB-1 and p-YB-1 expression in metastatic breast tumours and clinicopathologic characteristics.

Characteristics	YB-1 (%)		*p*	p-YB-1 (%)		*P*
	Positive	Negative		Positive	Negative	
Nº of Patients	14	36		24	24	
Age at diagnosis (years)						
Median (range)	60 (41 – 81)	54 (30 – 82)		60 (37 - 82)	54 (30 – 78)	
Adjuvant HT						
Yes	13 (92,9)	19 (52,8)		18 (75,0)	13 (54,2)	
No	0 (-)	15 (41,7)	**0,0039#**	5 (20,8)	9 (37,5)	0,2078#
Unknown	1 (7,1)	2 (5,6)		1 (4,2)	2 (8,3)	
HER2						
Negative	6 (42,8)	26 (72,2)		17 (70,9)	14 (58,4)	
Positive	4 (28,6)	9 (25,0)	0,4411#	5 (20,8)	8 (33,3)	0,5098 #
Unknown	4 (28,6)	1 (2,8)		2 (8,3)	2 (8,3)	
ER						
Negative	3 (21,4)	11 (30,5)		4 (16,7)	10 (41,7)	
Positive	7 (50,0)	24 (66,7)	>0,9999 #	18 (75,0)	12 (50,0)	0,1040 #
Unknown	4 (28,6)	1 (2,8)		2 (8,3)	2 (8,3)	
PR						
Negative	5 (35,7)	21 (58,3)		11 (45,8)	13 (54,2)	
Positive	6 (42,9)	13 (36,1)	0,4851#	12 (50,0)	8 (33,3)	0,3815 #
Unknown	3 (21,4)	2 (5,6)		1 (4,2)	3 (12,5)	
Metastasis site						
Bone only	2 (14,3)	8 (22,2)		1 (4,2)	8 (33,3)	
Visceral only	10 (71,4)	21 (58,3)	0,6889#	16 (66,7)	11 (45,8)	**0,0350#**
Bone & Visceral	2 (14,3)	7 (19,5)		7 (29,1)	5 (20,8)	

#Fisher's exact test.

Moreover, we observed a non-significant tendency to decreased OS in patients with positive nuclear p-YB-1 (YB-1: p=0,2824; HR= 0,597; 95%CI 0,258-1,379; p-YB-1: p=0,1766; HR= 0,601; 95%CI 0,286-1,262) (Figure 6.A and B).

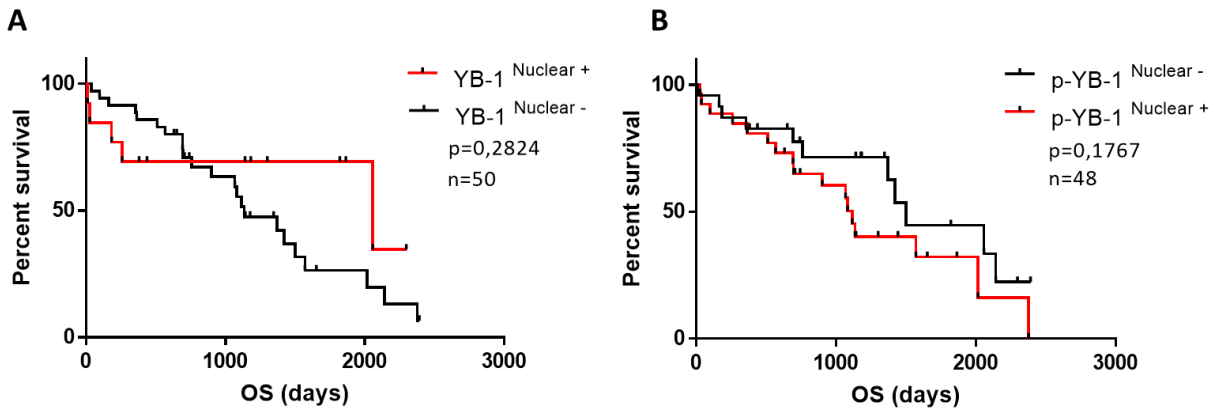

Figure 6. Positive nuclear p-YB-1 in metastasis is not associated with shorter OS. Kaplan-Meier Overall Survival (OS) curves according to **(A) YB-1 nuclear staining** (HR= 0,597; 95%CI 0,258-1,379) and **(B) p-YB-**

1 nuclear staining (HR= 0,601; 95%CI 0,286-1,262); P-Value and Hazard ratio (HR) was calculated using log-rank test and significance set at 0,05.

Next, we compared the expression of YB-1 and p-YB-1 in primary tumours and metastases. Median YB-1 H-Score was similar (p=0,3560) (Figure 7.A), while the levels of p-YB-1 were significantly reduced in metastases (p=0,0345) (Figure 7.B). In this cohort, 7 out of 12 patients with elevated p-YB-1 in primary tumour had a decrease in p-YB-1 expression in the metastasis sample. So, we questioned if the loss in p-YB-1 expression in metastases could be associated with the technical processing of samples affecting IHC. Sample processing may differ according to the specific metastatic tissue, for example, including sample decalcification in the case of bone metastases. However, there was no difference between metastases location in terms of median H-scores when comparing bone and non-bone metastases (Figure 7.C and D).

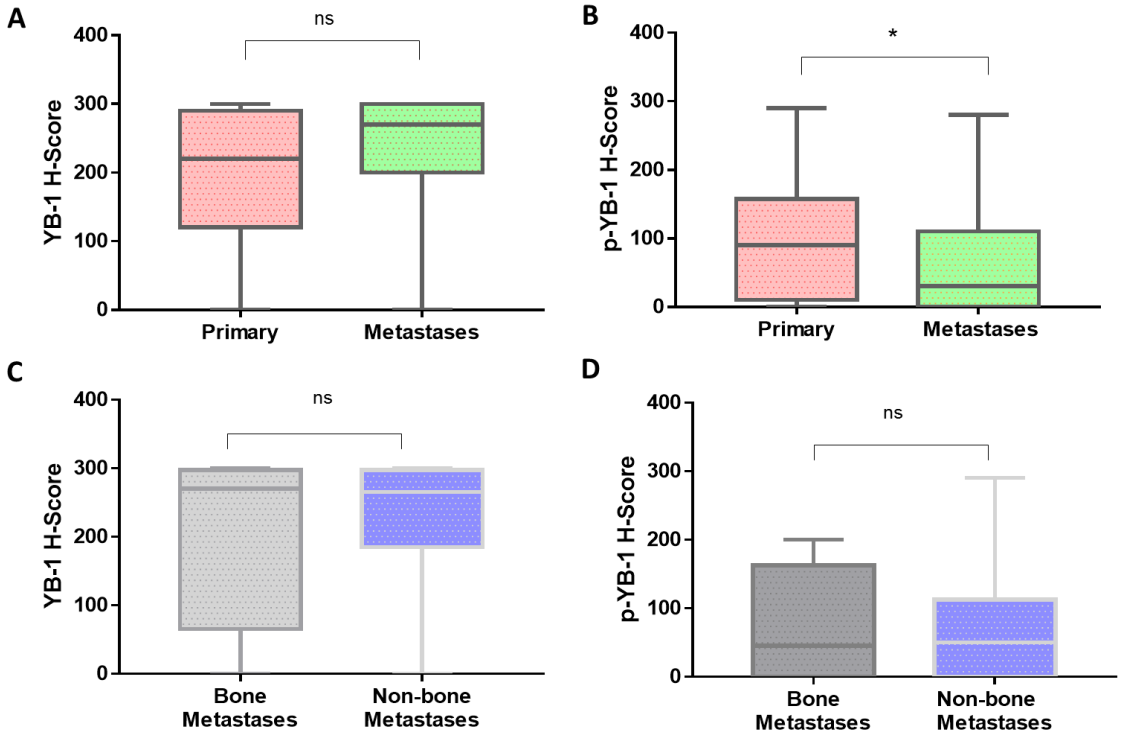

Figure 7. Box plot graphics of (A) YB-1 and (B) p-YB-1 H-score in primary tumours and paired metastases (n=51); and (C) YB-1 and (D) p-YB-1 H-score in bone or non-bone metastases. P-value was calculated using unpaired t-test and * p<0,05.

