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PhD Thesis in Molecular and Oncology Medicine

Exploring epigenetic profiling as prognostic/predictive markers of endocrine resistance in estrogen receptor positive breast cancer

Avaliação de perfis epigenéticos como marcadores prognósticos/preditivos de resistência endócrina no carcinoma da mama com receptores de estrogénio positivos

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“In the middle of difficulty lies opportunity”

Albert Einstein

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List of Abbreviations and Acronyms

AC - asymptomatic controls

AI – aromatase inhibitor

AJCC – American Joint Committee on Cancer

Akt - Protein kinase B

ANA – Anastrozole

ASCO – American Society of Clinical Oncology

AUC – Area Under the Curve

BC – Breast cancer

bp – Base pairs

BRCA1 – BRCA1, DNA repair associated

BRCA2 – BRCA2, DNA repair associated

ccfDNA - circulating cell free DNA

CCN – Cyclin

CDH – Cadherin

CDK – Cyclin-dependent kinase

CDKN - Cyclin-dependent kinase inhibitor

Cht – Chemotherapy

CI – Confidence interval

CpG - Cytosine-phosphate-Guanine

DCIS - Ductal carcinoma in situ

DFS - Disease-free survival

DMFS – Distant metastasis-free survival

DNA - Deoxyribonucleic acid

DNMT - DNA methyltransferase

DSS – Disease-specific survical

E2 – Estradiol

EDTA – Ethylenediamine teracetic acid

EFNA3 - Ephrin A3

EGFR - Epidermal growth factor receptor

EMT - Epithelial–mesenchymal transition

ER – Estrogen receptor

ERBB - Erb-B2 Receptor Tyrosine Kinase

ERFS - Endocrine resistance-free survival

ESMO – European Society for Medical Oncology

ESR1 - Estrogen Receptor 1

ET – Endocrine therapy

FGFR1 - Fibroblast Growth Factor Receptor 1

FGFRL1 - Fibroblast growth factor receptor-like 1

FOX - Forkhead Box

FOXA1 - Fork-head box A1

FULV – Fulvestrant

G – Grade

GATA3 - GATA Binding Protein 3

GEMIN4 - Gem (nuclear organelle)-associated protein 4

GRB7 - Growth Factor Receptor Bound Protein 7

GSTP1 - Glutathione S-Transferase Pi 1

H3K27me3 - Trimethylation of lysine 27 of histone 3

HDAC - Histone deacetylase

HDMs - Histone demethylases

HER2 - Human Epidermal growth factor Receptor 2

HMTs - Histone methyltransferases

HNRNPL - Heterogeneous Nuclear Ribonucleoprotein L

HoR – Hormone receptor

HR – Hazard ratio

IDC - Invasive ductal carcinoma

IGFR1 - Insulin-like growth factor 1 receptor

IHC - Immunohistochemistry

IPO Porto - Portuguese Oncology Institute of Porto

KIT - KIT Proto-Oncogene Receptor Tyrosine Kinase

KRT – Keratin

LAM – Laminin

LHRH - luteinizing hormone-releasing hormone

LNA - Locked Nucleic Acid

lncRNA – Long non-coding RNA

Lum – Luminal

MAPK - Mitogen-activated protein kinase

MET - MET Proto-Oncogene, Receptor Tyrosine Kinase

MiR-30f – MiR-30 family

MiRNA – MicroRNA

MKI67 - Marker Of Proliferation Ki-67

mRNA – Messenger RNA

MSP – Methylation Specific Methylation

MTDH - Metadherin

MYBL2 - MYB Proto-Oncogene Like 2

n.a. – not applicable

NBr – normal breast tissues

ncRNAs - Non-coding RNAs

NPV - Negative predictive value

nt - Nucleotide

OR - Odds ratio

OS – Overall survival

P25 – Percentile 25

PgR - Progesterone Receptor

PI3K - phosphoinositide3-kinase

pN+ - pathologic node positive breast cancer

pN- - pathologic node negative breast cancer

PPV - Positive predictive value

PSAT1 - Phosphoserine Aminotransferase 1

PTEN - Phosphatase and tensin homolog

qMSP - Quantitative real-time methylation specific PCR

RAD52 - RAD52 homolog, DNA repair protein

RASSF1A - Ras Association Domain Family Member 1

RB1 – Retinoblastoma 1

ROC - Receiver Operating Characteristic

RR -relative risk

RS - Recurrence score

RT – Radiotherapy

S – Sensitivity

SDS – Sodium Dodecyl Sulfate

TCGAN - The Cancer Genome Atlas Network

TGFBR1 - Transforming growth factor, beta-receptor 1

TMX – Tamoxifen

TNBC – Triple-negative breast cancer

TNM - Tumor, Node and Metastases

TP53 - Tumor protein p53

TP63 - Tumor Protein P63

TFF1 - Trefoil factor 1

UICC - Union for International Cancer Control

UNKN – Unknown

VIM – Vimentin

WGA - Whole genome amplification

WHO - World Health Organization

XBP1 - X-Box Binding Protein 1

yr – Years

YWHAZ - Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

ZEB - Zinc finger E-box-binding homeobox

ZNF217 - Zinc finger protein 217

Abstract

Breast cancer (BC) is the solid tumor that is most commonly diagnosed in women worldwide and it is considered a highly heterogeneous disease. Most BC express hormone receptors (luminal subtypes) which has diagnostic, prognostic and therapeutic implications. Endocrine treatment (ET) is adequate in every stage of the disease and so far, there are no reliable biomarkers for endocrine treatment resistance in use in clinical practice.

We proposed to assess the potential of prognostic/predictive value of epigenetic mechanisms (post-translational changes) in hormone receptor positive/luminal BC, namely methylation in circulating cell-free DNA, microRNAs and histone marker.

We expanded on the findings that *PSAT1* promoter methylation independently predicted for worse outcomes, namely disease-free survival (DFS). Furthermore, high *Fork-head box A1* (*FOXA1*) methylation levels independently predicted shorter disease-specific survival (DSS), a finding that, to best of our knowledge, had not been reported thus far. Remarkably, *FOXA1* expression was previously shown to associate with good prognosis and response to endocrine therapy in BC patients and, consequently, we consider promoter methylation the most likely mechanism underlying *FOXA1* downregulation in BC. Notably, the use of a multiplex assay for a three-gene panel that is able to accurately detect BC in ccfDNA, regardless of tumor subtype, constitutes a step forward in this field and allow for a swifter translation into routine clinical practice. Indeed, owing to its characteristics, this panel might not only be useful for BC detection, but also for disease monitoring which deserves further exploration.

On the other hand, our results also suggest a panel of miRNAs that might be tested in primary tumor tissues to assess the likelihood of recurrence and resistance to ET in newly diagnosed luminal BC. Our cohort displayed higher miR-182-5p and miR-200b-3p levels compared to normal breast, whereas miR-30b-5p, miR-30c-5p, miR-182-5p and miR-200b-3p downregulation associated with decreased distant metastasis-free survival (DMFS). Additionally, decreased expression of miR-200f, and related members, might be associated with Epithelial–mesenchymal transition (EMT) initiation, a marker of tumoral aggressiveness.

Lastly, our exploratory retrospective study that included 160 invasive breast cancer patients with a median 10-year plus follow-up an expression <85% of the epigenetic biomarker H3K27me3 was

associated to statistically significant worse prognosis (higher recurrence risk) and statistical tendency to a decreased survival in luminal A/B-like HER2-negative breast cancer. These results expand and strengthen previous reports and encourage prospective validation, potential clinical use and targeted therapy. To our knowledge, our results are the first using computer aided immunohistochemistry (IHC) scoring for H3K27me3 expression.

Overall, these results warrant further analysis, especially in a prospective mode with large cohorts with a long follow-up time. And although the mechanisms of endocrine-resistance remain largely unknown, we showed epigenetic profiling and cancer are becoming mainstream research and may soon be part of the clinical practice.

Resumo

O cancro da mama é o tumor sólido mais frequentemente diagnosticado em mulheres em todo o mundo e é considerada uma doença muito heterogénea. A maioria dos casos de cancro da mama expressam receptores hormonais (subtipos luminais), o que tem implicações diagnósticas, prognósticas e terapêuticas. O tratamento hormonal é adequado em todas as fases da doença, e até ao momento, não há biomarcadores fidedignos de resistência endócrina em uso na prática clínica.

Propusemo-nos a avaliar o potencial valor prognóstico/preditivo de mecanismos epigéticos (alterações pós-translacionais) em cancros da mama com receptores hormonais positivos/luminais, nomeadamente metilação em DNA circulante extra-celular (ccfDNA), microRNAs e marca de histona.

Aprofundamos os achados em que a metilação do promotor *PSAT1* prediz, de forma independente, piores resultados clínicos, nomeadamente sobrevivência livre de doença. Além disso, níveis elevados de metilação de *FOXA1*, de forma independente, predizem menor sobrevivência especifica da doença, uma associação que, tanto quanto se sabe, não reportada na literatura até ao momento. Em contraste, a expressão de *FOXA1* havia sido previamente associada a melhor prognóstico e resposta a terapêutica hormonal em doentes com cancro da mama e, portanto, consideramos que a metilação do promotor é muito provavelmente o mecanismo subjacente à sub-expressão de *FOXA1* no cancro da mama. Notavelmente, o uso de um ensaio *multiplex* de um painel com três genes que consegue de forma fidedigna detetar cancro da mama através de ccfDNA, independentemente do subtipo tumoral, constitui um avanço na área e permitir uma passagem rápida para a prática clínica diária. Aliás, pelas suas características, o painel pode não só ser útil na deteção de cancro da mama, mas também para monitorização de doença, o que merece mais investigação.

Por outro lado, os nossos resultados sugerem também que um painel de miRNAs que podem ser testados no tumor primário para avaliar o potencial de recidiva e resistência endócrina, em doentes recém-diagnosticadas com cancro da mama luminal. A nossa coorte evidenciou níveis elevados de miR-182-5p e miR-200b-3p, quando comparados com tecido mamário normal, enquanto a sub-

expressão de miR-30b-5p, miR-30c-5p, miR-182-5p e miR-200b-3p esteve associada a menor sobrevivência livre de metastização. Uma diminuição da expressão de miR-200f, e outros relacionados, pode estar associado a iniciação do processo de transição epitélio-mesênquima, um marcador de agressividade tumoral.

Por último, na nossa análise exploratória retrospectiva, que incluiu 160 doentes com cancros de mama invasores com uma mediana de mais de 10 anos de seguimento, uma expressão <85% do biomarcador epigenenético H3K27me3 associou-se, de forma estatisticamente significativa, a pior prognóstico (maior risco de recidiva) e tendência estatística para menor sobrevivência em cancros de mama tipo luminal A/B HER2 negativos. Estes resultados aprofundam e fortalecem dados prévios e motivam validação prospectiva, potencial uso clínico e terapia-alvo. Tanto quanto se sabe, são os primeiros resultados que utilizam software para avaliação de expressão imunohistoquímica de H3K27me3.

Globalmente, os resultados justificam mais análise, em particular de forma prospectiva com coortes maiores e com longos períodos de seguimento. E apesar dos mecanismos de resistência endócrina permanecem em larga medida desconhecidos, demonstramos que o perfil epigenético e cancro está a tornar-se tópico de investigação convencional e pode vir a integrar brevemente a prática clínica

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Statement

According to Art.º 8º do Decreto-Lei n.º 388/70, the following body of work is part of the Thesis:

Publication (first author)

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Mário Fontes-Sousa; João Lobo; Silvana Lobo; Sofia Salta; Maria Amorim; Paula Lopes; Luís Antunes; Susana Palma de Sousa; Rui Henrique; Carmen Jerónimo. **Digital imaging-assisted quantification of H3K27me3 immunoexpression in luminal A/B-like, HER2-negative, invasive breast cancer predicts patient survival and risk of recurrence.** *Under review*

Publication (co-author)

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Maria Amorim, João Lobo, Mário Fontes-Sousa, Helena Estevão-Pereira, Paula Lopes, Sofia Salta, Nuno Coimbra, Luís Antunes, Susana Palma de Sousa, Rui Henrique, Carmen Jerónimo. **Predictive and prognostic value of selected microRNAs in luminal breast cancer**. *Under review*

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Masters in Oncology Thesis

- Maria Rodrigues Amorim. "**Decoding the usefulness of miRNAs as biomarkers in breast cancer patients treated with endocrine therapy**". Dissertação de Candidatura ao grau de Mestre em Oncologia – Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto, 2017[4].

Oral communication (international)

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Oral communication (national)

- Mário Fontes Sousa, Silvana Lobo, Sofia Salta, Paula Lopes, João Lobo, Susana Sousa, Rui Henrique, Carmen Jeronimo; "**Marca epigenética H3K27me3 e risco de recidiva em Carcinoma da Mama Luminal A/B-like**" presented at the 14th Congresso Nacional de Oncologia, organization Sociedade Portuguesa de Oncologia, 26 - 29 de October 2017, Aveiro, Portugal.

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- Mário Fontes Sousa, Silvana Lobo, Sofia Salta, Paula Lopes, João Lobo, Susana Sousa, Rui Henrique, Carmen Jeronimo: "**Epigenetic biomarkers in breast cancer: preliminary results from H3K27m3 assessment in endocrine-treatment resistant breast cancers**" presented at 42nd ESMO Congress (ESMO Congress 2017), 11/09/2017, Madrid, Spain

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Introduction

Since George Thomas Beatson was the first to perform a bilateral oophorectomy on a woman with breast cancer (BC)[5] in 1895, over 120 years ago, endocrine therapy in breast cancer has been virtually used in every setting of hormone-positive breast cancer. So far, no biomarker (other than hormone receptors) has been uncovered to predict or monitor endocrine treatment resistance in clinical practice.

Breast cancer is the most commonly diagnosed malignancy in women worldwide and it is considered a highly heterogeneous disease[6], being a challenge for research, trials and clinical practice.

Most BC cases, about 70%, express hormone receptors (HoR; Estrogen receptor – ER; Progesterone receptor - PgR), being one of the drivers of cancer cell proliferation and subsequent tumor development [HoR-positive BC subtypes (luminal A and luminal B)]. These are intrinsic subtypes of BC[6], which translate into the clinical practice as ´Luminal A-like' [ER-positive, human epidermal growth factor 2 receptor (HER2) negative, Ki67 low, PgR high] and 'Luminal B-like' HER2-negative (ER-positive, HER2-negative, Ki67 high or PgR low) or 'Luminal B-like' HER2-positive (ER-positive, HER2-positive, any Ki67 and any PgR). The other subtypes [(HER2 overexpression - HER2-positive non-luminal – and 'Basal-like' (with the surrogate definition of 'triple negative' BC)] have no hormonal receptor expression (defined as less than 1% of the cells).

Thus, the steroid hormone estrogen, and its receptor, are not only critical for the development and maintenance of the female reproductive system but also has a fundamental role in breast cancer pathogenesis, prognosis and treatment options. For instance, general consensus agrees that the systemic therapy of early BC is mainly informed by expression of hormone receptors (together with HER2 status) and also for locally advanced or metastatic BC endocrine therapy is the preferred option except if clinically aggressive disease mandates a quicker disease response ("visceral crisis") or if there are doubts regarding endocrine responsiveness of the tumor, according to current guidelines[7]. Some of the hormone therapy agents frequently used in the clinical setting includes selective ER modulators (such as Tamoxifen) or receptor down-regulator (Fulvestrant), aromatase

inhibitors [Anastrozole or Letrozole (non-steroidal) and Exemestane (steroidal)] and ovarian function ablation [(luteinizing hormone-releasing hormone (LHRH) analogs, such as goserelin, triptorelin or leuprolide] or surgical bilateral oophorectomy. Other possibilities, such as progestins, estrogens or anabolic steroids are very individualized options and are seldom used nowadays.

For instance, tamoxifen, one of the most widely prescribed hormone therapy agents, has been effectively used for 40 years and has been proven to reduce the risk of disease recurrence. Nevertheless, 28% of luminal A-like and 43% of luminal B-like BC patients will exhibit intrinsic or acquired drug resistance and develop distant metastases, sometimes after 15 years of the initial diagnosis. This is an important issue, since endocrine-resistant disease makes up almost a quarter of all BC cases and represents one of the most significant obstacles in BC treatment, being chemotherapy one of the remaining options for treating these patients. Resistance to endocrine therapy can be interpreted clinically as cancer recurrence or progression shortly after completion or during endocrine therapy[7]. Disappointingly, molecular elements, i.e. biomarkers, that predict endocrine response or resistance in ER-positive BC patients remain poorly understood and are lacking in clinical practice. Therefore, there is an obvious and urgent need to improve both the way ER-positive BC patients are stratified as responders to endocrine therapy and how endocrine-resistant disease is managed therapeutically. This objective could be achieved with robust biomarkers that could be predictive of treatment response that, in the case of metastatic disease for example, could be profiled using noninvasive assays of the blood in the absence of a tumor biopsy. This would benefit both the patient and the clinician, since the dynamic nature of the process is not ideally matched to systematically use of invasive procedures to assess profile status.

Although HoR are used to classify and inform about prognostic and therapeutic options, there's not necessarily a correlation between the qualitative or quantitative expression of HoR and disease responsiveness to endocrine therapy. The clinical practice shows that many patients will exhibit positive HoR expression and yet are resistant to endocrine treatments (although, in general, the absence is informative about primary resistance to endocrine treatments) which creates a quite complex scenario for decision making. Additionally, The Cancer Genome Atlas Network (TCGAN) effort on BC showed that luminal BC are the most heterogeneous in terms of gene expression, mutation spectrum, copy number changes and patient outcomes[8].

Epigenetics, as defined by Jones & Baylin[9], are regulatory mechanisms not directly involving the underlying coding sequence, displaying a flexible and reversible event with high impact on tumorigenesis, and may provide the much-needed markers. Some Methylation or MicroRNAs biomarkers have already been described.

The first independently validated DNA-methylation marker for outcome prediction in primary breast cancer was *PITX2* (*Paired-like homeodomain transcription factor 2)*[10,11]. This transcription factor is regulated by Wnt/DVL/β-Catenin and *TGF-β* pathways. Results showed that 'low-risk' patients, identified as such by *PITX2* methylation status, would have sufficient benefit from adjuvant tamoxifen (possibly being spared from adjuvant chemotherapy treatment). Further research showed that hypermethylation of *PITX2* is positively associated with BC disease progression. More recently some ER-enhancers (*DAXX, RXRA, MSI2, NCOR2* and *C8orf46*) were described as markers for endocrine sensitivity, pointing that endocrine-resistant disease could be a combination of both acquired and intrinsic methylation differences.
It comes to no surprise, as mentioned before, since Tamoxifen in one of the most widely prescribed hormone therapy agents, some research has led to the development of related markers. *ESR1*, *ARH1* and *CYP1B1* are markers for treatment response in patients receiving and not receiving Tamoxifen as hormonal treatment and *PSAT1* (*Phosphoserine aminotransferase*), a marker to progression in patients treated with Tamoxifen. Interestingly, ESR1 gene, encoding estrogen receptor proved to be the best predictor of progesterone receptor status, whereas methylation of the PGR gene, encoding progesterone receptor, was the best predictor of estrogen receptor status.

In the endocrine resistance setting, the Mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways, have a substantial role in the perturbance of the epigenetic balance in cancer genomes[12], with growing support for combinatorial PI3K–AKT–mTOR-targeted and epigenetic therapies in cancer treatment. The European Medicines Agency (and later the Food and Drug Administration) have approved everolimus (mTOR inhibitor) for the treatment of postmenopausal women with advanced hormone receptor-positive, HER2-negative breast cancer in combination with exemestane, after failure of treatment with AI. Postatranslational modifications of histone tails have also been implicated in endocrine resistance but remain poorly understood.

However, the effectiveness of ET is limited - as high as 40% of unselected patients with primary breast cancer will experience disease recurrence while on ET adjuvant treatment[13,14]. Moreover, in the metastatic setting, acquired resistance to ET is virtually an universal feature, and is clinically defined by expert consensus, such as the 4th ESO-ESMO International Consensus Guidelines[7] and many efforts have been made to understand the biological mechanisms involved in acquisition of acquired resistance to ET. These, however, remain mostly elusive and no biomarkers have been validated in this setting despite intense drug development and approval.

Epigenetic mechanisms & breast cancer endocrine resistance

Epigenetics may be defined as mechanisms that regulate cell fate specifications, while the DNA remains unchanged[15]. Some of these mechanisms include DNA methylation, non-coding RNAs, chromatin remodeling and histone post-translational modifications or variants[13]. Collectively, these components constitute the epigenome machinery whose role is to define which information is available for transcription and for translation[15]. DNA methylation is performed by specific enzymes, the DNA methyltransferases (DNMTs) that introduce a methyl group at the 5' position of a cytosine ring inside CpG dinucleotides[16]. Globally, promoter methylation of genes is associated with transcription inhibition[16]. Furthermore, the N-terminal tails of histones may undergo post-translation modifications that subsequently impact the chromatin structure[17]. The most well-studied histone post-translation modifications are histone acetylation and histone methylation. Histone acetylation is associated with gene expression and is carried out by histone acetyltransferases (HATs), while histone deacetylation is accomplished by histone deacetylases (HDACs)[17]. Histone methylation, which depending on the residue and the number of methyl groups may lead either to transcription repression or activation[18], is catalyzed by histone methyltransferases (HMTs), while histone demethylation is performed by histone demethylases (HDMs)[17]. In addition to post-translational histone modifications, histone variants that can replace canonical histones are an additional level of epigenetic complexity and contribute to the shaping of the chromatin structure. Non-coding RNAs (ncRNAs) comprise a hidden layer of internal signals that control various levels of gene expression[19]. Among these, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are the most frequently reported in BC. lncRNAs are ncRNA molecules usually longer than 200 nucleotides that do not fit into known classes of small or structural RNAs[19] and may act as protein-DNA or protein-protein scaffolds, miRNA sponges, protein decoys, or

regulators of translation[20]. miRNAs are endogenous, small non-coding single-stranded RNAs with ~22 nucleotides in length, that exert a finely tuned regulation of gene expression at the post-transcriptional level[21] by binding to mRNA targets, inducing its cleavage or repressing its translation[21]. Over the last few years, convincing data has suggested that altered epigenetic regulation may be involved in tumor initiation, progression and cancer resistance to therapy, including endocrine resistance, particularly in BC. For instance, ER expression is currently one of the foremost predictive biomarkers of response to ET and altered expression of ER may be due to hypermethylation of CpG islands within its promoter, increased histone deacetylase activity in the ESR1 promoter or translational repression by miRNAs[22]. Since ER was found to be deleted in only 15-20% of endocrine-resistant BC, several

epigenetic mechanisms may be involved in the development of endocrine treatment-resistance[23], and some of these are depicted in Figure 1. For the selection of the most relevant bibliography, we conducted a PubMed® search using the terms 'endocrine resistance', 'breast cancer' and 'epigenetic mechanisms'. Reference lists from key articles were also searched for additional relevant data. The criteria for article selection were: written in English, central theme based on ET resistance on BC and epigenetic mechanisms.

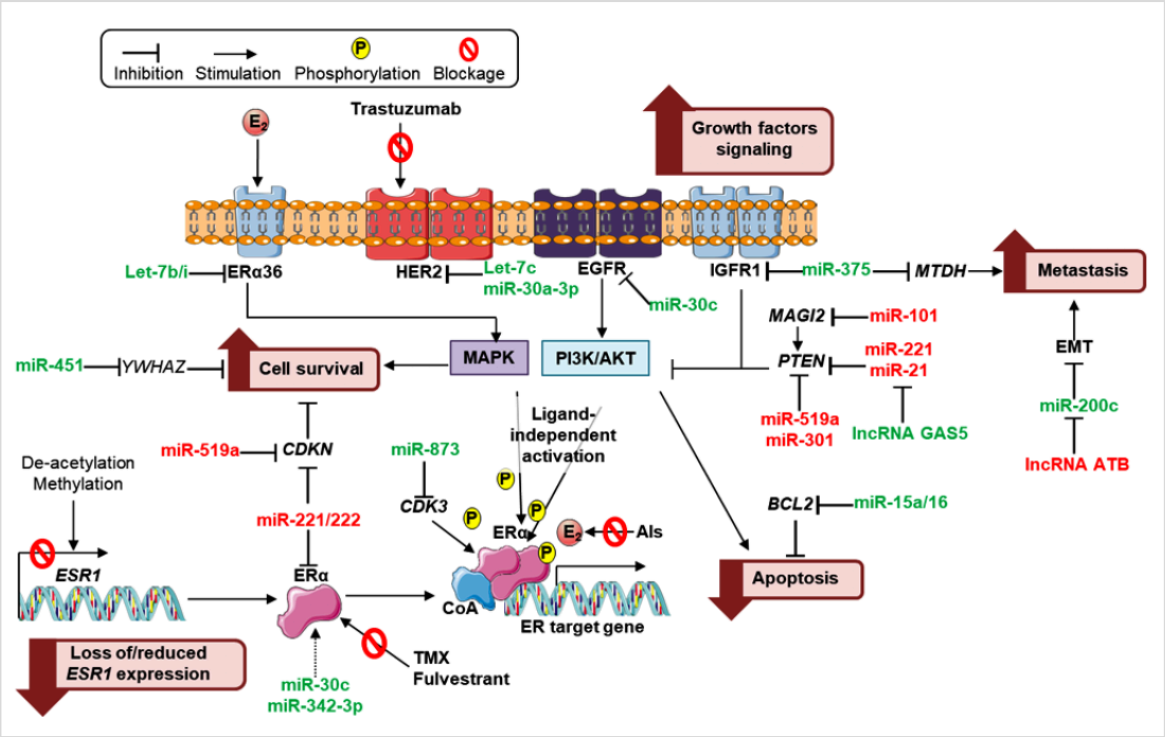


Figure 1. NcRNAs and their established targets involved in endocrine resistance. The ncRNAs and their targets involved in several mechanisms associated with endocrine resistance, along with their functional implication (in pink boxes), including loss of/reduced ESR1 expression, alternative growth-factor signaling inducing downstream signaling, including PI3K/Akt and MAPK signaling pathways, dysregulation of cell survival and apoptosis pathways, and increased metastasis. NcRNAs that confer sensitivity and resistance to endocrine therapies are depicted in green and red, respectively. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; EGFR, epidermal growth factor receptor; IGFR1, insulin-like growth factor 1 receptor; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ; MTDH, metadherin; MAGI2, membrane-associated guanylate kinase inverted 2; PTEN, phosphatase and tensin homolog; EMT, epithelial-mesenchymal transition; CDKN, cyclin-dependent kinase inhibitor; CDK3, cyclin dependent kinase 3; BRCAL2, B-cell CLL/lymphoma 2; PI3K/AKT, phosphoinositide 3-kinase/protein kinase B; ESR1, estrogen receptor 1; TMX, tamoxifen; AIs-aromatase inhibitors; E2, estradiol; miR, microRNA.

Original studies were selected based on the detail of analysis, mechanistic support of data, novelty, and potential clinical usefulness of the findings. Chemotherapy/radiotherapy-resistance, HER2-enriched subtype or 'triple negative' BC citations were excluded for being outside the scope of this review. DNA methylation. DNA methylation is one of the most common epigenetic changes and has been reported in multiple tumors, including BC[19,24]. This epigenetic alteration is inherently stable and has been proposed as a promising cancer biomarker in multiple cancers since it can be sampled from less invasive sources such as liquid biopsies (plasma or urine)[24,25,26(p)]. Thus, the role of DNA methylation as a predictor of ET resistance is a field of growing interest and has become the focus of several research teams[27–29] since it may improve BC patients' risk stratification. Notably,

Stone et al reported that in endocrine treated-resistant cell lines, DNA hypermethylation occurs predominantly at estrogen-responsive enhancers, leading to reduced ER binding and subsequently to expression downregulation. Furthermore, luminal subtype BC patients with relapsed disease exhibited significantly higher methylation levels at all enhancer loci studied[30]. By comparing anti-estrogen-resistant cell lines with the parental sensitive cell line, DNA methylation of the promoter region of genes was also suggested to play a role in the emergence of endocrine resistance[28,31] (Table 1). Multicenter studies, including several cohorts of BC patients were able to confirm these findings. Specifically, PITX2 methylation levels were consistently identified as a valuable biomarker to predict outcome in low-risk BC patients (ER-positive, node-negative) treated with surgery followed by adjuvant tamoxifen[10,32]. Nevertheless, multiple validations are still required before the implementation of these markers in the clinical setting (Table 1). Thus, to date, no clinical trials have assessed the clinical relevance of these candidate biomarkers.

Table 1. DNA methylation of the promoter region of genes as predictive biomarkers to different modalities of endocrine therapies along with their role and the biological samples used in each study.

Biomarker	Role	Agent	Samples
PTEN	Hypermethylation is associated with resistance	TMX	Cell lines
PTGER4	Hypomethylation is associated with resistance	EDT	
CDK10	Hypermethylation is associated with shorter PFS and OS	TMX	Cell lines and
HOXC10	Hypermethylation is associated with resistance	EDT, AIs and TMX	tumor tissues
ESR1 CYP1B1	High methylation levels are associated with a better outcome	TMX	Tumor tissues
ID4	Hypomethylation is associated with resistance		
NAT1	Hypermethylation is associated with resistance		
PITX2	Hypermethylation is associated with worse outcome and shorter MFS		
PR	Hypermethylation is associated with resistance		
PSAT1	Hypermethylation is associated with good clinical benefit		

PFS, progression-free survival; OS, overall survival; MFS, metastasis-free survival; TMX, tamoxifen; AIs, aromatase inhibitors; EDT, estrogen deprivation therapy; PTEN, phosphatase and tensin homolog; PTGER4, prostaglandin E receptor 4; CDK10, cyclin dependent kinase 10; HOXC10, homeobox C10; BRCA1, BRCA1 DNA repair associated; ESR1, estrogen receptor 1; CYP1B1, cytochrome P450 family 1 subfamily B member 1; ID4, inhibitor of DNA binding 4 HLH protein; NAT1, N-acetyltransferase 1; PITX2, paired like homeodomain 2; PR, progesterone receptor; PSAT1, phosphoserine aminotransferase 1.
Bibliographical references:[10,11,27,32–40]

As previously mentioned, decreased ER expression may be due to post-transcription regulation of miRNAs, including that of miR-221/222, whose overexpression has been associated with resistance to tamoxifen[41] and fulvestrant[42]. Conversely, miR-342-3p levels were revealed to be positively correlated with ER mRNA expression in human BC and associated with tamoxifen sensitivity[43,44]. miRNAs that regulate growth, survival, apoptosis, epithelial-mesenchymal transition (EMT) and metastasis of BC. cells may be implicated in loss of responsiveness to ET. In particular, PTEN downregulation due to specific miRNAs, permitting abnormal Pi3K/Akt pathway activation, promote estrogen-independent growth and survival of breast cancer cells leading to endocrine treatment resistance[45,46]. Several clinical trials are currently ongoing to evaluate the role of miRNAs as predictive biomarkers in BC. Specifically, trials such as NCT01231386* and NCT01722851*, aim to identify circulating miRNAs aiding at the identification of biomarkers of early response to neoadjuvant hormonal therapy, which may be used as potential targets for personalized therapies. Conversely, the NCT01612871* trial was set to explore a panel of circulating miRNAs that could aid to monitor the disease status of the patient while on adjuvant ET.

lncRNAs have also been associated with endocrine treatment resistance. Particularly, lncRNAs, breast cancer anti-estrogen resistance 4 (BRCAAR4) overexpression[47,48] and DSCAM antisense RNA 1 (DSCAM-AS1)[49], which contains an ER promoter binding motif, have been revealed to predict tamoxifen resistance in primary BC (Table 2 and Fig. 1).

* Details available at https://clinicaltrials.gov/ct2/home *(last accessed 27/02/2019)*

Table 2. Non-coding RNAs involved in response (sensitivity/resistance) to different modalities of endocrine therapies along with their putative targets/mechanism and the biological samples used in each study.