The analysis of the cohort of paired primary tumours and metastasis showed a switch in the molecular status from the primary to the metastatic tumour in 20 cases out of 51. To answer the question if YB-1 expression was associated with this switch, namely with ER and/or PR loss and HER2 gain, we looked for molecular receptors' alterations in cases with high YB-1 expression in the primary tumour (14/51) or metastasis (16/51), in comparison with cases with low YB-1 expression (Figure 8). Gain in HER2 (four cases) and loss of ER (five cases) were equally balanced between the groups; whereas loss of PR (eight cases) was predominant in cases with low YB-1 expression (7/8; 87,5%). The nuclear staining was also evaluated, and it was not associated with total YB-1 expression or alteration in molecular receptors' status. In this case, there were 24 positive cases for nuclear YB-1 and one correspond with a case of loss of HER2, two tumours corresponds to loss of ER and four with the loss of PR.

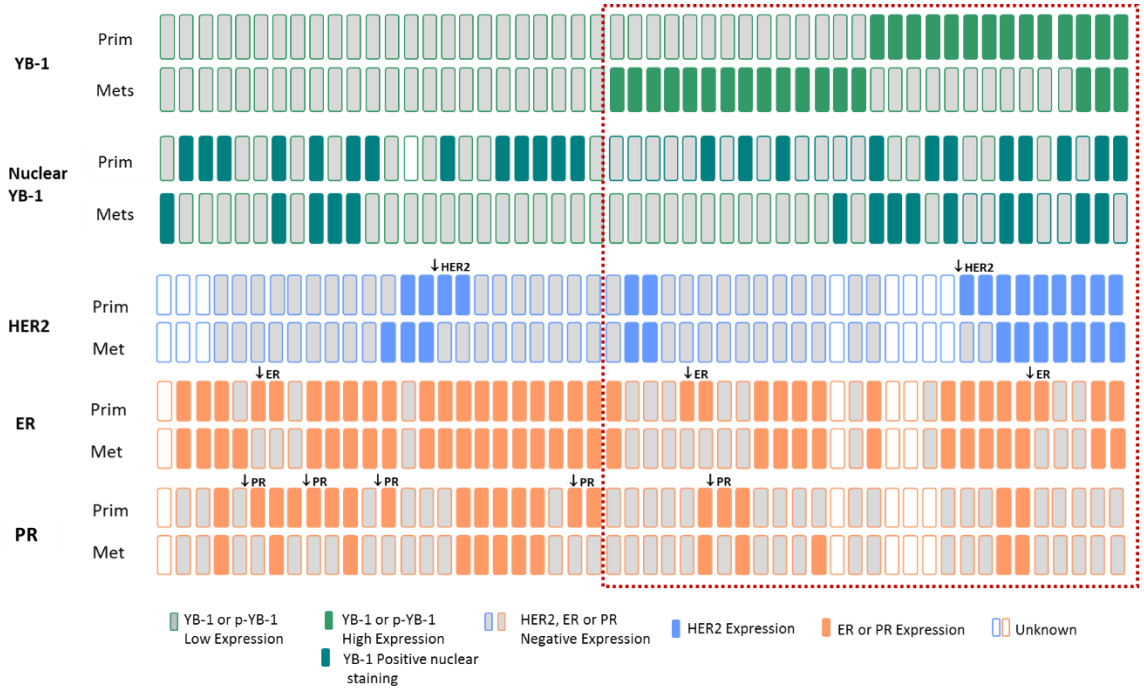

Figure 8. Schematic representation of YB-1, p-YB-1 expression and nuclear YB-1 in primary tumours and HER2, ER and PR clinical status in primary and metastatic tumours.

After, we extended the analysis for our entire cohort of primary tumours (n=78), where 20/78 were YB-1^{High} and 58/78 were YB-1^{Low}. The distribution of YB-1 expression, nuclear localization, HER2 expression and Hormone Receptors expression

are represented in Figure 9. The number of these cases remained balanced between tumours with low or high expression of YB-1; with 8/20 YB-1^{High} cases (40,0%) and 24/58 YB-1^{Low} (41,4%) cases presenting at least one alteration. HER2 alterations occurred in 8 metastases, ER alteration in 11 metastases, and PR alterations occurred in 20 metastases. However, molecular alterations were more frequent in metastases from YB-1^{Nuc+} primary tumours (11/24; 45,8%) than in metastases from YB1^{Nuc-} primary tumours (20/54; 37%).

Figure 9. Schematic representation of YB-1 expression and YB-1 nuclear staining in primary tumours and HER2, ER and PR clinical status in primary and metastatic tumours. Cases were ordered from the weakest (up) to the strongest staining (down) of total YB-1.

4.3. YB-1 expression and resistance to therapy in BC cell lines

4.3.1. Impact of YB-1 overexpression in sensitivity to therapy

To answer our question if YB-1 expression is affected by exposure to HT and/or related to acquired resistance, we used a panel of four Luminal BC cell lines (ER+HER2-: MCF-7 and T47D; ER+HER2+: BT474 and MDA-361). From herein, MDA-MB-361 cell line is designated by MDA-361. These cells express similar levels of YB-1 but less than the TNBC cell line MDA-MB-231 as expected (Figure 10). We also confirmed the expression of *ERBB2*, *ESR1*, *PGR* and *CCND1*, as well as the protein levels of YB-1, ER and HER2 in these cells, which was in accordance with their molecular subtype.

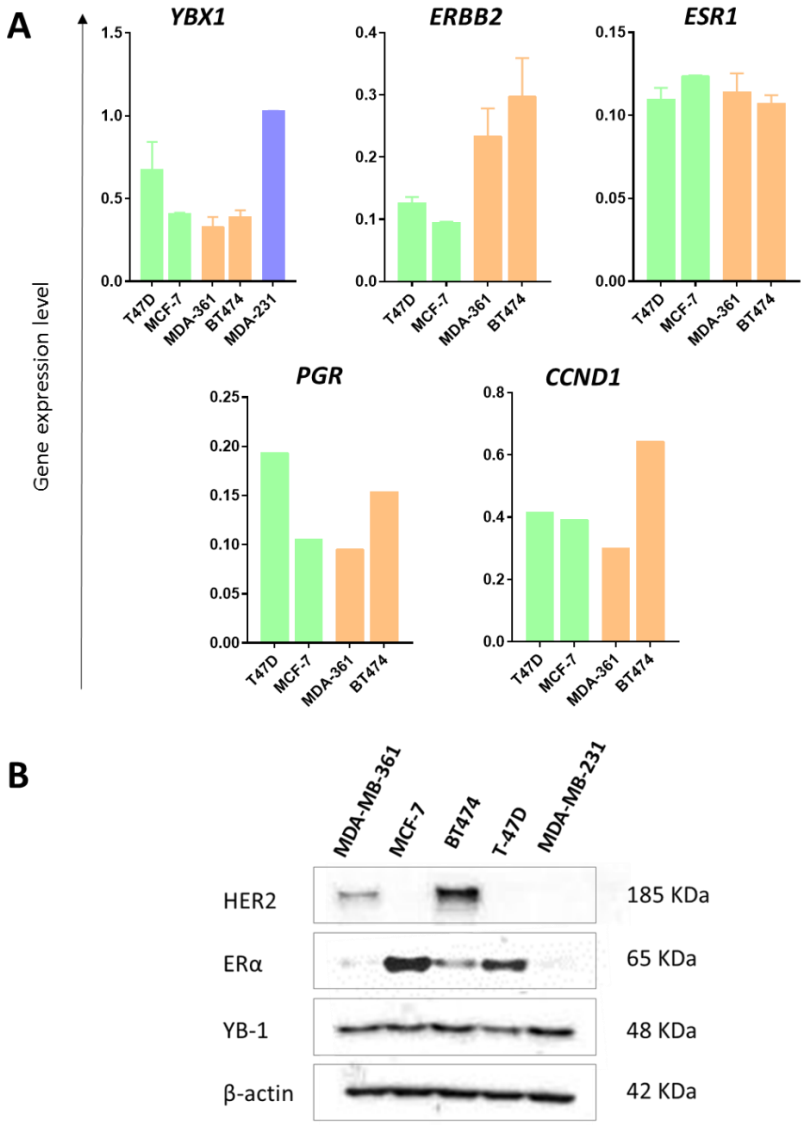

Figure 10. YBX1, ERBB2, ESR1, PGR and CCND1 mRNA and protein levels in a panel of BC cell lines. (A) Relative mRNA expression was quantified by RT-qPCR and normalized to GAPDH gene. The experiments

were done in triplicate (except PGR and CCND1) and results presented as the mean±SEM. **(B)** YB-1, ERα and HER2 protein expression was evaluated by Western Blot, using β-actin as loading control.