ET	Role	miRNA	Putative target	Agent	Samples
AntiE	Sensitivity	miR-375	*MTDH*	TMX	Cell lines
		miR-873	*CDK3*		
		miR-320a	*ARPP19, ESRRG*		
		Let-7b/i	*ESR1* (ER-α36 variant)		
		miR-451	*YWHAZ*		
		miR-17/20	*CCND1*		
		miR-148a	*ALCAM*		
		miR-152			
		miR-200c/b	*ZEB1/2*	TMX and FULV	
		miR-15a/16	*BRCAL2*	TMX	Cell lines and xenografts
		miR-342-3p	*BMP7, GEMIN4*		Cell lines and tumor tissues
		miR-26a	*EZH2*		Tumor tissues
		miR-30c	*EGFR*		
		miR-10a	-		
		miR-126			
	Resistance	miR-10b	*HDAC4*	TMX	Cell lines
		lncRNA DSCAM-AS1	Binding to the hnRNPL protein	Tumor tissue and cell lines	
		miR-519a	*CDKN1A, PTEN, RB1*		
		lncRNA BRCAAR4	-		
		miR-221/222	*ESR1, CDKN1B, CTNNB1*	TMX and FULV	
		miR-301	*FOXF2, PTEN, BBRCA3iso2, COL2A1*	TMX	Tumor tissue, cell lines and xenografts
		miR-155	*SOCS6*		
		miR-210	*EFNA3, E2F3, RAD52, FGFRL1, MET*		Tumor tissue
AIs	Sensitivity	Let-7f	*CYP19A1*	LET	Cell lines
		miR-125b	*ERBB2*	LET and ANA	Tumor tissues and cell lines
		let-7c			
	Resistance	miR-128a	*TGFBR1*	LET	Cell lines
		miR-181a	*BRCAL2L11*		Cell lines, xenografts and tumor tissue

miR, microRNA; lncRNA, long non-coding RNA; ET, endocrine therapies; AntiE, anti estrogen; AIs, aromatase inhibitors; ANA, anastrozole; FULV, fulvestrant; DSCAM-AS1, DSCAM antisense RNA 1; BRCAAR4, breast cancer anti-estrogen resistance 4; MTDH, metadherin; CDK, cyclin-dependent kinase; ARPP19, cAMP-regulated phosphoprotein 19; ESRRG, estrogen related receptor gamma; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ; CCND1, cyclin D1; ALCAM, activated leukocyte cell adhesion molecule; ZEB, zinc finger E-box-binding homeobox; BRCAL-2, B-cell lymphoma 2; BMP7, bone morphogenetic protein 7; GEMIN4, gem (nuclear organelle)-associated protein 4; EZH2, enhancer of zeste homolog 2; EGFR, epidermal growth factor receptor; HDAC4, histone deacetylase 4; HnRNPL, heterogeneous nuclear ribonucleoprotein L; CDKN, cyclin-dependent kinase inhibitor; PTEN, phosphatase and tensin homolog; RB1, retinoblastoma 1; ESR1, estrogen receptor 1; CTNNB1, catenin β 1; FOXF2, forkhead box F2; BBRCA3iso-2, BRCAL2 binding component 3 isoform 2; COL2A1, collagen type II alpha 1; SOCS, suppressor of cytokine signaling; EFNA3, ephrin A3; E2F3, E2F transcription factor 3; RAD52, RAD52 homolog DNA repair protein; FGFRL1, fibroblast growth factor receptor-like 1; MET, hepatocyte growth factor receptor; CYP19A1, cytochrome P450 family 19 subfamily A member 1; ERBB2, erb-b2 receptor tyrosine kinase 2; TGFBR1, transforming growth factor β-receptor 1; BRCAL2L11, BRCAL2 like 11; ZNF217, zinc finger protein 217.
Bibliographical references: [41,42,45,46,48–57,58(p7),59–69].

Chromatin remodeling, post-translational histone modifications and histone variants. Histone post-translation modifications induce chromatin landscape changes that subsequently favor ER repression, thus promoting other signaling pathways that could lead to endocrine resistance, as exemplified by Magnani et al that revealed how the genome's accessibility is altered in drug-resistant vs. drug-responsive BC cells[70]. Recently, expression of the H3K36 methyltransferase NSD2 was found to be higher in tamoxifen-resistant BC cell lines, associated with disease recurrence and worse survival[71]. Furthermore, H3K37me3 profiles enabled the identification of patients with poor outcome after aromatase inhibitor (AI) treatment[72]. Furthermore, it was recently demonstrated that transcription repression performed by ER co-repressors confer tamoxifen sensitivity through recruitment of HDACs to DNA[73]. This evidence suggests that loss of ER co-repressors may sensitize BC cells to the cytotoxic effects of HDACs inhibitors (HDACi). Notably, some clinical trials have demonstrated that HDACi appears to re-establish sensitivity to anti-estrogens in a subset of endocrine treated-resistant tumors[74,75]. In addition, the ENCORE-301, a randomized phase II trial[75] tested entinostat, an oral histone deacetylase inhibitor, in the endocrine-resistance, more specifically AI in post-menopausal women. The results revealed modest improvement in PFS but much greater improvement in overall survival (OS)-median OS improved to 28.1 months in the experimental arm vs. 19.8 months (HR, 0.59; 95% CI, 0.36 to 0.97; P=0.036). Ongoing clinical trials are further testing entinostat in monotherapy or in combination. Moreover, in custom-generated tamoxifen resistant cell lines, treatment with HDACi re-established sensitivity to tamoxifen through significant Bcl-2 downregulation, growth arrest and apoptosis[76]. Histone variants, such as H2A.Z, an H2A variant, have been shown to be intimately linked to estrogen signaling[77]. Notably, a study has already provided a link (yet uncharacterized) between H2A.Z and endocrine resistance by revealing that H2A.Z overexpression led to increased estrogen-independent proliferation[78]. Furthermore, another study demonstrated that the histone HIST1H2BE, an H2B variant, was overexpressed not only in endocrine-resistant cell lines, but also in AI-treated tumors from patients which relapsed compared to those that did not[79]. Furthermore, an emerging class of transcription factors named ‘pioneer factors’, appear to be key players in shaping chromatin structure through binding to chromatin prior to transcription factors, making it accessible for transcription factors, together with histone post-translation modifications and histone variants[50–52]. PBX1 is an example of this class-its expression levels have been associated with reduced metastasis-free survival in ER-positive BC patients[80]. Furthermore, a gene expression signature based on NOTCH-PBX1 activity was found to discriminate BC patients that are responsive

to ET from those which are not. Notably, PBX1 knockdown was sufficient to arrest ER-resistant BC cell growth[70]. These and other chromatin remodeling complexes associated with endocrine resistance are summarized in Table 3 along with their putative role and the biological samples in which they were characterized.

Table 3. Chromatin remodeling, post-translational histone modifications and histone variants involved in response (sensitivity/resistance) to endocrine therapies along with their putative epigenetic mechanism and role in response.

Biomarker	Epigenetic mechanism	Role	Samples
H3K27me3 profiles	Post-translational histone modification	H3K27me3 profile predicts the treatment outcomes for first-line AIs	Tumor tissues
PBX1	Chromatin remodeling	Resistance to ET	Cell lines and tumor tissues
HDAC6	Post-translational histone modification	Sensitivity to TMX by deacetylation of alpha-tubulin	
HIST1H2BE	Histone variant	Overexpressed in AI-resistant tumors/cell lines compared to AI-sensitive tumors/cell lines	
NSD2	Post-translational histone modification	Histone H3K36 methyltransferase that confers resistance to TAM by upregulating key glucose metabolic enzyme genes	
H3K27me3 demethylation	Post-translational histone modification	Resistance to ET due to BrCal-2 expression	Cell lines
H2A.Z	Histone variant	Increased H2A.Z expression promotes cellular proliferation, namely when E2 levels are low and during TMX treatment	

PBX1, PBX homeobox 1; HDAC6, histone deacetylase 6; HIST1H2BE, histone cluster 1 H2B family member E; NSD2, nuclear receptor binding SET domain protein 2; H2A.Z, H2A histone family member Z; ET, endrocrine therapies; TMX, tamoxifen; AIs, aromatase inhibitors.
Bibliographical references: [71,72,78–83].

In conclusion, for such a significant prevalence of endocrine treatment resistance in BC, predictive and diagnostic biomarkers in this setting are surprisingly lacking in clinical practice, thus identified as an unmet need, imposing serious limitations on clinical practice.

There's convincing emerging data that epigenetic mechanisms may prove useful for this purpose, and in a methodological point of view, they could be used as non-invasive predictive biomarkers of treatment-resistance, providing affordable and sequential monitoring during the course of treatment.

The concept of early detection (preclinical) of therapy-resistance is compelling, as it could assist clinicians in choosing the most appropriate individualized follow-up and therapeutic strategy. Furthermore, some epigenetic modifications in addition to conveying information concerning

prediction of response, are also appealingly targetable, in particular due to their reversible nature. The clinical usefulness of these findings, however, is still elusive, mostly due to lack of standardization in methodology, limiting reproducibility. Promising results have been arising in clinically meaningful trials, such as ENCORE-301. A useful approach would be the integration of the candidate biomarkers into a panel, enabling its validation in a clinical trial setting, therefore the inception of the present work.

Objectives

To assess the prognostic/predictive value of epigenetic mechanisms in HoR positive/luminal BC by undertaking the following accomplishments:

1. A DNA methylation-based test for breast cancer detection in circulating cell-free DNA *(published data)*
2. Predictive and prognostic value of selected microRNAs in luminal breast cancer *(data under review)*
3. Epigenetic biomarker H3K27me3 software aided expression assessment in luminal A/B-like HER2-negative invasive breast cancer for survival and recurrence risks *(data under review)*

Thus, as it can be noted, three independent epigenetic mechanisms have been explored in order to expand the field in several fronts: DNA methylation, microRNAs and histone markers. Their respective rationale, methodology, results, discussion and conclusion will follow.

1. A DNA methylation-based test for breast cancer detection in circulating cell-free DNA

Introduction

Implementation of mammography-based BC screening increased the proportion of cancers detected at an early-stage, contributing to a decrease in BC-related mortality[84]. Nevertheless, this screening strategy, is hampered by frequent false positive results leading to overdiagnosis and subsequent overtreatment. Also, its usefulness in women in with dense breast tissue remains controversial[85,86]. Although grade, stage, histological and molecular subtype are currently used to risk-stratify BC patients, divergent outcomes and therapeutic responses are common[87]. Furthermore, currently used prognostic and predictive biomarkers, such as hormone receptor or *Erb-b2 receptor tyrosine kinase 2* (ERBB2) status have a limited power to predict recurrence and therapeutic response[88]. Hence, despite all improvements in early detection, patients' stratification and treatment, BC remains the foremost cause of cancer-related mortality among women, mostly due to disease recurrence and/or metastasis development[89]. In recent years, several biomarkers for early diagnosis have been proposed. However, despite their less invasive nature[90–92], improved tumor characterization[8,93–95] or better patient stratification[33,92] have been proposed, but with limited success.

Because aberrant DNA methylation is considered a cancer-associated event, characterization of tumor-specific methylome has become the focus of multiple studies[96]. Interestingly, aberrant promoter methylation of several tumor suppressor genes was found in BC precursor lesions, indicating that DNA methylation might be an early event in breast carcinogenesis[97–100]. Moreover, DNA methylation has been proposed as a valuable cancer detection and prognosis biomarker owing to its link with tissue-specific gene silencing[24,25,92,101,102]. Tumor-specific DNA methylation may also be detected in circulating cell-free DNA (ccfDNA) from liquid biopsies[103] and its potential for early cancer detection was already reported[102,104–106], representing a minimal-invasive test[107]. Herein, we aimed to define a DNA methylation-based test to improve or complement early detection strategies and to enable better BC patients' prognostic stratification. Thus, methylation levels of seven gene promoters [*Adenomatosis polyposis coli (APC), BRCA1, DNA repair associated (BRCA1)*, *Cyclin D2 (CCND2)*, *Fork-head box A1 (FOXA1)*, *Phosphoserine Aminotransferase 1* (*PSAT1)*, *Ras association domain family 1 isoform A (RASSF1A)* and *Secretoglobin family 3A member 1 (SCGB3A1*)] previously reported as dysregulated in BC and conveying diagnostic and/or prognostic

information[33,90–92,108] were firstly assessed in tissue for confirmation of cancer-specificity and prognostic significance. Then, the best performing gene panel was tested in plasma ccfDNA to determine its BC detection performance.

Experimental Section

Patients and Samples Collection

Two independent cohorts of BC patients were included in this study. Cohort #1 was comprised of 137 patients, primarily submitted to surgery, from 1996 to 2001, at the Portuguese Oncology Institute of Porto (IPO Porto), with frozen tissue available. For control purposes, normal breast tissue (NBr) was collected from reduction mammoplasty of contralateral breast of BC in patients without BC hereditary syndrome. After surgical resection and examination, samples were immediately frozen at -80°C. Relevant clinical and pathological data was retrieved from the patients' clinical charts. Five μm frozen sections were cut and stained by hematoxilin-eosin for histological evaluation by an experienced pathologist.

Cohort #2 was composed of 44 BC patients, primarily diagnosed between 2015 and 2017 at IPO Porto, which voluntarily provided blood samples prior any treatment. For control purposes, blood samples were also obtained from 39 asymptomatic controls (AC). The blood samples were collected in two EDTA tubes and centrifuged at 2,000 rpm for 10 minutes at 4°C for plasma separation. Plasma was immediately frozen at -80°C until further use. Relevant clinical data was collected from clinical records.

This study was approved by the institutional review board (Comissão de Ética para a Saúde – CES 120/2015) of IPO Porto, Portugal. All patients and controls enrolled had signed an informed consent.

Immunohistochemistry

Immunohistochemistry (IHC) allowed for identification of BC molecular subtype of each case in cohort #1, using corresponding formalin-fixed paraffin-embedded tissue. Commercially available antibodies for Estrogen Receptor (ER) (Clone 6F11, mouse, Leica, Newcastle, UK), Progesterone Receptor (PR) (Clone 16, mouse, Leica, Newcastle, UK), ERBB2 (Clone 4B5, rabbit, Roche,

Tucson, AZ, USA) and Ki67 (Clone MIB-1, mouse, Dako, Glostrup, Denmark) were used. IHC was carried out in BenchMark ULTRA (Ventana, Roche) using ultraView Universal DAB Detection Kit (Ventana, Roche, Tucson, AZ, USA) according to manufacturer's instructions.

IHC staining was evaluated by an experienced pathologist according to College of American Pathologists' recommendations. Each case was categorized according to ESMO guidelines [6]. Cutoff values were set for Ki67 (high proliferative rate if ≥ 15% positive cells) and PR (high expression if ≥ 25% positive cells).

DNA Extraction

Genomic DNA was extracted from tumor and normal tissues by the phenol–chlorophorm method at pH 8, as previously described[109]. Samples were first submitted to overnight digestion in a bath at 55°C, using buffer solution SE (75 mM NaCl; 25 mM EDTA), SDS 10% and proteinase K, 20 mg/ml (Sigma-Aldrich®, Schnelldorf, Germany). After digestion, extraction was performed with phenol/chloroform (Sigma-Aldrich®, Schnelldorf, Germany, Merck, Darmstadt, Germany) followed by precipitation with 100% ethanol.

CcfDNA was extracted from 2 mL of plasma using QIAamp MinElute ccfDNA (Qiagen, Germany), according to manufacturers' recommendations. The ccfDNA was eluted in 20 μL of ultra-clean water (Qiagen, Germany).

Bisulfite Treatment and Whole genome amplification (WGA)

Bisulfite conversion was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA), according to manufacturer's instructions. One μg of DNA obtained from fresh frozen sections was used. Modified DNA was eluted with 60 μL of sterile distilled water. In plasma samples, 20 μL of ccfDNA was used for bisulfite modification. Modified ccfDNA was eluted in 10 μL of sterile distilled water. For control purposes, 1 μg of CpGenome™ Universal Methylated DNA (Millipore, Temecula, CA, USA) was also modified, according to the method described above and eluted in 20μL of M-elution buffer. All samples were stored at -80°C until further use. Whole-genome amplification of sodium bisulfite modified ccfDNA was carried out using the EpiTect Whole Bisulfitome Kit (Qiagen, Germany) according to manufacturer's recommendations. The amplified ccfDNA final volume was 65 μL.

Quantitative Methylation-Specific PCR (QMSP)

Modified DNA was used as template for QMSP. Overall, seven gene promoters (*APC, BRCA1, CCND2, FOXA1*, *PSAT1, RASSF1A* and *SCGB3A1*) were assessed in BC tissue samples. Primers used specifically amplify methylated bisulfite converted complementary sequences. *β-actin* (*ACTβ*) was used as reference gene to normalize for DNA input in each sample[92]. Reactions were performed in 96-well plates using Applied Biosystems 7500 RealTime System (Thermo Fisher Scientific, USA) using 2 μL of modified DNA and 5 μL of 2X KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, MA, USA). All the samples were run in triplicates and the melting curves were obtained for each case/ gene. Owing to the limited amount of ccfDNA plasma samples, three gene promoters were selected (*APC, FOXA1, RASSF1A*) in addition to the reference gene (*ACTβ*) for assessment of methylation using multiplex QMSP with TaqMan probes having different fluorescent reporters and Xpert Fast Probe (GRISP, Porto, Portugal), whereas *SCGB3A1* methylation levels were assessed in a separated QMSP reaction.

Modified CpGenome™ Universal Methylated DNA was used in each plate to generate a standard curve, allowing for quantification as well as to ascertain PCR efficiency. All plates disclosed efficiency values between 90-100%. For each gene, relative methylation levels in each sample were determined by the ratio of the mean quantity obtained by QMSP analysis for each gene and the respective value of the internal reference gene (*ACTβ*), multiplied by 1000 for easier tabulation (methylation level = target gene/reference gene × 1000).

Statistical Analysis

The frequency, median and interquartile range of promoter methylation levels of normal tissue/control samples and plasma samples were determined. Non-parametric tests were performed to determine statistical significance in all the comparisons made. Specifically, Kruskall-Wallis test was used for comparisons between three or more groups, whereas Mann-Whitney U test was used for comparisons between two groups.

For each gene, ROC curves were built to assess respective performance as tumor biomarker. Moreover, specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were determined. For this purpose, the cut-off established was the highest value

obtained by the ROC curve analysis [sensitivity + (1-specificity)]. To categorize samples as methylated or unmethylated, a cut-off value was chosen based on Youden's J index obtained by the ROC curve analysis for each gene[110,111]. For combination of markers, the cases were considered positive if at least one of the individual markers was positive. Logistic regression models were built in order to evaluate the potential of confounding factors as age in our BC detection model.

Spearman nonparametric correlation test was used to assess the association of methylation levels and age. Disease-specific survival curves and disease-free survival curves (Kaplan–Meier with log rank test) were computed for standard clinicopathological variables and for categorized methylation status. A Cox-regression model comprising all significant variables (multivariable model) was computed to assess the relative contribution of each variable to the follow-up status.

Two-tailed P-values were derived from statistical tests, using a computer assisted program (SPSS Version 20.0, Chicago, IL), and results were considered statistically significant at $p < 0.05$, with Bonferroni´s correction for multiple tests, when applicable. Graphics were assembled using GraphPad 6 Prism (GraphPad Software, USA).

Results

Clinical and Pathological Data of Tissue Cohort

Relevant clinical and pathological data are presented in Tables 1.1 and 1.2. Although patients' age did not differ between the two cohorts, a significant difference was observed between BC patients and controls age (Cohort #1: p =0.007, Cohort #2: p =0.001).

Table 1.1 – Clinical–pathological data of normal breast tissue, and (NBr) breast cancer (BC) patient's (Cohort#1).

Clinicopathologic features	BrC	NBr
Patients (no.)	137	28
Age median (range)	62 (33-88)	54 (30-70)
Molecular subtype (no).		
Luminal A-like	29	n.a.[a]
Luminal B-like	74	
ERBB2 overexpression-like	12	
TNBC[b]	22	
Histological Type		
Invasive Ductal Carcinoma	116	n.a.
Invasive Lobular Carcinoma	8	
Special Subtype Carcinomas	5	
Mixed Type Carcinoma	8	
Grade (no.)		
G1	13	n.a.
G2	63	
G3	56	
n.a.	5	
Estrogen Receptor Status		
Positive	102	n.a.
Negative	35	
Progesterone Receptor Status		
Positive	66	n.a.
Negative	71	
ERBB2 Receptor Status		
Positive	25	n.a.
Negative	112	
Pathological T Stage (no.)		
pT1	42	n.a.
pT2	84	
pT3	7	
pT4	2	
pTx	2	
Pathological N Stage (no.)		
pN0	52	n.a.
pN1	45	
pN2	17	
pN3	19	
pNx	4	
Stage (no.)		
I	21	n.a.
II	72	
III & IV	43	

n.a. – not applicable; TNBC – Triple Negative Breast Cancer; ERBB2, Erb-b2 receptor tyrosine kinase.

Table 1.2 – Clinical and pathological data of asymptomatic controls (AC) and BC patients (Cohort#2).

Clinicopathologic features	BrC	AC
Patients (n)	44	39
Age median (range)	63 (37-91)	52 (46-65)
Molecular subtype (no).		
Luminal	41	n.a.[a]
TNBC[b]	2	
Histological Type		
Invasive Ductal Carcinoma	33	n.a.
Others	11	
Grade (no.)		
G1	6	n.a.
G2	18	
G3	19	
Not determined	1	
Estrogen Receptor Status		
Positive	40	n.a.
Negative	3	
Not determined	1	
Progesterone Receptor Status		
Positive	37	n.a.
Negative	6	
Not determined	1	
ERBB2 Receptor Status		
Positive	3	n.a.
Negative	40	
Not determined	1	
Pathological Stage (no.)		
I	16	n.a.
II	19	
III	9	
Not determined	1	

[a] n.a. – not applicable; [b] TNBC – Triple Negative Breast Cancer; c Breast cancer diagnosis by cytology, patient refused treatment.

Assessment of BC and NBr Tissue Samples Methylation Levels

To assess cancer-specificity, promoters' methylation levels of *APC, BRCA1, CCND2, FOXA1*, *PSAT1, RASSF1A* and *SCGB3A1* were evaluated in Cohort #1 (BC and NBr tissue samples). Overall, BC samples displayed higher *APC*, *CCND2, FOXA1, PSAT1, RASSF1A,* and *SCGB3A1* methylation levels than NrB samples (p<0.001 for all genes, Table 1.3), whereas no differences were found for *BRCA1*, which was not further tested.

Table 1.3 – Frequency of positive cases [n(%)] and distribution of methylation levels of cancerrelated genes in tissues from Cohort#1 [gene/ACTB x1000 median (IQR)].

Genes	NBr		BrC		p value
	n (%)	Median (IQR[a])	n (%)	Median (IQR)	
APC	0/28 (0%)	1.172 (0.4732-3.875)	70/137 (51%)	24.10 (1.429-233.8)	<0.0001
BRCA1	21/28 (75.0%)	0.0830 (0.0043-0.2785)	135/137 (98.5%)	0.1376 (0.0519-0.4138)	0.111
CCND2	2/28 (7.1%)	0.0285 (0.0001-0.2944)	99/137 (72.3%)	5.552 (0.3338-31.29)	<0.0001
FOXA1	5/28 (17.9%)	12.25 (7.725-19.00)	85/137 (62.0%)	28.33 (13.51-61.73)	<0.0001
PSAT1	14/28 (50.0%)	66.83 (25.33-248.7)	125/137 (91.2%)	222.15 (103.7-351.5)	<0.0001
RASSF1A	1/28 (3.6%)	13.90 (2.277-31.47)	108/137 (78.8%)	329.84 (141.4-560.7)	<0.0001
SCGB3A1	0/28 (0%)	1.218 (0.2794-5.715)	89/137 (65.0%)	217.27 (3.225-577.8)	<0.0001

a IQR – Interquartile range; p value obtained from Mann-Whitney test.

Subsequently, ROC curve analysis was performed, and an empirical cutoff value was determined for each gene (*APC:* 16.99, *CCND2:* 0.4171 for, *FOXA1:* 21.57, *PSAT1:* 48.05, *RASSF1A:* 114.5 and *SCGB3A1:* 67.18). All genes displayed an Area Under the Curve (AUC) superior to 0.70. *APC* and *SCGB3A1* disclosed 100% specificity for cancer detection, whereas *PSAT1* showed the highest sensitivity (91.97%). *RASSF1A* demonstrated the best individual performance, with 78.83% sensitivity and 96.43% specificity (Table 1.4). Several gene combinations were tested, and the best detection performance was achieved for the panel comprising *APC, FOXA1, RASSF1A* and *SCGB3A1,* displaying 97.81% sensitivity, 78.57% specificity and 94.50% accuracy (Table 1.4, Figure 1.1). Due to age's difference between patients and controls, a multivariable model was constructed using logistic regression with the most informative

genes and age. In this model, age did not show a significant impact in BC detection (p=0.2227). Moreover, biomarker performance was carried out restricted to BC patient's with similar age to controls (p=0.136, Mann-Whitney for age). Similar results were obtained in biomarker performance.

Table 1.4– Performance of promoter gene methylation as biomarkers for detection of Breast Cancer in tissue samples

Genes	Sensitivity %	Specificity %	PPV[a] %	NPV[b] %	Accuracy %
APC	51.09	100.0	100.0	29.47	59.39
CCND2	72.26	92.86	98.02	40.63	75.76
FOXA1	62.04	82.14	94.44	30.67	65.45
PSAT1	91.24	50.00	89.93	53.85	84.24
RASSF1A	78.83	96.43	99.08	48.21	81.82
SCGB3A1	64.96	100.0	100.0	36.84	70.91
APC/FOXA1 RASSF1A/ SCGB3A1	97.81	78.57	95.71	88.00	94.55

[a]PPV – Positive Predictive Value; [b]NPV – Negative Predictive Value.

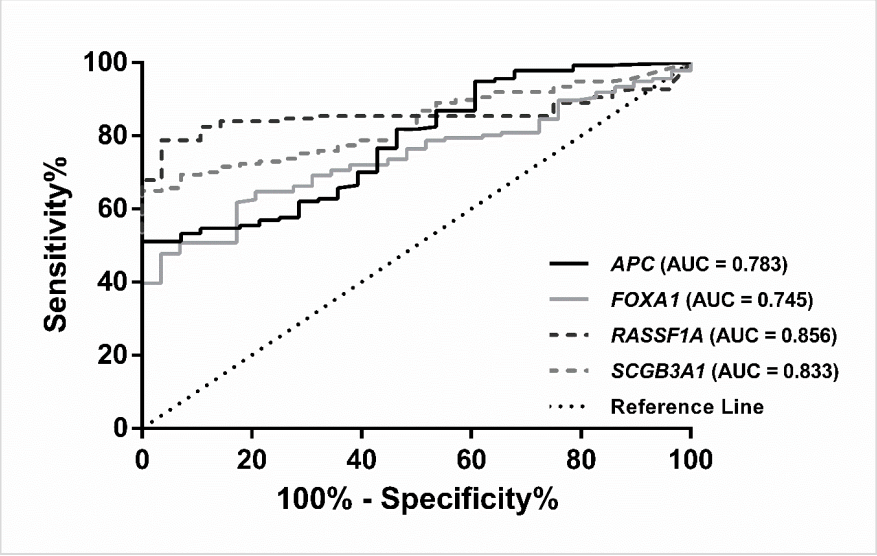


Figure 1.1 – Receiver Operating Characteristic Curve of the four-gene panel (*APC*, *FOXA1*, *RASSF1A* and *SCGB3A1*) in breast cancer tissues from Cohort#1).

Association between Promoter Methylation Levels, Molecular Subtypes and Standard Clinicopathological Parameters in cohort #1

No significant differences in promoter methylation levels were apparent according to molecular subtype, tumor grade, pathological stage or ERBB2 status in tissue samples. Nevertheless, in BC patients, but not in controls, a significant correlation was found between *CCND2* and *RASSF1A* methylation levels and age (R=0.194, p=0.023 and R=0.223, p=0.009, respectively). Additionally, a significant association was found between histological subtype and *APC* and *SCGB3A1* methylation levels: special subtype carcinomas disclosed the lowest *SCGB3A1* methylation levels in comparison to all the other histological subtypes (p=0.016) and lower *APC* methylation levels comparing with invasive lobular carcinomas (p=0.0293).

Additionally, *FOXA1* and *RASSF1A* methylation levels associated with hormone receptor status. ER+ and PR+ BC displayed significantly lower *FOXA1* methylation levels than ER- and PR- BC (p= 0.0084) or ER+ BC (p= 0.0319). Contrarily, ER+ and PR+ BC showed higher *RASSF1A* methylation levels than ER+ BC (p= 0.0017). No statistical differences were observed for the remainder genes and for cohort #2.

Survival Analysis

Survival analysis was only carried out for Cohort #1 due to the short-time of follow-up of Cohort#2. In the former Cohort (#1), 10 years of follow-up was considered for analysis. During this period, 37 patients (27.0%) had deceased, 24 of which due to BC (17.5% of all cases). At the time of the last follow-up, 8 patients (5.8%) were alive with cancer and 92 patients (67.2%) were alive with no evidence of cancer.

Clinicopathological features were grouped according to: Grade (G1&G2 vs. G3), pT stage (pT1, pT2 and pT3&pT4), pN Stage (N0&N1 vs. N2&N3) and stage (I, II and III&IV).

Higher tumor grade and pN stage and low *PSAT1* methylation levels categorized by percentile 75 significantly associated with worse disease-free survival (DFS) in Cox regression univariable analysis (Table 1.5). Nonetheless, in multivariable analysis, however, only *PSAT1* methylation levels and pN stage remained independent predictors of DFS (Table 1.5).

Table 1.5 – Cox regression models assessing the potential of clinical and epigenetic variables in the prediction of disease-free survival for 122 patients with Breast Cancer and disease-specific survival for 127 patients with Breast Cancer.

Disease-Free Survival	Variable	HR[a]	CI[b] (95%)	*p* value
Univariable	Grade			
	G1	1		
	G2 & G3	2.054	1.029 – 4.098	0.041
	pN Stage			
	N0 & N1	1		
	N2 &N3	3.894	1.940 – 7.812	<0.001
	PSAT1			
	>P75[c]	1		
	≤ P75	3.707	1.133 – 12.127	0.030
Multivariable	Grade			
	G1	1		
	G2 & G3	1.490	0.717 – 3.096	0.286
	pN Stage			
	N0 & N1	1		
	N2 &N3	4.345	2.114 – 8.930	<0.001
	PSAT1			
	>P75[c]	1		
	≤ P75	3.613	1.077 – 12.123	0.038
Disease-Specific Survival	Variable	HR[a]	CI[b] (95%)	*p* value
Univariable	Grade			
	G1	1		
	G2 & G3	2.725	1.155 – 6.428	0.022
	pN Stage			
	N0 & N1	1		
	N2 &N3	4.061	1.814 – 9.089	0.001
	FOXA1			
	≤P75	1		
	>P75	2.678	1.200 – 5.978	0.016
Multivariable	Grade			
	G1	1		
	G2 & G3	2.005	0.082 – 4.866	0.124
	pN Stage			
	N0 & N1	1		
	N2 &N3	4.855	1.981 – 10.611	<0.001
	FOXA1			
	≤P75[d]	1		
	>P75	2.710	1.161 – 6.324	0.021

[a]HR – Hazard Ratio; [b]CI – Confidence Interval; [c]P75 – Percentile 75 of methylation levels of *PSAT1;* [d]P75 – Percentile 75 of methylation levels of *FOXA1.*

Concerning disease-specific survival (DSS), in univariable model, pN stage and grade significantly associated with prognosis. Moreover, BC patients with high *FOXA1* promoter levels of methylation (P>75) had shorter DSS (Table 1.5). In the Cox regression multivariable model, only *FOXA1* methylation levels and pN stage retained significance for DSS prediction (Table 1.5).

Biomarker Detection Performance in ccfDNA liquid biopsies (Cohort#2)

The 4-gene panel (*APC, FOXA1, RASSF1A,* and *SCGB3A1*) identified in Cohort#1 was tested in ccfDNA extracted from plasma samples of Cohort#2. *APC*, *FOXA*1 and *RASSF1A* promoter methylation levels significantly differed between BC patients and AC (p=0.008, p<0.001 and p=0.017, respectively), whereas no significant differences were found for *SCGB3A1* (p=0.127). Thus, *SCGB3A1* was not further analyzed. An empirical cutoff value was determined for each gene using ROC curve analysis (*APC:* 3.446, *FOXA1:* 64.38 and *RASSF1A:* 30.00). *FOXA1* disclosed the best individual performance, with 68.18% sensitivity and 82.05% specificity (Table 1.6). Nevertheless, the three-gene panel achieved 81.82% sensitivity and 76.92% specificity (Table 1.6, Figure 1.2). Similar to Cohort#1, a biomarker performance analysis restricted by the maximum age of the controls was performed (p=0.777, Mann-Whitney for age). The biomarker performance was similar.

Table 1.6 – Performance of promoter gene methylation as biomarkers for detection of Breast Cancer in plasmas samples

Genes	Sensitivity %	Specificity %	PPV[a] %	NPV[b] %	Accuracy %
APC	27.27	94.87	85.71	53.62	59.04
FOXA1	68.18	82.05	81.08	69.57	74.70
RASSF1A	13.64	100.0	100.0	50.65	54.22
APC/FOXA1/RASSF1A	81.82	76.92	80.00	78.95	79.52

[a]PPV – Positive Predictive Value; [b]NPV – Negative Predictive Value

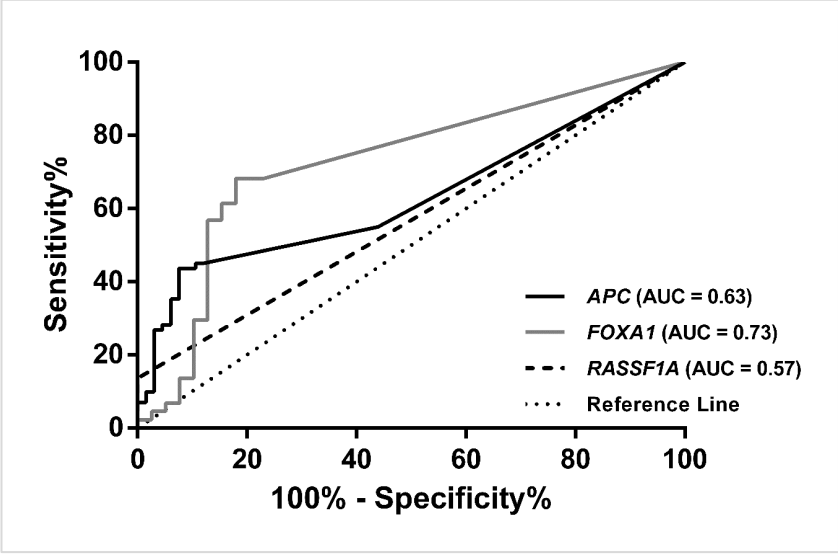


Figure 1.2 – Receiver Operating Characteristic Curve of the three-gene panel (*APC, FOXA1* and *RASSF1A*) in plasma samples from breast cancer patients from Cohort#2.