Next, we selected the MCF-7 cell line to overexpress YB-1 by lentiviral transduction. YB-1 overexpression (OE) was confirmed by RT-qPCR (Figure 11.A) and Western Blot (Figure 11.B). We obtained a 3-fold increase in YB-1 mRNA; and 1,55-fold and 1,63-fold increase in YB-1 total and phosphorylated protein levels, respectively. We also characterized the expression of Hormone Receptors (ER and PR), HER2, and the ER-downstream target gene *CCND1* (cyclin D1) in YB-1 OE cells (herein designated MCF-7$^{YB\text{-}1\ OE}$), by RT-qPCR and/or Western blot (Figure 11.A and 11.B, respectively). MCF-7$^{YB\text{-}1\ OE}$ cells have decreased expression of ER, without significant decrease in *CCND1* expression. In accordance, we did not observe a difference in proliferation rate of MCF-7$^{YB\text{-}1\ OE}$ cells when comparing to parental MCF-7 cells (Figure 11.C).

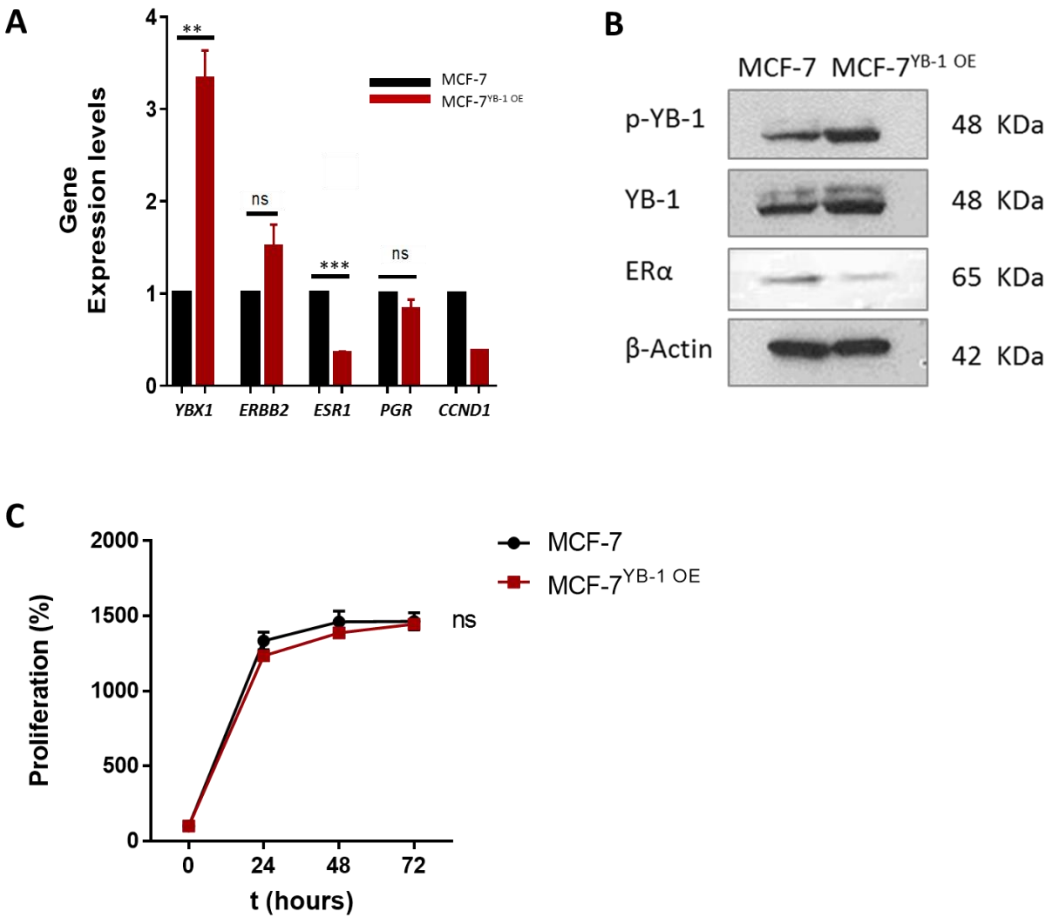

Figure 11. YB-1 overexpression in MCF-7 cell line decreases ER expression. (A) Relative mRNA expression was quantified by RT-qPCR and normalized to GAPDH gene. Gene fold change was determined using the 2-ΔΔCt method. p-value was calculated using unpaired t-test and significance set

at 0,05. (YBX1: p=0,0099; ERBB2: p=0,1641; ESR1: p<0,0001; PGR:p=0,2432) (B) Protein expression was evaluated by Western Blot and β-Actin was used as loading control. (C) Proliferation was assessed by Alamar blue viability assay. Experiments were performed in triplicate, and results presented as the mean±SEM. p-value was calculated using two-way ANOVA and significance set at 0,05.

We later assessed the effect of YB-1 OE over MCF-7 sensitivity to five different drugs, used to treat Luminal BC, namely the HT drugs tamoxifen and fulvestrant; the anti-HER2 lapatinib, since we observe a slight increase in *ERBB2* mRNA expression; the mTOR inhibitor everolimus; and the CDK4/6 inhibitor palbociclib. Cells were exposed to different drugs' concentrations for seven days, after which cell viability was measured by Alamar blue assay (Figure 12). MCF-7$^{YB\text{-}1\ OE}$ cells were less sensitive to HT (tamoxifen: p=0,0192; fulvestrant: p=0,0433); and more sensitive to lapatinib (p=0,0065), whereas no differences were observed concerning everolimus and palbociclib.

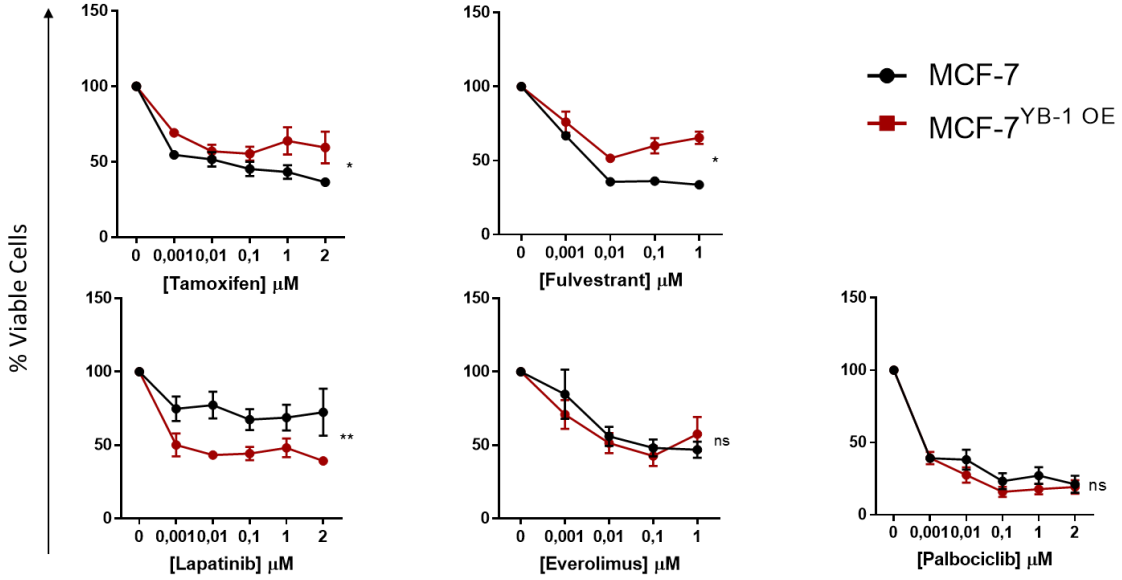

Figure 12. Overexpression of YB-1 decreases sensitivity to HT but sensitizes cells to anti-HER2 lapatinib. Cells were seeded in 96-well plates and exposed to different drugs for seven days. Medium was replaced every two days, and viability was assessed by Alamar blue assay. Results are the mean of three independent assays, with 4 replicates per assay, and presented as the mean±SEM. *p-value* was calculated using paired t-test, *p<0,05 and **p<0,01. Hormone therapy: Tamoxifen (p=0,0192) and Fulvestrant (p=0,0433); Anti-HER2: Lapatinib (p=0,0065); mTOR inhibitor: Everolimus; CDK4/6 inhibitor: Palbociclib.

Together, these data show that YB-1 expression is implicated in the modulation of important receptors, like HER2 and ER, and affects cell responsiveness to therapy. Therefore, we further aimed to test if under therapy, YB-1 overexpressing cells are selected and implicated in acquired resistance.

4.3.2. Modulation of YB-1 expression and sensitivity profiles after long-term exposure to low-dose therapies

Next, to select resistant clones, cells were exposed for five months to low doses of the different drugs. The best dose, that killed approximately 50% of cells, was chosen after assessing the sensitivity of each cell line to the different drugs to be tested (Figure 13).

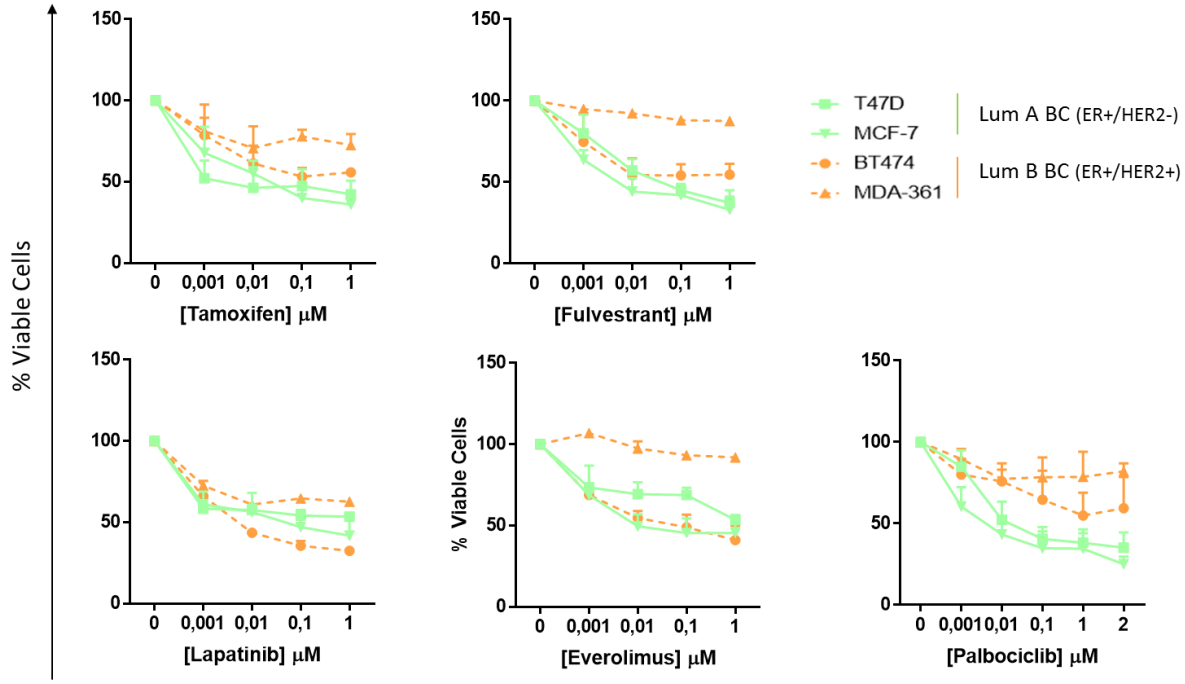

Figure 13. **Baseline sensitivity profiles assessed by Alamar blue viability assay.** Cells were seeded in 96-well plates and exposed to different drugs for seven days. Results are the mean of three independent assays, with four replicates per assay, and presented as the mean±SEM. Hormone therapy: Tamoxifen and Fulvestrant; Anti-HER2: Lapatinib; mTOR inhibitor: Everolimus; CDK4/6 inhibitor: Palbociclib.

Based on these results, the four parental cell lines were exposed to a fixed concentration of 10nM tamoxifen (TAM), 10nM fulvestrant (FULV), 100nM lapatinib (LAP), 100nM everolimus (EVE) and 10nM of palbociclib (PALB) for five months. During this period, a first evaluation of the sensitivity profiles was performed after 3 months. When comparing untreated with treated cells, designated as "_R" cells, we found there was not a significant difference (Figure 14). Missing combinations are due to difficulty in maintaining some cells in culture, for different technical reasons, including MDA-361 cells exposed to fulvestrant or everolimus, or BT474 exposed to everolimus and palbociclib.

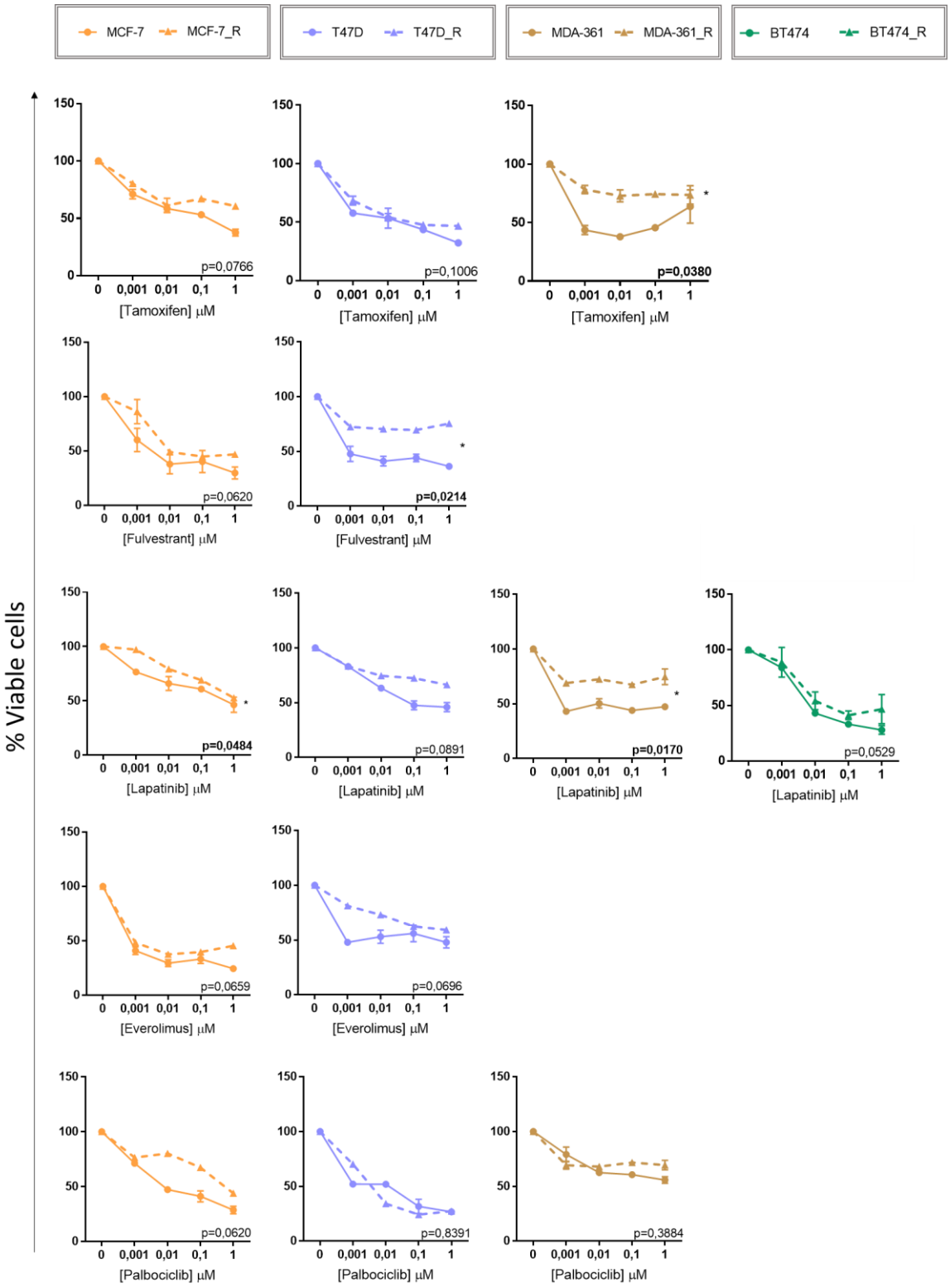

Figure 14. Sensitivity profiles after 3 months of drug's exposure assessed by Alamar blue viability assay. Cells were seeded in 96-well plates and exposed to different drugs for seven days. Results are the mean of four replicates of one assay and presented as the mean±SEM. *p-value* was calculated using paired t-test and *p<0,05. Hormone therapy: Tamoxifen and Fulvestrant; Anti-HER2: Lapatinib; mTOR inhibitor: Everolimus; CDK4/6 inhibitor: Palbociclib

Based on this interim evaluation, we continued the experiment with MCF-7 and T47D cells, exposed to tamoxifen and fulvestrant; and the four cell lines exposed to lapatinib. After five months of continuous exposure to these drugs we re-assessed the sensitivity profiles to each drug. All treated cells were less sensitive to drugs, in comparison to the respective parental cells (untreated), although MCF-7 cells exposed to fulvestrant and T47D cells exposed to lapatinib did not reached a statistically significant difference (Figure 15).