Discussion

Mammographic-based screening has contributed to a 28-45% reduction in BC mortality[86,112], disclosing 70% sensitivity and 92% specificity for BC detection[85]. Owing to its limitations, the need for novel detection methods, with improved accuracy and allowing for stratification of BC aggressiveness has been emphasized[112]. In recent years, the methylome has emerged as the basis for diagnostic and prognostic biomarkers, which might be used in DNA extracted from liquid biopsies[113,114]. Considering published studies on gene promoter methylation in BC, we aimed to define the best gene panel for detection and prognosis in tissue samples, as well as BC detection in ccfDNA.

From the seven most promising candidates, six (*APC, CCND2, FOXA1, PSAT1, RASSF1A* and *SCGB3A1*) confirmed its cancer-specificity, discriminating normal from cancerous tissues, although with variable performance, paralleling previous observations from our team and others[91,92,115]. Interestingly, a panel combining *APC*, *FOXA1*, *RASSF1A* and *SCGB3A1* disclosed the highest accuracy for BC detection (94%). *APC, RASSF1A* and *SCGB3A1* promoter methylation have been previously tested in a diagnostic setting of fine-needle aspiration biopsy samples[91,92], whereas *FOXA1* expression has been associated with BC subtype and prognosis[116,117], but not diagnosis. This result compares well with other gene promoter methylation panels that have been reported, disclosing 60-80% sensitivity and 78-100% specificity, and differences in performance are most likely related to biological sample type (tissue vs. bodily fluids) and methylation assessment methods[118].

Since a major goal of this study was to define a panel for BC detection, ideally its performance should be homogenous regardless of molecular subtype. Thus, we used IHC for tumor subtyping, although acknowledging its limitations in TNBC /basal-type classification and luminal A vs. luminal B discrimination[119–121]. Interestingly, no association was found between gene promoter methylation and BC molecular subtype, suggesting that the gene panel might be effective across molecular subtypes. Some studies have associated DNA methylation and specific molecular subtypes, but these have used a similar proportion of all subtypes or have only analyzed a specific subtype[95,122,123], or even used different methods[8,93,124,125]. Our results, however, are based on a consecutive series of cases, which were not selected according to subtype, and, thus, ERBB2-like and TNBC tumor subtypes are, naturally, in a smaller proportion, which might limit statistical analysis. Nevertheless, *APC* and *SCGB3A1* promoter methylation levels associated with specific histological subtypes, confirming previous observations[126]. Interestingly, *FOXA1* and *RASSF1A* promoter methylation levels

associated with hormone receptor status. Although the reason for these associations is unclear, similar results for *RASSF1A* have been reported[27,127]. On the other hand, the higher *FOXA1* promoter methylation observed in hormone receptor negative BC is in accordance with reported *FOXA1* hypermethylation in basal BC cell lines[108,128].

Because liquid biopsies represent a promising method for minimally-invasive early cancer detection[103,107], we tested the selected gene panel in ccfDNA. Interestingly, three genes retained diagnostic significance (*APC*, *FOXA1* and *RASSF1A*), whereas *SCGB3A1* did not discriminate BC patients from controls. These results are in accordance with another study[129] and might be due to differences in sample number and methylation assessment methods[130]. Moreover, the frequency of gene methylation in cohort#2 was similar to that previous reported in ccfDNA (Table 1.7)[104,130–138].

Table 1.7 – Frequency of positive cases [n(%)] for methylation levels of cancer-related genes in ccfDNA ([a] No information about single gene methylation; [b] The cut-off used in the panel was different the one used in the single gene analysis).

Genes/Panel	Controls (healthy donors)		Patients		References
	n	%	n	%	
HIC-1/RARβ2/RASSF1A[a]	0/10	0%	18/20	90%	[135]
APC	0/38	0%	8/47	17%	[131]
GSTP1	0/38	0%	12/47	26%	
RARβ2	3/38	8%	12/47	26%	
RASSF1A	2/38	5%	15/47	32%	
APC/GSTP1/RARβ2/RASSF1A	5/38	13%	29/47	62%	
ATM	0/14	0%	13/50	26%	[133]
RASSF1A	0/14	0%	7/50	14%	
ATM/RASSF1A	0/14	0%	18/50	36%	
RARβ2	8/125	6%	103/119	87%	[130]
RASSF1A	6/125	5%	39/119	33%	
SCGB3A1	0/125	0%	36/119	30%	
Twist	10/125	8%	65/119	55%	
RARβ2/RASSF1A/ SCGB3A1/Twist	23/125	18%	117/119	98%	
ITIH5	7/135	6%	19/138	14%	[104]
DKK3	2/135	2%	41/138	30%	
RASSF1A	25/135	26%	64/138	47%	
ITIH5/DKK3/RASSF1A	42/135	31%	92/138	67%	
SFN	143/245	58%	197/268	74%	[134]
P16	41/245	17%	60/268	33%	
hMLH1	35/245	14%	75/268	28%	
HOXD13	6/245	2%	37/268	14%	
PCDHGB7	116/245	48%	149/268	56%	
RASSF1A	25/245	10%	46/248	17%	
SFN/P16/ hMLH1/HOXD13/ PCDHGB7/RASSF1A[a]	68/245	28%	213/268	80%	
ESR1	35/74	47%	80/106	75%	[132]
14-3-3-σ	28/74	38%	69/106	65%	
ESR1/14-3-3-σ[b]	33/74	45%	86/106	81%	
GSTP1	2/87	2%	4/101	4%	[137]
RARβ2	0/87	0%	7/101	7%	
RASSF1A	4/87	5%	12/101	12%	
GSTP1/RARβ2/RASSF1A	6/87	7%	22/101	22%	
DAPK1	0/12	0%	23/26	88%	[138]
RASSF1A	1/12	8%	18/26	69%	
DAPK1 /RASSF1A	1/12	8%	25/26	96%	
APC	1/19	5%	23/79	30%	[136]
ESR1	2/19	11%	16/79	20%	
RASSF1A	0/19	0%	28/79	35%	
APC/ESR1/RASSF1A	3/19	16%	42/79	53%	
APC	**2/39**	**5%**	**12/44**	**27%**	–
FOXA1	**7/39**	**18%**	**30/44**	**68%**	
RASSF1A	**0/39**	**0%**	**6/44**	**14%**	
APC/FOXA1/RASSF1A	**9/39**	**23%**	**36/44**	**82%**	

Nonetheless, the three gene-panel identified BC with sensitivity, specificity and accuracy higher than 75%. Thus, this panel disclosed a better combination of sensitivity and specificity than most published studies using plasma or serum samples (Table 1.8), excepting those of Skvortosova et al. (three-gene panel in plasma) and Kim et al. (four-gene panel in serum)[104,130–138]. Nevertheless, the same authors tested a very limited set of samples (BC N=20, fibroadenomas N=15 and healthy donors=10). Importantly, we used a 4-color multiplex assay that, when compared with the most widely reported two-color multiplex assays represents a faster method and requires less amounts of DNA, thus facilitating its use in clinical routine[113,114,139–141]. Hence, this gene-panel may constitute an appealing alternative to conventional diagnostic methods due to its less-invasive characteristics and to also detect also women with a dense breast.

Table 1.8 – Comparison of sensitivity and specificity of previously published panels with values obtained

Panels	Sensitivity (%)	Specificity (%)	Specimen type	Methods	References
HIC-1/RARβ2/RASSF1A	90	100	Plasma	MSP[a]	[135]
APC/GSTP1/RARβ2/RASSF1A	62	87	Plasma	QMSP[b]	[131]
ATM/RASSF1A	36	100	Plasma	QMSP[b]	[133]
RARβ2/RASSF1A/ SCGB3A1/Twist	98.3	81.6	Serum	Two-steps QMSP[b]	[130]
ITIH5/DKK3/RASSF1A	67	72	Serum	QMSP[b]	[104]
SFN/P16/ hMLH1/HOXD13/ PCDHGB7/RASSF1A	79.6	72.4	Serum	QMSP[b]	[134]
ESR1/14-3-3-σ	81	55	Serum	QMSP[b]	[132]
GSTP1/RARβ2/RASSF1A	22	93	Serum	One-step MSP[a]	[137]
DAPK1 /RASSF1A	96	71	Serum	MSP[a]	[138]
APC/ESR1/RASSF1A	53	84	Serum	QMSP[b]	[136]
APC/FOXA1/RASSF1A	81.82	76.92	Plasma	Multiplex QMSP[b]	—

[a] MSP - Methylation-Specific PCR; [b] QMSP - Quantitative Methylation-Specific PCR

Although BC displays high mortality and recurrence rate, clinical course is heterogeneous and perfecting disease prognostication might improve patient management. Interestingly, lower *PSAT1* promoter methylation independently predicted for worse DFS. The potential of *PSAT1* methylation to predict BC recurrence has been previously reported in early diagnosed luminal-type BC.

Furthermore, a correlation between high *PSAT1* methylation levels, on the one hand, and low *PSAT1* mRNAs levels and better outcome, on the other, were disclosed[33]. Interestingly, high *PSAT1* expression were associated with poor outcome in nasopharyngeal carcinoma[142]. These data are in accordance with our findings. Furthermore, high *FOXA1* methylation levels independently predicted shorter DSS, a finding that, to best of our knowledge, has not been reported, thus far. Remarkably, *FOXA1* expression was previously shown to associate with good prognosis and response to endocrine therapy in BC patients[116,117], and, thus, promoter methylation is the most likely mechanism underlying *FOXA1* downregulation in BC. In Cohort#1, *RASSF1A* methylation levels did not show prognostic value, which is in accordance with some previous publications[143–145]. Nonetheless, other studies have found *RASSF1A* hypermethylation as a poor prognosis marker in BC, associating with shorter DFS and DSS[92,101,146]. This discrepancy might be due to differences in sample type and methodologies. Because a meta-analysis suggested that *RASSF1A* methylation is, indeed, associated with worse DFS and DSS[147], additional studies are needed to definitively establish the prognostic value of *RASSF1A* promoter methylation in BC.

The retrospective nature of Cohort#1, the limited sample size of Cohort#2 and the age differences between BC patients and controls in both series constitute the main limitations of our study. Nonetheless, it should be emphasized that the use of a multiplex assay for a three-gene panel that is able to accurately detect BC in ccfDNA, regardless of tumor subtype, constitutes a step forward in this field and allow for a swifter translation into routine clinical practice. Indeed, owing to its characteristics, this panel might not only be useful for BC detection, but also for disease monitoring which deserves further exploration.

2. Predictive and prognostic value of selected microRNAs in luminal breast cancer

Introduction

Adjuvant systemic therapy in BC is aimed at reducing disease recurrence by eradicating potential micrometastatic tumors present at diagnosis and at the curative intent treatment setting, can be comprised from one to three modalities: chemotherapy, anti-HER2 therapy and endocrine therapy (ET). ER and HER2 status are used as predictive factors to select patients for specific adjuvant therapies (Table 2.1).

Table 2.1. Breast Cancer molecular subtypes characterization[8,87,88,148–153]

Breast cancer subtypes		Clinicopathological surrogate markers	Signature genes	Adjuvant systemic therapeutic options
Luminal A		ER+ PR high * HER2- Ki-67 low **	*ESR1* and/or *PGR*, *KRT8*/18, *GATA3*, *XBP1*, *FOXA1* and *ADH1B*	ET alone in most of cases + Cht if high tumor burden (≥N3, ≥T3)
Luminal B	**HER2-**	ER+ HER2- Ki-67 high or PR low	*ESR1* and/or *PGR*, *KRT8*/18, *FGFR1*, *ERBB1*, *MKI67 and*/or *CCNE1*, *CCNB1* and *MYBL2*	ET + Cht for the most of cases
	HER2+	ER+ HER2+ Any Ki-67 Any PR		ChT + anti-HER2 + ET for all patients
Basal-like		ER- PR- HER2-	*KRT5*/6, *KRT17*, *ERBB1* and/or *KIT*, FOXC1, TP63, *CDH3*, VIM and *LAM*	ChT
HER2-enriched		HER2+ ER- PR-	*ERBB2* and *GRB7*	ChT + anti-HER2

* Suggested cut-off value is 20% ** Ki-67 scores should be interpreted in the light of local laboratory median values. Abbreviations: ER – Estrogen Receptor; PR – Progesterone Receptor; HER2 - Human Epidermal Growth Factor Receptor 2; *ESR1* - Estrogen Receptor 1; *PGR* - Progesterone Receptor; *KRT* – Keratin; *GATA3* - GATA Binding Protein 3; *XBP1* - X-Box Binding Protein 1; *FOX* - Forkhead Box; *ADH1B* - Alcohol Dehydrogenase 1B (Class I), Beta Polypeptide; *FGFR1* - Fibroblast Growth Factor Receptor 1; *ERBB* - Erb-B2 Receptor Tyrosine Kinase; *MKI67* - Marker Of Proliferation Ki-67; *CCN* – Cyclin; MYBL2 - MYB Proto-Oncogene Like 2; *MYBL2* - MYB Proto-Oncogene Like 2; *KIT* - KIT Proto-Oncogene Receptor Tyrosine Kinase; *TP63* - Tumor Protein P63; *CDH* – Cadherin; *VIM* – Vimentin; *LAM* – Laminin; *GRB7* - Growth Factor Receptor Bound Protein 7; Cht – Chemotherapy; ET – Endocrine Therapy; N – Nodal Stage; T – Tumor Size.

ET, which blocks ER activation, is recommended for patients with ER-positive disease, to stop or slow the growth of hormone-sensitive BC[154]. Most luminal A tumors, except those with the highest risk of relapse, do not require adjuvant chemotherapy, whereas most luminal B tumors, especially those with HER2 overexpression, benefit from chemotherapy in addition to HER2 pathway blockade[155,156]. Although ET results in substantial improvement of patients' outcome, resistance to

treatment has become a major limitation[153], affecting 30-40% of ER-positive BC patients, with all those treated in the metastatic setting eventually progressing[23,157]. According to 4th ESO–ESMO International Consensus Guidelines, and as mentioned before, endocrine resistance may be defined as primary endocrine resistance when patients relapse within the first 2 years of adjuvant ET, or as secondary (acquired) endocrine resistance, when patients relapse while on adjuvant ET after the first 2 years of treatment or within the 12 months after completing treatment[7].

MicroRNAs (miRNAs), a class of small (~22 nucleotides) non-coding single-stranded RNAs, have shown promise for assisting in clinical management of BC, as diagnostic, prognostic or predictive biomarkers[19], namely by their assessment in liquid biopsies (plasma, serum, urine)[158]. Indeed, several studies associated miRNAs deregulation with endocrine resistance and prognosis in luminal BC[53,159–161]. Whereas decreased ER expression and endocrine resistance may be due to miR-221/222 overexpression[42,51,162], miR-342-3p expression positively correlated with ER mRNA transcript levels, being downregulated in tamoxifen refractory BC[43]. Moreover, miRNAs regulating growth, survival and apoptosis of BC cells may also be implicated in loss of responsiveness to ET by endowing tumor cells with alternative proliferative and survival stimuli[163]. Indeed, miR-519a associated with worse prognosis of luminal BC patients, directly targeting the transcripts of cyclin dependent kinase inhibitor 1A (CDKN1A) and phosphatase and tensin homolog (PTEN), allowing for enhanced signaling of the phosphoinositide3-kinase (PI3K) growth and survival pathway[46] and reducing sensitivity and tumor cell apoptosis in response to apoptotic stimuli[164]. Furthermore, miRNA-mediated endocrine resistance might be related with epithelial-to-mesenchymal transition (EMT) and metastatic potential of BC cells, as members of the miR-200 family (miR-200f) were found downregulated in endocrine-resistant BC vs. endocrine-sensitive cell lines, acting as major regulators of EMT[54,165].