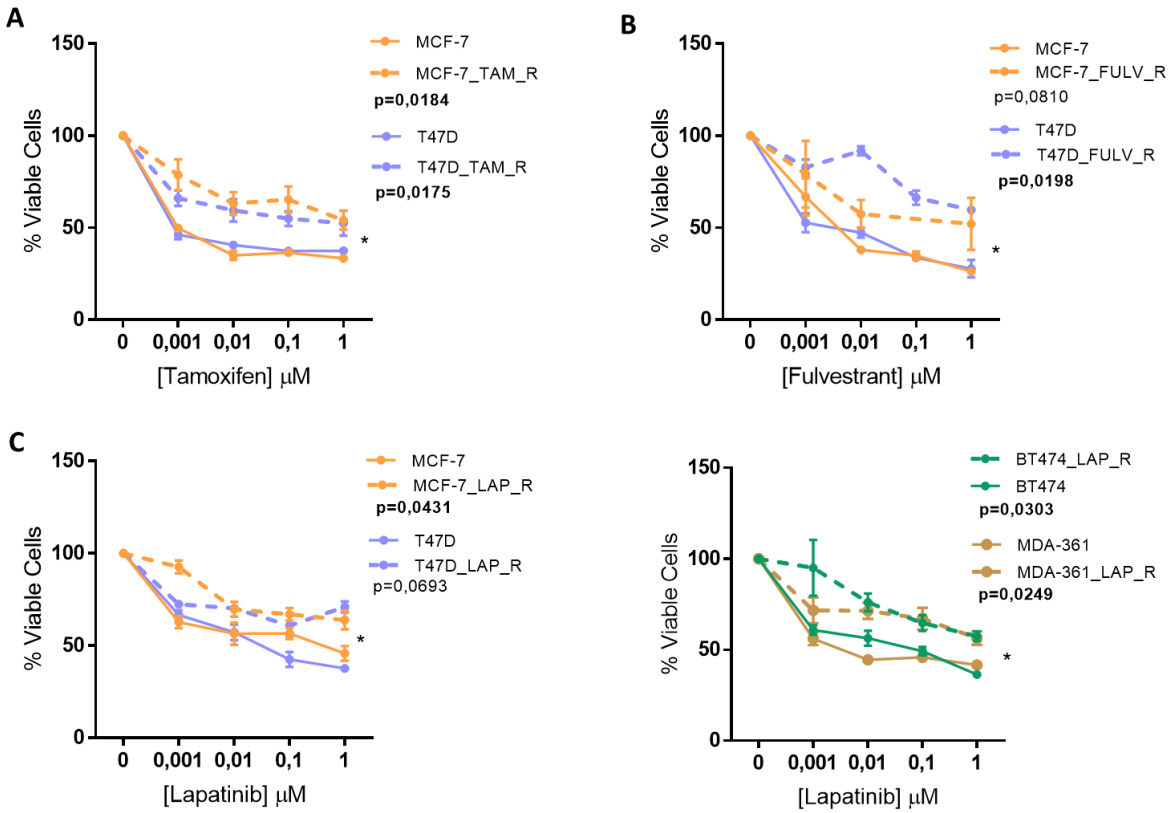

Figure 15. Sensitivity profiles after 5 months of drug's exposure assessed by Alamar blue viability assay. (A) Tamoxifen; (B) Fulvestrant; **(C) Lapatinib**. Viability Assays was measured by Alamar Blue assay after 7 days of exposure to drugs. The experiments were done with four replicates per assay and presented as the mean±SEM. *p-values* were calculated using paired t-test and *p<0,05.

Next, we assessed whether YB-1, ER, HER2, PR and cyclin D1 were differentially expressed in resistant cells (time of exposure t=5 months) in comparison to parental ones (t=0), by RT-qPCR and Western blot. In cells resistant to tamoxifen we observed a

decrease in hormone receptors expression (ER and PR) in MCF-7 cells, but an increase in T47D cells (Figure 16.A and Figure 17). Upon exposure to fulvestrant, T47D cells show an increase in *ERBB2* expression which did not translated into increased amount of protein (Figure 16.B and Figure 17), and both Luminal A cell lines had a major down-regulation of *PGR* (Figure 16.B). Regarding lapatinib treated cells (Figure 16.C), it was observed an up-regulation in all genes in T47D cells, a down-regulation in hormone receptors in MCF-7 cells, and increased YB-1 expression in T47D and MDA-361 cells. At the protein level, we confirmed the up-regulation of YB-1 and ER in T47D cells resistant to tamoxifen and fulvestrant, and the down-regulation of ER in MCF-7 cells resistant to tamoxifen and fulvestrant; but not the increase in YB-1 in T47D and MDA-361 cells resistant to lapatinib (Figure 17). Expression of cyclin D1 followed the differential expression of its major regulator ER.

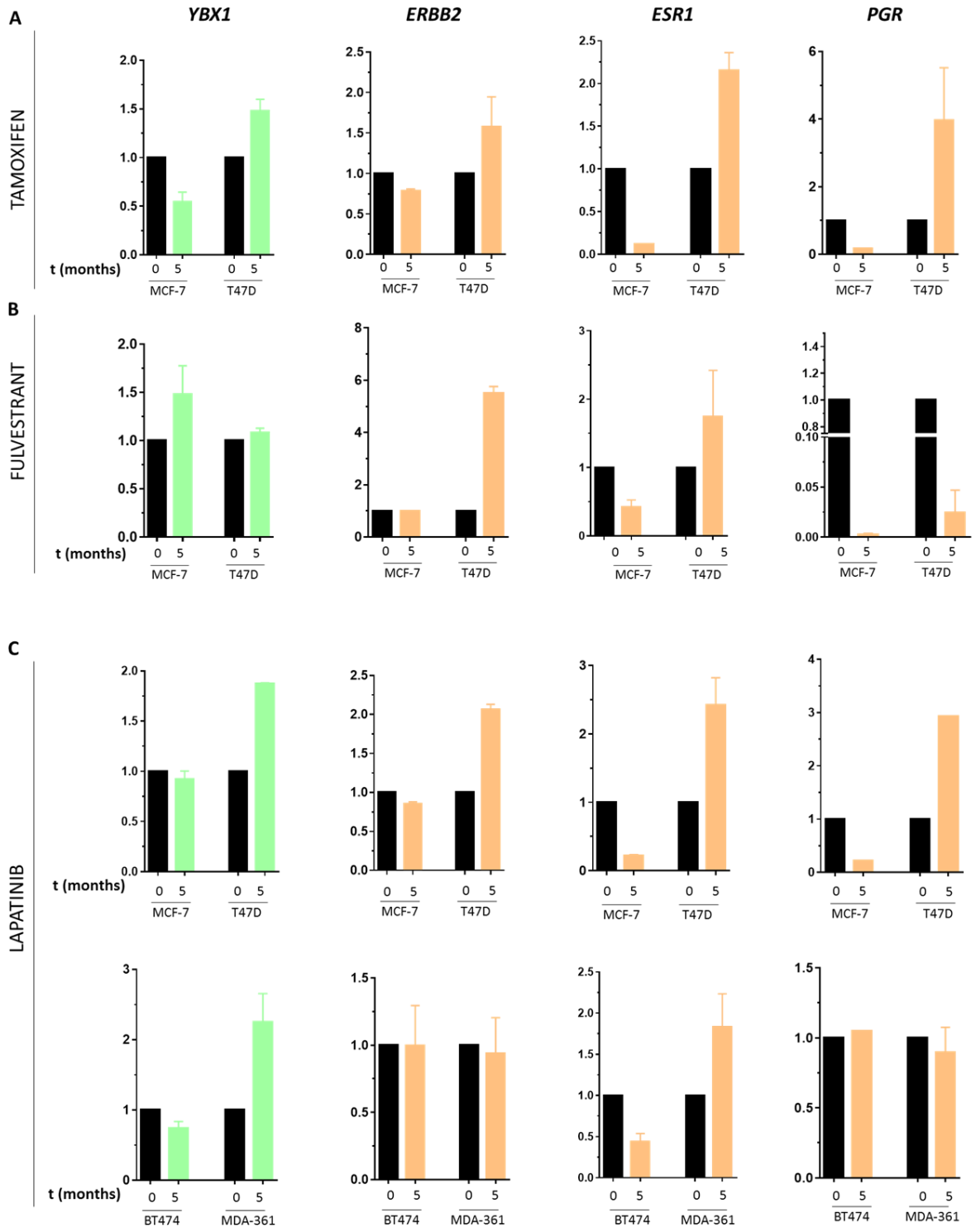

Figure 16. ***YBX1*, *ERBB2*, *ESR1* and *PGR* expression levels in cells resistant to (A) tamoxifen (B) fulvestrant and (C) lapatinib.** mRNA of gene expression was quantified by RT-qPCR and normalized to 18S gene. Gene fold change was determined using the $2^{-\Delta\Delta Ct}$ method and expression was normalized to untreated parental cells (0). The experiment was done in triplicate and results presented as the mean±SEM.

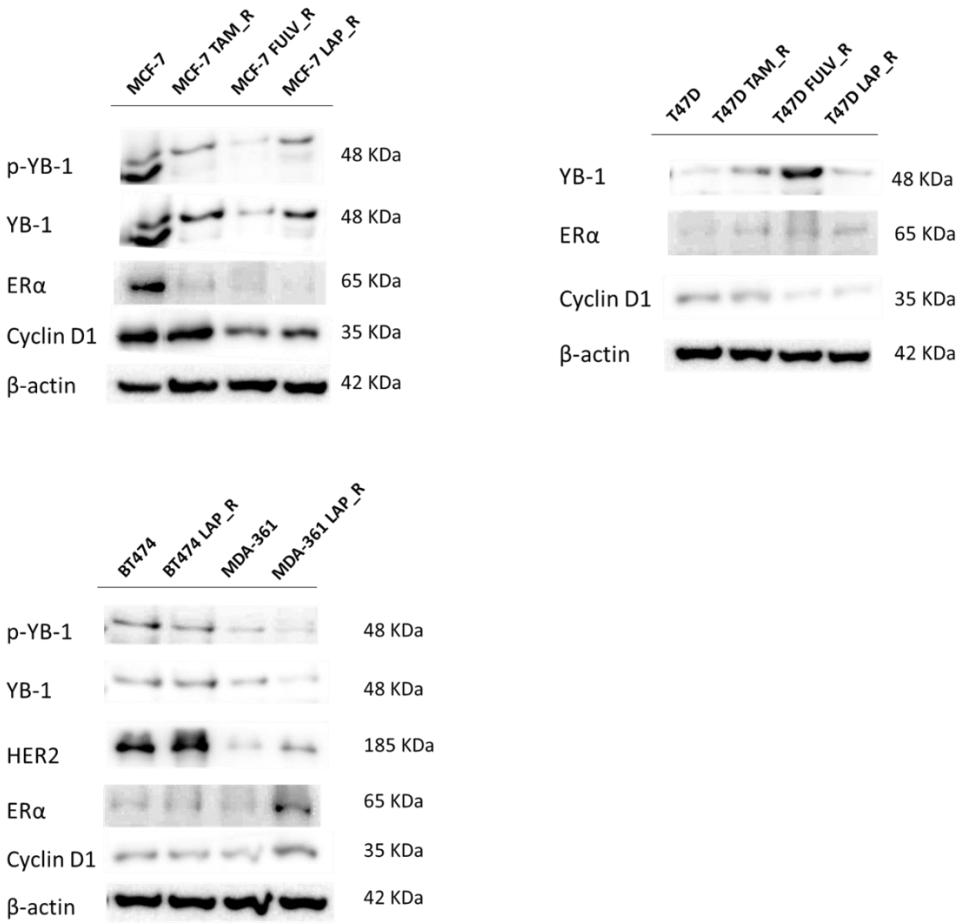

Figure 17. Protein expression in tamoxifen, fulvestrant, and lapatinib resistant cells. p-YB-1, YB-1, HER2, ER and CyclinD1 protein expression was evaluated by Western Blot and β-Actin was used as loading control.

Since YB-1, ER and Cyclin D1 are associated with proliferation, we also assessed the proliferation rate of resistant cells in comparison to untreated parental cell lines. In general, proliferation was not significantly affected in resistant cells (Figure 18.A).

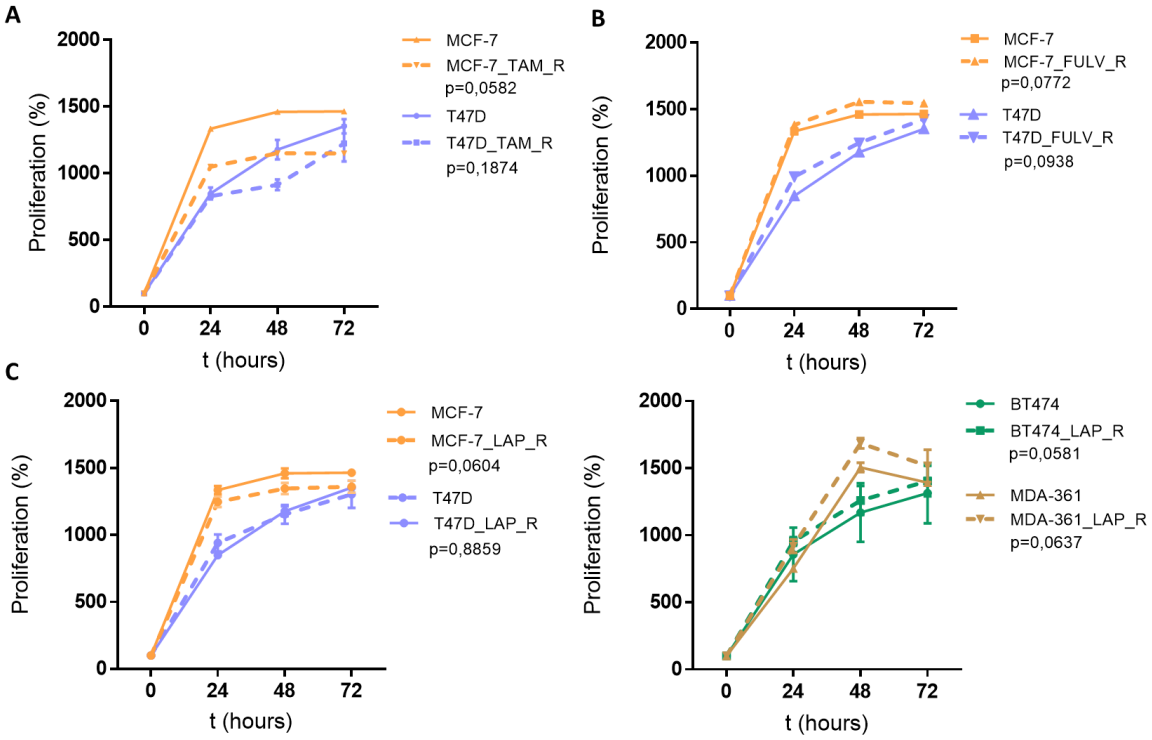

Figure 18. Proliferation rates in cells resistant to (A) Tamoxifen; (B) Fulvestrant; and (C) Lapatinib. Proliferation was measured by Alamar Blue assay for 3 days. The experiments were done with four replicates and results presented as the mean SEM. p-value were calculated using paired t-test and significance set as 0,05..

Since the proliferation of ER+ cells is estradiol-dependent, we questioned if the lower expression of ER in MCF-7 TAM_R and FULV_R cells (Figure 17) could be associated with the lower proliferation rate. Therefore, we assessed the sensitivity to 17β-estradiol of MCF-7 TAM_R cells in comparison with MCF-7 parental cells. MCF-7 TAM_R cells were less sensitive to 17β-estradiol depletion, although still sensitive to β-estradiol addition (Figure 19).

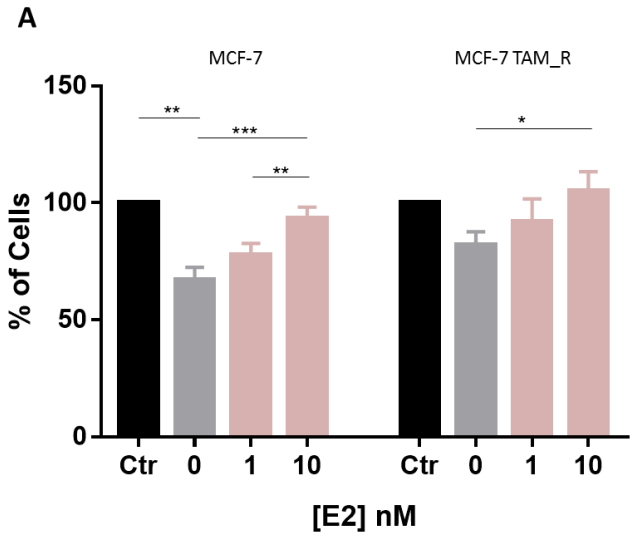

Figure 19. Estradiol sensitivity assay. After β-estradiol complete depletion from culture medium, cells were cultured in the presence of 0nM, 1nM or 10nM β-estradiol for 5 days. Viable cells were measured by Alamar Blue Assay. *p<0,05, **p<0,01 ***p<0,001 and p-value were calculated using One-way ANOVA.