Herein, we aimed to identify miRNAs able to predict endocrine resistance among luminal BC patients undergoing ET, through the comparison of expression levels between BC samples of patients that develop endocrine-resistance in long term follow-up with those that did not develop endocrine-resistance. This might allow for the stratification of luminal BC cases into a low-risk subgroup, for whom additional adjuvant systemic treatment can be safely omitted, and patients who are at high-risk for recurrence potentially allowing the detection of resistance to ET at an early stage.

Material and methods

Patients and samples collection

For this study, 136 BC tissue samples were prospectively collected, after informed consent, from patients with luminal BC and without metastasis at diagnosis, aged between 40 and 75 years, submitted to adjuvant ET (with or without other adjuvant modalities), after first line surgical treatment, from 1995 to 2002 at the Portuguese Oncology Institute of Porto (IPO-Porto). Furthermore, 26 normal breast tissue samples were collected from reduction mammoplasties of contralateral breast of BC patients. All specimens were obtained from patients without BC hereditary syndrome and showed no evidence of preneoplastic/neoplastic lesions. After surgical resection, samples were immediately frozen at -80°C. Relevant clinical and pathological data was retrieved from patients' charts. Five-μm frozen sections were cut and stained with hematoxylin-eosin (H&E) staining for confirmation of BC by an experienced pathologist, ensuring that samples contained at least 70% of tumor cells, and confirm that tissues obtained from reduction mammoplasties harbored normal epithelial cells. This study was approved by institutional ethical committee (CES-IPOFG-120/015).

Breast cancer subtyping

IHC was performed to identify the molecular subtype of each tumor tissue included in this study. Commercially available antibodies were used for ER (Clone 6F11, mouse, Leica), PR (Clone 16, mouse, Leica), HER2 (Clone 4B5, rabbit, Roche) and Ki-67 (Clone MIB-1, mouse, Dako). IHC was carried out in BenchMark ULTRA (Ventana, Roche) using ultraView Universal DAB Detection Kit (Ventana, Roche) according to manufacturer's instructions. Each case was evaluated by an experienced pathologist and was classified according to the College of American Pathologists recommendations[166]. Each case was categorized according to ESMO guidelines[88]. Cutoffs for Ki-67 and PR expression were 15% and 25% of positive cells, respectively.

RNA extraction from fresh frozen tissues

Total RNA was extracted from fresh frozen tissues using the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations. RNA concentrations and purity ratios were ascertained using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA samples were stored at -80°C.

MicroRNAs cDNA synthesis

cDNA synthesis was performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using miRCURY LNA™ Universal RT microRNA PCR (Exiqon, Vedbaek, Denmark) following manufacturer's instructions. cDNA samples were then stored at -20°C.

Global focus microRNA PCR panel

Global miRNAs' expression was evaluated using a Cancer Focus microRNA PCR Panel, 384 well (V4.R) (Exiqon). Each plate, besides containing 80 lyophilized LNA™ miRNA primer sets focusing on cancer relevant human miRNAs, also contained interplate calibrators, candidate reference genes [miRNAs and small nuclear RNAs (snRNAs)] and one water blank. In each well, it was added 0.05 μL of cDNA previously synthesized, 5 μL of SYBR® Green master mix (Exiqon) and 4.95 μL of nuclease-free water (Exiqon). Quantitative reverse transcription polymerase chain reactions (RT-qPCR) were performed in the LightCycler 480 instrument (Roche Diagnostics, Manheim, Germany) according to the following conditions: 95°C for 10 minutes and 45 cycles at 95°C for 10 seconds and 60°C for 1 minute.

The median values of miR-103a-3p, miR-207, miR-191-5p and SNORD38B were used for normalization, as these genes were the most stably expressed candidate reference genes (data not shown). Differences in expression values for target miRNAs were calculated using the 2-ΔΔCT method. The selection of deregulated miRNAs for further validation was performed considering prominent fold change, good sensitivity for qRT-PCR detection (Ct values, in general, below 30), and novelty.

Individual assays

Initially, cDNA samples were diluted 80x in sterile distilled water (B. Braun, Melsungen, Germany). Then, on ice, per each well of a 384-well plate it was added: 5 μL of NZYSpeedy qPCR Green Master Mix (2x) (NZYTECH, Portugal), 1 μL of miRNA specific primer mix (microRNA LNA™ PCR primer set, Exiqon), and 4 μL of previously diluted cDNA. Each amplification reaction was performed in triplicate on a LightCycler 480 instrument (Roche Diagnostics, Manheim, Germany). Each plate also contained 2 negative template controls. RT-qPCR protocol consisted in a denaturation step at 95°C for 2 minutes, followed by 40 amplification cycles at 95°C for 5 seconds

and 60°C for 20 seconds. Melting curve analysis was performed according to instrument's manufacturer recommendations.

SNORD38B was used as a reference gene for data normalization, as this gene was the most stably expressed over the whole range of the samples used for the global expression assay. Notwithstanding, the stability SNORD38B expression was empirically validated in additional samples. Relative miRNAs expression in each sample was calculated by the 2-ΔΔCT method.

Statistical analysis

To ascertain statistical significance for continuous variables comparisons made between independent samples, non-parametric Mann-Whitney U tests were performed. Fold changes for single miRNAs were calculated using the 2-ΔΔCT method[167]. Spearman nonparametric correlation test was performed to assess the association between continuous variables. Chi-square test or Fisher's exact test were used as appropriate to compare proportions between two groups.

Some clinicopathological features were grouped, including pT stage (T1&T2 and T3&T4), pN stage (N0&N1 and N2&N3) and grade [grade (G)1&G2 and G3]. Age was categorized into four groups (≤44; 45-64; 65-74; ≥75), and miRNA expression levels were categorized according to 25th or 75th percentile. For the survival analysis, Cox-regression univariable and multivariable models were computed to assess standard clinicopathological variables and miRNAs prognostic value. Hazard Ratios (HR) along with respective 95% Confidence Interval (95%CI) were reported. Multivariable Cox models only included the statistically significant variables. Kaplan-Meier with log rank test was used to construct and compare survival curves according to categorized miRNAs expression levels. Endocrine resistance-free survival (ERFS) was defined as the time between surgery and the recurrence dates. Recurrences occurring after 12 months of completing ET were not considered events for this analysis. Disease-free survival (DFS) was defined as the time between surgery date and recurrence date. Distant metastasis-free survival (DMFS) was defined as the time between surgery and the development of distant metastases.

Statistical analysis was performed using SPSS software (SPSS Version 20.0, Chicago, IL) and two-tailed p-values were considered statistically significant when $p<0.05$. Graphs were built using GraphPad 6 Prism (GraphPad Software, USA).

Results

Characteristics of study populations

The discovery cohort, used for global expression assay analysis, consisted of four luminal A and four luminal B tumors from patients which relapsed, and the same number of patients that did not relapse after adjuvant ET. Patients that relapsed during adjuvant ET or within the first 12 months of completing adjuvant ET were considered endocrine-resistant (Table 2.2).

Table 2.2. Clinical and pathological data of luminal tumors included in the discovery cohort.

	Molecular Subtype	Age at diagnosis	Grade	Stage	ChT	RT	Recurrency Site	Endocrine-resistant
Patients who relapsed	Luminal A	82	G2	IIIA	NO	NO	Liver	YES
		41	G3	IIA	YES	YES	Bone	YES
		60	UNKN	IA	NO	YES	Contralateral breast	NO
		43	G2	IIB	YES	YES	Lymph nodes	NO
	Luminal B	65	G3	IIIC	YES	YES	Lung	YES
		63	G2	IIIA	NO	YES	Bone	YES
		67	G2	IIB	NO	NO	Bone	NO
		66	G3	IIIA	NO	NO	Locoregional	NO
Patients who did not relapse	Luminal A	70	G3	IIB	NO	YES	n.a.	n.a.
		68	G2	IIB	NO	YES		
		69	G2	IIIA	NO	NO		
		69	G2	IA	NO	YES		
	Luminal B	65	G3	IIIC	YES	YES		
		72	G3	IIIC	NO	YES		
		70	G1	IIB	NO	YES		
		73	G1	IIIC	NO	YES		

Abbreviations: ChT – Chemotherapy; RT – Radiotherapy; UNKN – Unknown; n.a. – Not Applicable.

The validation cohort was composed by 162 patients, from which 136 fresh frozen luminal BC tissues and 26 normal breast tissues were collected. From the 136 luminal BC, 40 derived from patients which recurred and 96 from patients that did not. Among 40 patients with BC recurrence, 22 were considered endocrine-resistant. Clinical and pathological characteristics of patients and controls included in this study are shown in Table 2.3. Endocrine-sensitive and endocrine-resistant groups did not differ significantly concerning age distribution ($p=0.127$). As expected, among endocrine-resistant BC cases, luminal B tumors were more common ($p=0.004$), and consequently, the same trend was depicted for HER2-positivity ($p=0.024$) and high Ki-67 index ($p<0.001$). Moreover, this group also showed more moderate- and high-grade (G2 and G3) BC cases ($p<0.001$). For the remaining clinicopathological features or treatment modalities no significant differences were depicted.

Table 2.3. Clinical and pathological data of luminal tumors and normal breast samples included in the validation cohort.

Clinipathological features	Endocrine-Sensitive	Endocrine-Resistant	NBr
Patients (n)	114	22	26
Age median (range)	61.5 (43-73)	60 (41-75)	54 (40-70)
	61.0 (41-75)		
Molecular subtype (%)			
Luminal A	53 (46.5)	3 (13.6)	n.a.
Luminal B	61 (53.5)	19 (86.4)	
Histological type (%)			
Invasive carcinoma of NST (IDC)	99 (86.8)	19 (86.4)	
Invasive lobular carcinoma	6 (5.3)	2 (9.1)	n.a.
Other special subtype carcinoma	1 (0.9)	1 (4.5)	
Mixed type carcinoma	8 (7.0)	0 (0.0)	
Progesterone receptor *status* (%)			
Positive	96 (84.2)	15 (68.2)	n.a.
Negative	18 (15.8)	7 (31.8)	
HER2 receptor *status* (%)			
Positive	10 (8.8)	6 (27.3)	n.a.
Negative	104 (91.2)	16 (27.3)	
Ki-67 index (%)			
<15%	89 (78.1)	7 (31.8)	n.a.
>15%	20 (17.5)	11 (50.0)	
UNKN	5 (4.4)	4 (18.2)	
Grade (%)			
G1	19 (16.7)	0 (0.0)	
G2	57 (50.0)	9 (40.9)	n.a.
G3	31 (27.2)	11 (50.0)	
Not determined	7 (6.1)	2 (9.1)	
Pathological T Stage (%)			
pT1	34 (29.8)	5 (22.7)	
pT2	56 (49.1)	14 (63.6)	n.a.
pT3	3 (2.6)	0 (0.0)	
pT4	5 (4.4)	1 (4.5)	
Not determined	16 (14.0)	2 (9.1)	
Pathological N Stage (%)			
pN0	42 (36.8)	8 (36.4)	
p N1	43 (37.7)	8 (36.4)	n.a.
p N2	9 (7.9)	3 (13.6)	
p N3	5 (4.4)	1 (4.5)	
Not determined	15 (13.2)	2 (9.1)	
Adjuvant RT			
Yes	85 (74.6)	19 (86.4)	n.a.
No	19 (16.7)	3 (13.6)	
Not determined	10 (8.8)	0 (0.0)	
Adjuvant ChT			
Yes	39 (34.2)	12 (54.5)	n.a.
No	59 (51.8)	8 (36.4)	
Not determined	16 (14.0)	2 (9.1)	

Abbreviations: NBr – Normal Breast Tissues; NST – No Special Type; IDC – Invasive Ductal Carcinoma; HER2 - Human Epidermal Growth Factor Receptor 2; G – Grade; RT – Radiotherapy; ChT – Chemotherapy; n.a.- Not Applicable.

Global focus microRNA PCR Panel analysis

In the global expression assay, one luminal A case with recurrence was excluded from the analysis, due to low RT-qPCR success rate (25% of the miRNAs did not amplify, and the remaining showed Ct values higher than 30). Likewise, three (miR-202-3p, -206 and -20b-5p) out of the 80 miRNAs

were excluded due to low real-time PCR success rates. MiRNAs with fold variation values higher than 1 were selected, resulting in a panel comprising 56 miRNAs.

Gene-specific assays

From the global expression assay analysis, miR-30b-5p, miR-181a-5p, miR-182-5p, miR-200b-3p and miR-205-5p were selected for further validation. All these miRNAs disclosed prominent fold change and good sensitivity for qRT-PCR detection. MiR-30b-5p was chosen because several studies focused on other members of the miR-30 family (miR-30f) and, to the best of our knowledge, its predictive potential for endocrine therapy had not been assessed previously[168–172]. MiR-181a-5p and miR-200b-3p were selected to confirm the reported association with endocrine-resistance in in vitro studies[54,173–176]. Furthermore, miR-182-5p was also selected to better ascertain its role in endocrine resistance due to controversial results in global focus microRNA PCR panel, since it was overexpressed in luminal B tumors from recurrent patients and downregulated in luminal A tumors from recurrent patients. Finally, miR-30c-5p was chosen as a positive control since higher expression levels of this miRNA had been positively associated with benefit of ET, in multivariable analysis, in advanced ER-positive BC[53].

To determine "baseline" miRNA expression, 26 normal breast tissues were also analyzed, and we found that miR-181a-5p ($p=0.0007$), miR-182-5p ($p<0.0001$) and miR-200b-3p ($p<0.0001$) expression levels were significantly higher whereas miR-205-5p expression levels were significantly lower ($p=0.0056$) in luminal BC tissues (Figure 2.1). No differences were depicted for the remainder miRNAs.

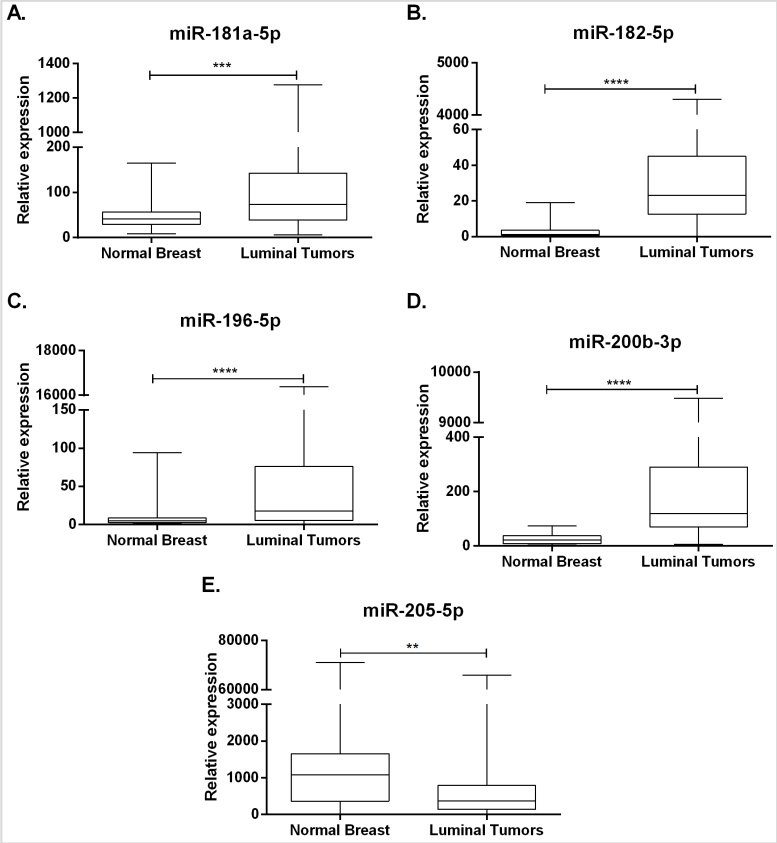


Figure 2.1. Box-plots of miR-181a-5p (A), miR-182-5p (B), miR-196-5p (C), miR-200b-3p (D) and miR-205-5p (E) relative expression levels in luminal tumor tissues and normal breast tissues. A ** denotes p-value <0.01, a *** denotes p-value <0.001 and a **** denotes p-value <0.0001 by non-parametric Mann-Whitney U test. Y-axis denotes 2-ΔΔCT values multiplied by 1000.

Furthermore, miR-30c-5p (p=0.0041), miR-30b-5p (p=0.0396) and miR-200b-3p (p=0.0293) were significantly downregulated in tumor tissues from endocrine-resistant BC compared to endocrine-sensitive tumors (Figure 2.2), while no differences were depicted for the remainder miRNAs.