3.3.3. Sensitivity profile of tamoxifen and fulvestrant resistant cells to alternative therapeutic options

Since Luminal A cells resistant to tamoxifen and fulvestrant were found to have molecular alterations in YB-1, ER, HER2 and/or cyclin D1, we chose to evaluate the response of those cells to alternative therapies like lapatinib, everolimus and palbociclib. MCF-7 cells resistant to fulvestrant were more sensitive to everolimus and to palbociclib; and there was also a trend for sensitivity to palbociclib in T47D cells resistant to fulvestrant (Figure 20).

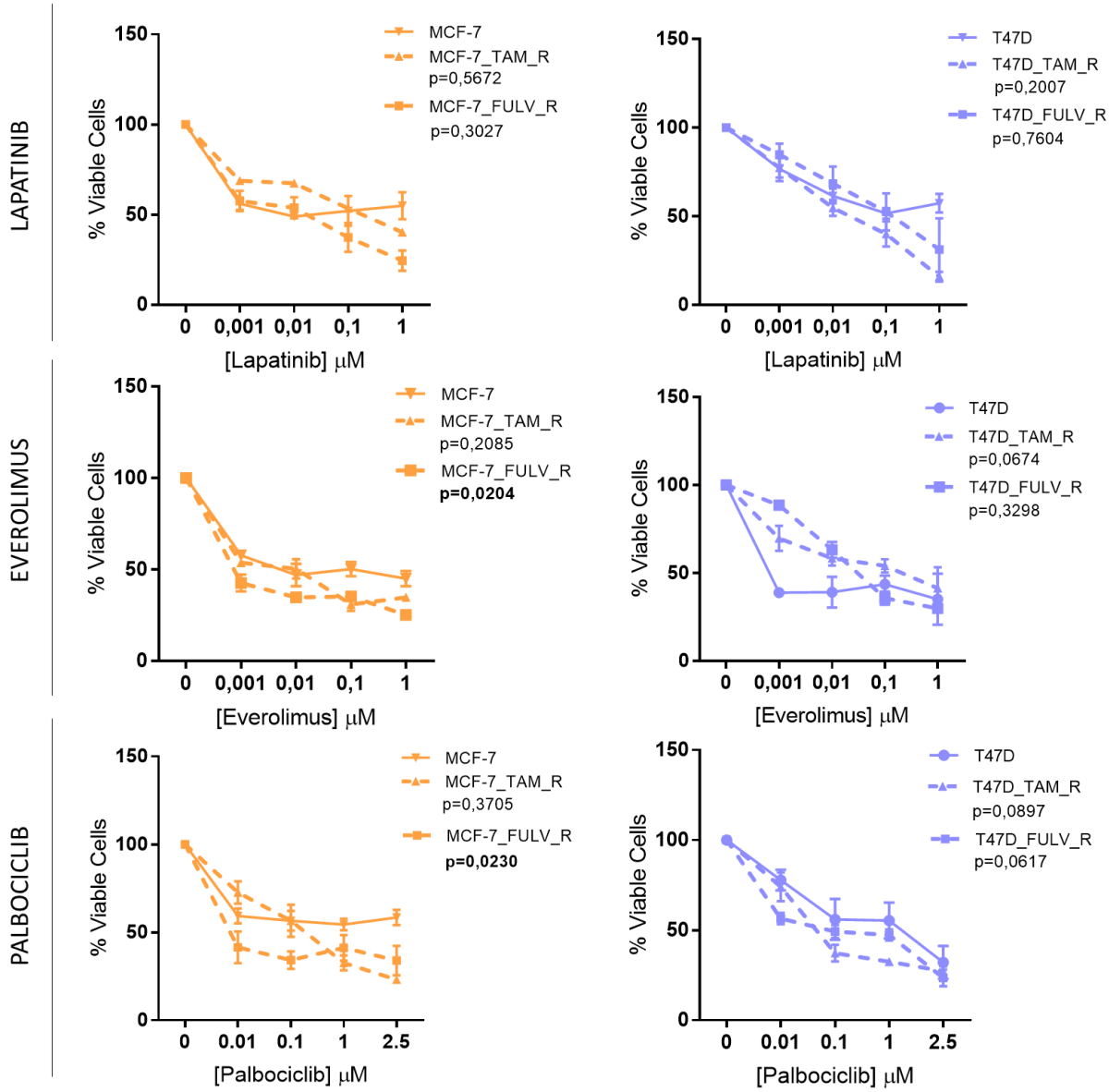

Figure 20. Sensitivity profiles assessed by Alamar blue viability to (A) anti-HER2, (B) mTOR inhibitor and (C) CDK4/6 inhibitor. Viability Assays was measured by Alamar Blue assay after 7 days of exposure to drugs. Cells were seeded in 96-well plates and exposed to drugs for seven days. Results are the mean of four replicates and presented as the mean±SEM. *p-value* were calculated using paired t-test and significance set at 0,05.

5. DISCUSSION

BC is the most diagnosed cancer among women worldwide, and Luminal BC is the most frequent subtype corresponding to 60% of new cases per year.[1] Despite significant therapeutic improvements in recent years, about 30% of women will develop metastatic BC.[4] Since resistance to therapy will dictate the success of metastatic care, it is extremely important to address resistance mechanisms and to find reliable predictive biomarkers.

YB-1 affects the expression of a variety of genes related with oncogenic signalling pathways[45,56,61] and was recently described as a transcriptional modulator of ER and HER2 expression, involved in resistance to HT.[63] Therefore, we were interested in study its role as a possible prognostic and predictive biomarker in Luminal BC.

Different studies have shown that high levels of YB-1 are associated with poor prognosis[60,61] especially when YB-1 is located in the nucleus. In this way, we started by analysing the impact of YB-1 and p-YB-1 in RFS and OS in a cohort of 80 BC patients. In this cohort, and in line with other reported analyses[43,66], elevated YB-1 was associated with a negative ER status; and there was also a trend for association between low YB-1 and visceral-only metastasis (Table 5). Accordingly, and also as previously described[60], elevated YB-1 was a biomarker of worst clinical outcome, translated into decreased OS (Figure 3.A). Although some studies have related the levels of YB-1 with metastasis development[60], in this cohort there was no association with RFS (Figure 3.C and D). To our knowledge this was the first study of p-YB-1 as a prognostic biomarker. We did not find any association with clinicopathologic characteristics or clinical outcome.

YB-1 regulates different cellular functions accordingly to its cellular location. Once activated by phosphorylation, cytoplasmic YB-1 translocates to the nucleus where it acts as transcription factor.[34] So, we looked for associations between nuclear YB-1 and p-YB-1 and clinicopathological characteristics. In this cohort, there was an association between nuclear p-YB-1 and negativity of ER (Table 6). Then, we assessed the impact of nuclear YB-1 or p-YB-1 in OS and RFS, and both YB-1 and p-YB-1 positive nuclear staining showed a trend towards decreased OS, particularly in the first years of

follow-up (Figure 4.A and B). As the median OS of this cohort was 4,93 years, we performed a 5-year survival analysis, showing that nuclear YB-1 and p-YB-1 are biomarkers of decreased 5-years OS (Figure 4.C and D).

We also analysed a cohort of 51 paired metastases. No association between YB-1 or p-YB-1 and clinical characteristics was found (Table 7). However, elevated YB-1 was again a biomarker of worst OS (Figure 5.A). Concerning p-YB-1 there were no differences between the two arms, but it should be noted that there were only five patients in the YB-1^{High} arm and all of them were alive in the last follow-up (Figure 5.B). Putting primary tumours aside, we next assessed the impact of nuclear YB-1 and p-YB-1 in this cohort of metastases. We found an association between nuclear YB-1 and adjuvant HT; and between nuclear p-YB-1 and visceral metastasis (Table 8). This suggests that adjuvant HT may drive the selection of clones with elevated YB-1 expression. Concerning clinical outcomes, there was no association between nuclear YB-1 or p-YB-1 with OS after relapse (Figure 6).

We observed that p-YB-1^{High} cases were a minority, and to discard the hypothesis that this was influenced by technical processing of samples, as decalcification of bone metastases, we assessed the levels of YB-1 and p-YB-1 in bone versus non-bone metastasis, which were similar (Figure 7).

It is known that the expression of HER2, ER and PR can change from primary tumours to metastasis. Since YB-1 has been described as a regulator of ER and HER2 expression *in vitro*, we performed the first analysis of association between YB-1 expression and HER2, ER and PR status in the clinical setting. We identified the cases with alteration in molecular status of HER2, ER and/or PR, but in our cohort, there was no obvious association with YB-1 or p-YB-1 expression. Nevertheless, all cases with alteration and YB-1^{High} corresponded to loss of ER/PR and gain of HER2 (Figure 8 and Figure 9). It is important to validate these results in a larger cohort.

Overall, our analysis in a cohort of 80 primary BC tumours and 51 paired metastases corroborates the importance of YB-1 as a biomarker of poor prognosis and shows that it is implicated in ER negativity and HER2 positivity. Moreover, we provide the first data that shows that adjuvant HT may be implicated in the selection of clones

with elevated YB-1 expression and this may be associated with the poor outcome of YB-1^{High} patients.

Therefore, we proposed to understand if and how YB-1 expression is associated with acquired resistance to HT. We used an *in vitro* model, with a panel of four Luminal BC cell lines (ER+HER2-: MCF-7 and T47D; ER+HER2+: BT474 and MDA-361) (Figure 10). As mentioned before, YB-1 upregulation has been previously associated with decreased ER and increased HER2 expression, in ER+ BC cells T47D.[63] Accordingly, our results showed that YB-1 overexpression in MCF-7 cells led to a slight increase in *ERBB2* and a significant decrease in *ESR1* (Figure 11.A and B). The expression of *PGR* was not affected by upregulation of YB-1. Since *ESR1* and *CCND1* were decreased, we also asked whether proliferation would be influenced. However, no effect on proliferation was observed (Figure 11.C).

As expected upon the decrease in ER, cells overexpressing YB-1 show intrinsic decreased sensitivity to tamoxifen and fulvestrant; furthermore, MCF-7$^{YB\text{-}1\ OE}$ was more responsive to the anti-HER2 Lapatinib (Figure 12). Lapatinib is a tyrosine kinase inhibitor that inactivates HER2 signalling pathway. Therefore, an increase in HER2 expression is predictive of better response.

We also hypothesised that YB-1 could be associated with acquired resistance to HT. It has already been demonstrated that T47D Luminal BC cells treated with two HT drugs have high level of *YBX1* expression.[63] Thus, we exposed the four cell lines MCF-7, T47D, MDA-361 and BT474 to a low concentration of tamoxifen, fulvestrant, lapatinib, everolimus and palbociclib for five months, to select the resistant ones (Figure 13). The drugs were chosen based on clinical relevance. Tamoxifen and fulvestrant are standard of care therapies for ER+ BC; and Lapatinib, Everolimus and Palbociclib are second line treatments for metastatic BC.

There are three molecular targets particularly relevant for the treatment of metastatic Luminal BC. In the case of HER2 overexpression[67], the use of the anti-HER2 tyrosine kinase inhibitor lapatinib has shown efficacy in combination with HT[15]. PI3K/AKT/mTOR pathway is in most cases mutated and constitutively hyperactivated[21,55]; and some studies have shown that patients who have decontrolled mTOR pathway benefit from mTOR inhibitors, like

everolimus.[22,23] Finally, CDK4/6 inhibitors have been identified as a good option to treat metastatic ER+HER2- BC, in combination with HT.[33]

An interim three months evaluation of sensitivity (Figure 14), and technical aspects related to culture maintenance dictated that MCF-7 and T47D cells, cultured in the presence of tamoxifen, fulvestrant and lapatinib; and BT474 and MDA-361 cells cultured in the presence of lapatinib were used for the final 5 months analysis (Figure 15). All cell lines exhibited decreased sensitivity to the respective selection drugs.

After that, we evaluated the expression of YB-1 and analysed if it was associated with differential expressions of ER, PR and HER2, as it has been reported before.[60,63] PR is a hormone receptor activated by progesterone and it is often expressed in Luminal BC and, as ER, promotes cell development and proliferation.[68] Also, it has been associated in a negative manner with the expression of YB-1.[34] Although we did not observe a significative difference in the expression of YB-1 or HER2 in MCF-7 cells resistant to HT, ER and PR decreased. However, T47D cells exposed to HT showed an increase in YB-1, HER2 and ER (Figure. 16 and Figure. 17). We also observed an increase in YB-1, *ERBB2, ESR1* and *PGR* in T47D cells resistant to lapatinib, but downregulation of ER and PR in lapatinib resistant-MCF-7 cells. Further studies should be done to clarify both the results obtained in this analysis and the impact of YB-1 in receptors' expression. However, in cells with acquired resistance to therapy, YB-1 probably does not affect exclusively ER and HER2 expression. A study has shown that high expression of YB-1 in BC is accompanied by hyper activation of KRAS, inducing the activation of HIF1α, associated with poor prognosis.[72]

Additionally, MCF-7 resistant to HT had a lower proliferation rate than parental cells (Figure 18). Since these cells, which are estradiol dependent[69,70], had decreased ER expression and a lower proliferation rate, we measured cell proliferation in the absence or presence of 17β-Estradiol (E2). Our data shows that proliferation of parental MCF-7 cells was decreased in the absence of E2 and that cells are responsive to E2, increasing the proliferation rate. However, HT-resistant MCF-7 cells were not so dependent on E2 (Figure 19), which can suggest that, although they also rely on the activation of ER-pathway, other pathways will compensate the decrease in ER expression and subsequent signalling.

Metastatic BC is a challenge for clinicians and researchers and until now many mechanisms have been discovered and targeted to treat patients. An *in vitro* study has previously demonstrated that anti-HER2 therapies could be an alternative treatment for HT-resistant Luminal A BC (HER2-) with YB-1 overexpression.[63] In the same line, we tested the sensitivity of MCF-7 and T47D HT-resistant cells to lapatinib. MCF-7 resistant to fulvestrant are a quite more sensitivity to lapatinib. Moreover, we also tested the sensitivity of these cells to everolimus and palbociclib. In general, although not statistically significative, HT-resistant clones have a trend to be more sensitive to alternative drugs (Figure 20).

6. CONCLUSIONS AND FUTURE PERSPECTIVES

YB-1 overexpression has been described as an oncogenic driver and marker, associated with drug resistance and poor clinical outcome in BC. As a negative ER modulator, YB-1 is mainly expressed in TNBC. Also, among BC, YB-1 expression has been related with decreased OS. Accordingly, in our cohort we demonstrated that high expression of YB-1, as well as nuclear YB-1, were associated with negative ER molecular status and with decreased OS. Interestingly, metastases from patients who benefited from adjuvant HT were enriched in elevated YB-1 expression.

Our *in vitro* study, using MCF-7 cells for the first time, corroborates that increasing YB-1 in Luminal BC results in the downregulation of *ESR1,* and less available ER in cells. This alteration is most likely responsible for the observed resistance to HT, tamoxifen and fulvestrant. On the other hand, cells which have been exposed to HT and anti-HER2 drugs, showed differences in the expression of receptors ER, PR and HER2; however, we did not see significant differences on the levels of YB-1. Importantly, we also have demonstrated that HT-resistant cells remained sensitive to alternative therapies such as lapatinib, everolimus and palbociclib, that are used in clinical practice for metastatic BC.

All results considered, our findings are important for future YB-1 studies in the context of BC, as we suggest that overexpressed YB-1 and its nuclear localization can be predictive biomarkers for HT resistance.

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8. SUPPLEMENTARY DATA

Table S 1. Population characteristics of BC samples

Characteristics	Cohort
Nº of Patients	80
Age at diagnosis (years)	
Median (range)	52
Menopausal Status (n, %)	
Premenopausal	29
Postmenopausal	46
Unknown	5
TNM Stage	
I	5
II	19
III	22
IV	14
Unknown	20
T	
1	22
2	27
3	4
4	9
Unknwon	18
N	
0	20
1	13
2	9
3	10
Unknown	28
Tumor Grade	
1	1
2	38
3	26
Unknown	15
HER2	
Negative	45
Positive	24
Unknown	11
ER	
Negative	21
Positive	54
Unknown	5
PR	
Negative	43
Positive	32
Unknown	5
Metastasis site	
Bone only	18
Visceral only	43
Bone & Visceral	18
Unknown	1