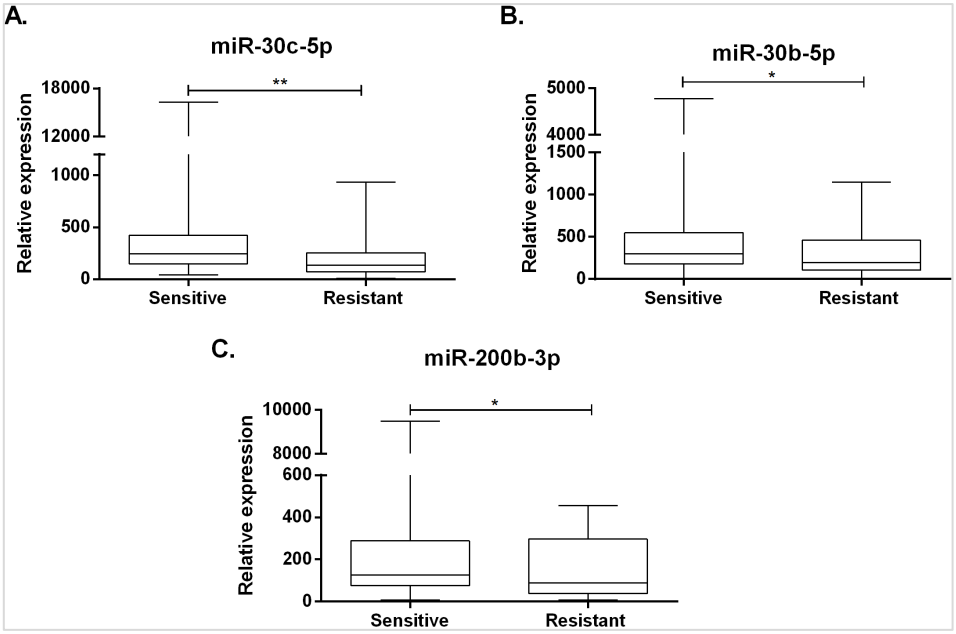


Figure 2.2. Box-plots of miR-30c-5p (A), miR-30b-5p (B) and miR-200b-3p (C) expression levels in tumor tissues from endocrine-sensitive and –resistant patients. A * denotes *p*-value <0.05 and a ** denotes *p*-value <0.01 by non-parametric Mann-Whitney U test. Y-axis denotes $2^{-\Delta\Delta C_T}$ values multiplied by 1000.

Association between miRNAs expression and clinicopathological features

MiR-30c-5p expression levels were significantly associated with PR-positive and HER2-negative tumors (p=0.0314 and p=0.0462, respectively). Moreover, miR-30b-5p expression levels were also higher in HER2-negative tumors (p=0.0447). Additionally, high grade (G3) BC displayed significantly lower miR-205-5p levels (p=0.0268) compared to G1/G2 BC (Figure 2.3).

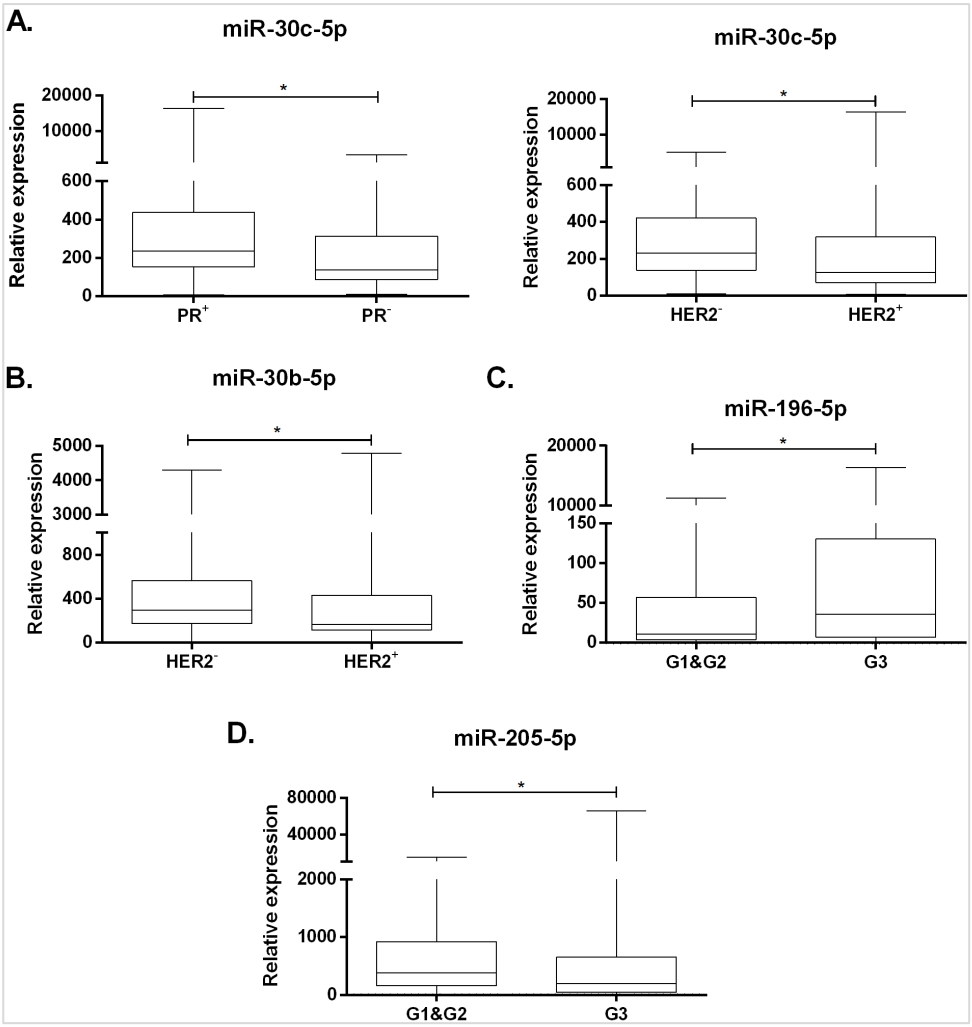


Figure 2.3. Box-plots of miR-30c-5p (A) expression levels according to PR-status (left) and HER2-status (right), miR-30b-5p (B) expression according to HER2-status, and miR-196a-5p (C) and miR-205-5p (D) expression according to grade. A * denotes p-value <0.05 by non-parametric Mann-Whitney U test. Y-axis denotes 2-ΔΔCT values multiplied by 1000.

Survival analyses

All survival analyses were restricted to 15 years of follow-up. The median follow-up time was 121 months (17.6-180 months). At 15 years of follow-up, 79 (58.1%) patients were alive. Of these, 76 patients (55.9%) were alive with no evidence of cancer and 3 patients (2.2%) with cancer. Additionally, 57 patients (41.9%) had deceased, 31 of which due to BC (22.8%).

Firstly, ERFS was calculated and, in univariable analysis, most standard clinicopathological parameters were significantly associated with ERFS. Specifically, HER2-positivity (HR = 3.46, p=0.010), high Ki-67 index (HR=5.82, p<0.001), high grade (G3) (HR=2.69, p=0.028) and luminal B subtype (HR=5.11, p=0.009) disclosed worse ERFS. Furthermore, lower miR-30c-5p, miR-30b-

5p, miR-182-5p and miR-200b-3p levels predicted decreased ERFS (Table 2.4, Figure 2.4). In multivariable analysis, all miRNAs identified in the univariable model remained independent predictors of improved ERFS adjusted to molecular subtype (Table 2.4). To disclose the potential of miRNAs expression level as predictors of ERFS for each molecular subtype, a stratified analysis by luminal subtype was performed (Table 6). However, miRNAs only retained statistical significance in luminal B tumors.

Likewise, to assess the miRNAs prognostic value, DFS analysis was also performed. In an univariable analysis, HER2-positivity (HR = 3.33, p=0.0002), high Ki-67 index (HR=2.48, p=0.010) and high grade (G3) (HR=2.21, p=0.016) associated with worse DFS, as expected. Interestingly, lower miR-30c-5p, miR-30b-5p, miR-182-5p and miR-200b-3p expression levels associated with decreased DFS (Table 2.4, Figure 2.5). Nonetheless, only miR-200b-3p and miR-182-5p were independent prognostic predictors adjusted for HER2 status in the multivariable model (Table 2.4). After stratifying the analysis according to HER2 status, both miRNAs retained statistical significance in both HER2-positive and HER2-negative BC (Table 2.5).

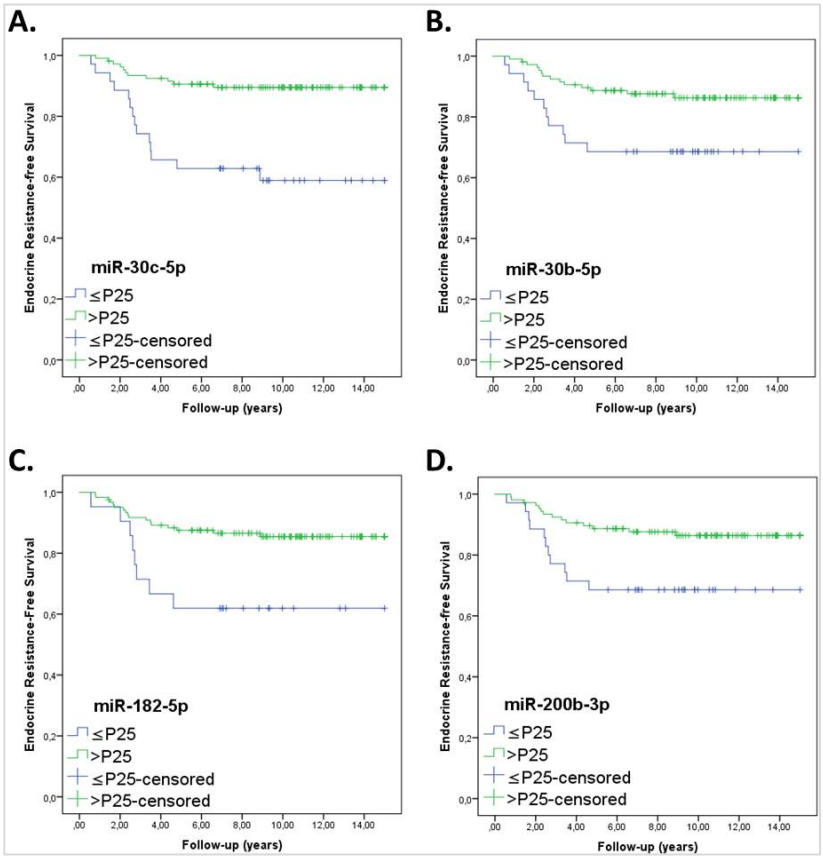


Figure 2.4. Endocrine Resistance-free survival curves of miR-30c-5p (A), miR-30b-5p (B), miR-182-5p (C) and miR-200b (D). Abbreviations: P25 – percentile 25.

Finally, DMFS was also performed, disregarding locoregional recurrences. In the same line as for DFS, HER2-positivity (HR = 3.39, p=0.001), high Ki-67 index (HR=2.27, p=0.029) and high grade (G3) (HR=2.25, p=0.020) associated with worse DMFS, in a univariable analysis. Besides, lower miR-30c-5p, miR-30b-5p, miR-182-5p and miR-200b-3p expression levels also associated with decreased DMFS (Table 2.4). In multivariable analysis, miR-182-5p retained statistical significance adjusted for HER2 status and tumor grade, whereas miR-200b-3p only retained statistical significance for HER2 status (Table 2.4). After stratifying analysis according to HER2 status and grade, miR-182-5p retained statistical significance in both low/intermediate and high-grade cancers, as well as in HER2-negative tumors, whereas miR-200b-3p retained statistical significance in HER2-positive BC (Table 2.5).

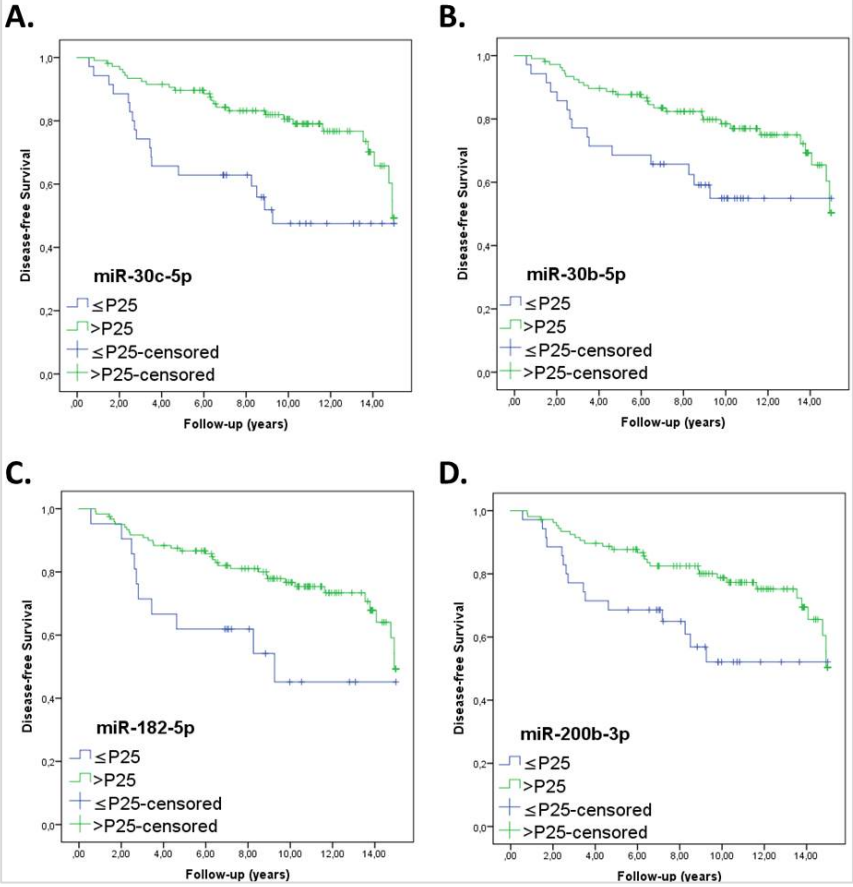


Figure 2.5. Disease-free survival curves (Kaplan–Meier with log rank test) of miR-30c-5p (A), miR-30b-5p (B), miR-182-5p (C) and miR-200b (D). Abbreviations: P25 – percentile 25.

Table 2.4. Univariable and multivariable cox regression models assessing the association between microRNAs expression levels and clinical outcome.

Model	Outcome	Variable	HR (95% CI)	*p*-value
Univariable Analysis	ERFS	miR-30c-5p expression categorized ≤P25 >P25	 1 0.311 (0.135-0.717)	0.006
		miR-30b-5p expression categorized ≤P25 >P25	 1 0.362 (0.156-0.838)	0.018
		miR-182-5p expression categorized ≤P25 >P25	 1 0.194 (0.081-0.464)	< 0.001
		miR-200b-3p expression categorized ≤P25 >P25	 1 0.217 (0.091-0.518)	0.001
	DFS	miR-30c-5p expression categorized ≤P25 >P25	 1 0.426 (0.223-0.815)	0.010
		miR-30b-5p expression categorized ≤P25 >P25	 1 0.412 (0.208-0.817)	0.011
		miR-182-5p expression categorized ≤P25 >P25	 1 0.213 (0.101-0.452)	< 0.001
		miR-200b-3p expression categorized ≤P25 >P25	 1 0.226 (0.110-0.465)	< 0.001
	DMFS	miR-30c-5p expression categorized ≤P25 >P25	 1 0.467 (0.234-0.932)	0.031
		miR-30b-5p expression categorized ≤P25 >P25	 1 0.465 (0.224-0.964)	0.040
		miR-182-5p expression categorized ≤P25 >P25	 1 0.284 (0.126-0.644)	0.003
		miR-200b-3p expression categorized ≤P25 >P25	 1 0.287 (0.131-0.628)	0.002
Multivariable Analysis	ERFS	miR-30c-5p expression categorized [1] ≤P25 >P25	 1 0.353 (0.152-0.818)	0.015
		miR-30b-5p expression categorized [1] ≤P25 >P25	 1 0.367 (1.497-17.112)	0.019
		miR-182-5p expression categorized [1] ≤P25 >P25	 1 0.181 (0.075-0.434)	< 0.001
		miR-200b-3p expression categorized [1] ≤P25 >P25	 1 0.218 (0.091-0.522)	0.001
	DFS	miR-182-5p expression categorized [2] ≤P25 >P25	 1 0.194 (0.091-0.415)	< 0.001
		miR-200b-3p expression categorized [2] ≤P25 >P25	 1 0.246 (0.119-0.511)	< 0.001
	DMFS	miR-182-5p expression categorized [3] ≤P25 >P25	 1 0.191 (0.081-0.454)	< 0.001
		miR-200b-3p expression categorized [2] ≤P25 >P25	 1 0.314 (0.143-0.691)	0.004

[1] Cox regression model adjusted for molecular subtype. [2] Cox regression models adjusted for HER2 *status*; [3] Cox regression model adjusted for grade and HER2 *status*. **Abbreviations**: ERFS - Endocrine Resistance-free Survival; DFS - Disease-free Survival; DMFS - Distant Metastasis-free Survival.

Table 2.5. Cox regression models stratified according to the clinicopathological features with statistical significance in the multivariable analysis.

Outcome	Layering Variable	Variable	HR	*p*-value
ERFS	Luminal A	miR-30c-5p expression categorized ≤P25 >P25	-	0.555
	Luminal B	miR-30c-5p expression categorized ≤P25 >P25	1 0.344 (0.140-0.847)	0.020
	Luminal A	miR-30b-5p expression categorized ≤P25 >P25	-	0.661
	Luminal B	miR-30b-5p expression categorized ≤P25 >P25	1 0.344 (0.140-0.848)	0.020
	Luminal A	miR-182-5p expression categorized ≤P25 >P25	-	0.689
	Luminal B	miR-182-5p expression categorized ≤P25 >P25	1 0.145 (0.058-0.364)	< 0.001
	Luminal A	miR-200b-3p expression categorized ≤P25 >P25	-	0.699
	Luminal B	miR-200b-3p expression categorized ≤P25 >P25	1 0.178 (0.071-0.445)	< 0.001
DFS	HER2-negative	miR-182-5p expression categorized ≤P25 >P25	1 0.179 (0.058-0.364)	0.002
	HER2-positive	miR-182-5p expression categorized ≤P25 >P25	1 0.197 (0.058-0.364)	0.004
	HER2-negative	miR-200b-3p expression categorized ≤P25 >P25	1 0.235 (0.073-0.750)	0.014
	HER2-positive	miR-200b-3p expression categorized ≤P25 >P25	1 0.311 (0.113-0.858)	0.024
DMFS	Grade 1&2	miR-182-5p expression categorized [1] ≤P25 >P25	1 0.249 (0.076-0.819)	0.022
	Grade 3	miR-182-5p expression categorized [1] ≤P25 >P25	1 0.168 (0.044-0.642)	0.009
	HER2-negative	miR-182-5p expression categorized [2] ≤P25 >P25	1 0.235 (0.089-0.625)	0.004
	HER2-positive	miR-182-5p expression categorized[2] ≤P25 >P25	-	0.053
	HER2-negative	miR-200b-3p expression categorized ≤P25 >P25	-	0.066
	HER2-positive	miR-200b-3p expression categorized ≤P25 >P25	1 0.219 (0.054-0.884)	0.033

[1] Cox regression model adjusted for HER2 *status*. [2] Cox regression models adjusted for grade. **Abbreviations**: ERFS - Endocrine Resistance-free Survival; DFS - Disease-free Survival; DMFS - Distant Metastasis-free Survival; HER2 – Human Epidermal Growth Factor 2 Receptor.

Discussion

BC remains the most common malignancy in women and a major cause of morbidity and mortality[177]. De-escalation of both systemic and local adjuvant treatment, paralleling trends in surgery, is critical to provide patient-tailored treatment and avoid harmful side effects[88,178]. Indeed, identification of luminal BC patients with low recurrence risk after or while on ET, for which additional adjuvant systemic treatment can be safely omitted, is clinically important. Additionally, the identification of high-risk luminal BC patients requiring more aggressive treatment regimens might further reduce recurrence and subsequent metastatic disease, currently affecting approximately 40% of luminal BC patients after adjuvant ET[23,157,179]. Thus, identification of biomarkers providing predictive and prognostic information in this group of patients is clinically relevant. Assessment of specific miRNAs expression deregulation, which has been associated with several mechanisms underlying endocrine resistance and sensitivity[159,161] might provide such kind of information. Nonetheless, most of those studies have been performed in cancer cell lines and display several limitations, including absence of epithelial-stromal and tumor-host interactions, that could modulate sensitivity *in vivo* [180]. Conversely, tissue analysis from patients treated with ET may allow for broader insight into biologically and clinically relevant miRNAs that may serve as markers of response or resistance to ET. Thus, we focused on the identification of aberrantly expressed miRNAs in endocrine-resistant BC, exploring its predictive and prognostic value in luminal BC patients treated with adjuvant ET.

The first step of this study consisted on the profiling of miRNAs expression patterns, looking for differences between endocrine-sensitive and endocrine-resistant luminal BC. Hence, miR-30c-5p, miR-30b-5p, miR-181a-5p, miR-182-5p, miR-200b-3p and miR-205-5p were selected for validation in a larger set of luminal BC and normal breast tissues. Upregulation of miR-181a-5p and miR-182-5p and downregulation of miR-205-5p in this BC tissue cohort was consistent with previous publications[181–183], providing indirect validation of our methodological approach. Contrarily, downregulation of miR-200b-3p in tumor compared to normal tissues has been previously reported[184,185]. However, these studies have used non-cancerous breast tissues harboring carcinoma as controls, which may not represent truly normal breast tissues. Our results have also successfully confirmed the biomarker potential of miR-30c-5p, which was downregulated in endocrine-resistant BC patients and independently predicted better ERFS in luminal B BC patients. Moreover, miR-30c-5p expression correlated with PR-positivity and HER2-negativity, two of the most important

predictive factors for ET sensitivity[186]. In fact, higher PR expression has suggested better sensitivity to ET and activation of HER2 signaling has been known as one of the factors most prominently contributing to endocrine resistance[187,188]. Likewise, miR-30b-5p and miR-200b-3p displayed the same trend and together with miR-182-5p, also independently predicted for improved ERFS in luminal B BC patients. The lack of significance in luminal A subtype might be due to the small number of cases and events in our series. Importantly, we were able to validate in primary BC the association between miR-200b-3p and endocrine-resistance, previously reported in in vitro models[54]. Interestingly, several members of miR-30f have been reported as markers of favorable prognosis in BC[168–171,189] and our study also revealed that miR-30b-5p might be predictive of response to ET. Finally, concerning miR-182-5p, our results extended previous observations on the correlation with clinical benefit from therapy with tamoxifen in advanced-stage BC, only showed in univariable analysis[53].

In addition to their predictive value, miR-30b-5p and miR-30c-5p also displayed prognostic potential in univariable analysis. Lower levels of these miRNAs were associated with decreased DFS and DMFS. MiR-30f members and their role as tumor suppressor during BC a have been previously reported[169,170]. Indeed, decreased levels of miR-30f, and related members, has been associated with poor relapse-free survival[189]. Remarkably, we have also showed that miR-182-5p and miR-200b-3p are not only predictive, but also independent prognostic markers in multivariable analysis. Downregulation of these miRNAs was associated with decreased DFS in both HER2-positive and HER2–negative BC and both miRNAs independently predict DMFS in HER2-negative and HER2-positive cancers, respectively. The role of miR-200b-3p as a prognostic marker in BC is not a novelty[184,185]. Indeed, members of miR-200f are known to act as enforcers of epithelial phenotype through either Zinc finger E-box-binding homeobox (ZEB)-dependent or –independent pathways[190]. Intriguingly, most in vitro studies consistently attributed an oncogenic role to miR-182-5p[191,192]. Though, higher miR-182-5p expression levels were associated with poor clinical outcome in BC patients[162], contrarily to our findings. It should be noted, however, that miR-182-5p is a member of a miRNA family comprising three homologous, coordinately expressed, miRNAs (miR-183, miR-182 and miR-196) that are clustered in chromosome 7q32.2 and that members of this cluster have been linked to both pro- and anti-metastatic behavior in BC, suggesting that miR-183/96/182 cluster members may have divergent functions which are regulated in a context- and tissue-dependent manner[182,193,194]. Furthermore, the 7q32.2 locus has been considered a metastasis suppressor locus, enduring genetic copy number losses in BC progression[195]. Thus, the association

between miR-182-5p downregulation and worse prognosis probably results from a complex molecular scenario and additional studies are required to discriminate which members of the miR-183/96/182 cluster may contribute and to which extent to BC prognosis.

BC tissues displayed higher miR-182-5p and miR-200b-3p levels compared to normal breast, whereas miR-30b-5p, miR-30c-5p, miR-182-5p and miR-200b-3p downregulation associated with decreased DMFS. Once development of solid neoplasms results from multiple sequential steps in which malignant cells undergo widespread modifications to successfully migrate and colonize other organs, we are tempted to speculate a context-dependent role of these miRNAs that may contribute to the emergence of malignant phenotype. Indeed, decreased miR-200f members expression might be associated with EMT initiation enabling cells with invasive features, whereas subsequent upregulation might be associated with MET, facilitating metastization at distance[196,197].

Globally, our results suggest a panel of miRNAs that might be tested in primary tumor tissues to assess the likelihood of recurrence and resistance to ET in newly diagnosed luminal BC. Nevertheless, these miRNAs need to be carefully validated, ideally in multicenter studies, to generate more conclusive results. Furthermore, in vitro studies, including gain and loss of function assays following in vitro treatment with ET, are also critical to functionally characterize the role of these miRNAs. As future perspective, we intend to evaluate the potential role of these miRNAs in tumor dissemination. Additionally, we also intend to assess the expression of these miRNAs in liquid biopsies, evaluating their potential as non-invasive biomarkers. Indeed, miRNAs in circulation would enable the repeated noninvasive monitoring of miRNA expression profile changes during treatment's course, which could allow for early detection of ET resistance and/or recurrence, potentially improving the management and care of luminal BC patients.

3. Epigenetic biomarker H3K27me3 software aided expression assessment in luminal A/B-like HER2-negative invasive breast cancer for survival and recurrence risks

Introduction

Biological role of H3K27me3 and pre-clinical data on cancer cells

H3K27me3 stands for trimethylation of lysine 27 of histone 3 relates and corresponds to a histone modification mechanism (post-translational mechanisms).

One must report to the regulation of (cancer) stem cells and the Polycomb family of genes – that are epigenetic transcriptional repressors and key regulators of cell fate[198,199] – to introduce H3K27me3, which is specifically related to Polycomb Repressive Complexes 2 (PRC2). In the core of PRC2 complexes exist methyltransferases (notably EZH2) that catalyze the trimethylation of histone H3, thus participating in the control of gene expression patterns[199,200].

There is also support[12] that the activation of PI3K/AKT/mTOR signaling may be a trigger of trimethylation of histone H3, which is a known pathway involved in endocrine-therapy resistance in breast cancer – with therapeutic agents approved for this setting (eg. Everolimus).

Clinical data on H3K27me3 and immunohistochemistry (IHC) scoring for human breast cancer

In the clinical setting, until 2008, there were no published reports about the significance of changes of H3K27 methylation in solid tumors. The initial findings suggested that low expression of H3K27me3 could be a predictor of poor outcome in breast tumors[201] in a Chinese cohort of 142 breast cancer patients, 43 of which were estrogen receptor positive with a median age of 51 years. The median observation time for overall survival was 50 months (~4.2 years). Two pathologists assessed the level of H3K27me3 staining independently. The median of expression (30%) was used as cut-off to determine dichotomic categories of low-expression and high expression. The authors showed a 46% 5-yr survival in cases with low H3K27me3 expression versus 72% among the rest (P=0.005).

A Swedish team published[202] the results regarding two sets of estrogen receptor positive patients: set I - node negative, N=112; set II - premenopausal N=89 (from a prospective trial in which around half the patients were treated with Tamoxifen for two years and the other half did not

received any adjuvant treatment). An investigator, under a pathologist guidance, assessed IHC (blinded to clinical data). In this case, intensity values were classified as 1 to 3, with 1 being weak, and 3 strong. The final score was the average between two calculated scores per case. The differences in distant disease-free survival, censored at 5 years follow-up, with patients with low abundance having a shorter survival (P=0.01, log-rank) than those with intermediate or high levels (P=0.004, log-rank).

Later, H3K27me3 expression and clinical correlates were described in a Korean cohort[203], expanding on previously published observations[201]. For the IHC assessment, the team used N=146 of invasive breast cancer patients, of which 102 were estrogen receptor positive (considered if ≥ 10% positive estrogen receptors) with a median age of 46 years. The median follow-up time was 6.2 years. Two pathologists assessed the level of H3K27me3 staining independently. For IHC staining purposes an intensity value score was used that ranged between 1 and 3, with 1 being weak, and 3 strong. The authors reported that high H3K27me3 expression was associated with longer OS (P<0.001, log-rank). Another study reported[204] that H3K27me3 positivity was associated with lower grade tumors and the luminal A subtype, with a follow-up time over 20-years. In this particular case, dichotomic positivity score was defined at 50% expression.

Gene expression patterns were also reported[205] and H3K27me3 allowed for stratification into good and poor prognostic groups independent of known breast cancer gene signatures (N=95). Similarly, H3K27me3 genome-wide chromatin-binding profiles (N=72) predicted the treatment outcomes for first-line aromatase inhibitors[72].

More recently, a subtype classification and risk of breast cancer by histone modification profiling was performed[206] that included a small number of clinical samples for validation (luminal subtypes N=6) and later in a cohort subclassified using a commercial genetic panel (luminal subtypes N=47) suggest H3K27me3-proximal gene classifiers were significantly correlated with relapse-free survival (P < 0.0001, log-rank).

Data on experimental therapy using H3K27me3 as drug target

A phase 1 first-in-human study[207] of histone methyl transferase inhibitor described an interaction with H3K27me3, but did not include breast cancer patients.

In breast cancer cell lines it was suggested that inactivation of GSK3β was significantly correlated with higher level of H3K27 trimethylation[208], thus potentially targetable. It was also published[209] that in breast cancer cells and xenograft model that the drug GSK126 induced marked re-expression of

genes, including ones related to H3K27me3 and yet other teams published[210–212] that by using the inhibitory effect of experimental drug GSKJ4 in cancer stem cells - by incrementing H3K27me3 levels – showed that it resulted in suppression of stemness factors.

Material and Methods

Review of published data

A resource search was conducted through PUBMED® using the key words "H3K27me3 breast cancer" that retrieved 101 results (by November 15th, 2017). Data considered of interest was reviewed.

Population of the study

A total of 363 post-surgical female breast cancer samples corresponding to the same number of patients were identified at the Portuguese Institute of Oncology of Porto biobank. These patients were treated at our institution between January 1995 and December 2002. All tissue samples were classified by an experienced pathologist (blinded to previous classification). We excluded neoadjuvant treated patients (N=24) 'triple negative' cancers (N=56), HER2-positive (N=26), stage IV (N=7) and cases with insufficient information/non-assessable material (90).

Therefore our sample entailed N=160 tissue samples with luminal A/B-like HER2-negative invasive breast cancer from female patients, stage I-III.

The median observation time for overall survival was 128.2 months (~10.7 years, range 4–254.9 months). Date cut-off for follow-up was November 15th, 2017.

All of the collected samples were used with informed consent under protocols approved by institutional ethics committee. The individual clinical file was used to retrieve the study variables for each patient, complemented by electronic clinical file and cancer registry, whenever possible.

Definition of clinicopathological variables and endpoints

Positive hormonal receptor breast cancer was defined as Estrogen or Progesterone receptors ≥ 1% as per international guidelines[213] and HER2 positivity according to standard recommendations[214].

Regarding the T stage definition, the cases would have been classified according to Union for International Cancer Control (UICC) / American Joint Committee on Cancer (AJCC) manuals from the 4th edition through the 6th, in which T stage remained generally consistent (T1 ≤ 2 cm; T2 > 2

cm but ≤ 5 cm; T3 > 5 cm; T4 any size with extension to chest wall or skin, including inflammatory carcinoma of the breast). These definitions remain almost unchanged until even in the most recent 7th or 8th editions. On the other hand, N staging has varied, so we considered pathological node negative cases (pN-) and pathological node positive cases (pN+) and further specified if equal or more than 4 metastasized nodes.

Recurrence was defined has evidence of loco-regional or at distance evidence of breast cancer disease > 4 months from diagnosis and after surgical treatment. Early recurrence was considered when ≤ 5 years within surgery date and late recurrence > 5 years from surgery date. Endocrine-treatment resistance was classified according to international consensus guidelines for advanced breast cancer (ABC 3)[215].

Sample storage and procedures

Expression of H3K27me3 was determined by IHC in formalin fixed paraffin embedded tissues.

Evaluation of H3K27me3 IHC expression

A Pathologist, blinded to clinicopathological variables, selected invasive breast cancer areas for each sample for further H3K27me3 immunostaining analysis. A team Investigator, also blinded to clinicopathological variables, then used GenASIs™ software, a computer IHC scoring aid, for cell-expression assessment (Image 3.1) and only staining identified in the cell nuclei was considered. A customized profile from positive control was used. Two pre-specified conditions were considered: ≥ 5 frames analyzed/case ≥ 3.000 cells analyzed/case.

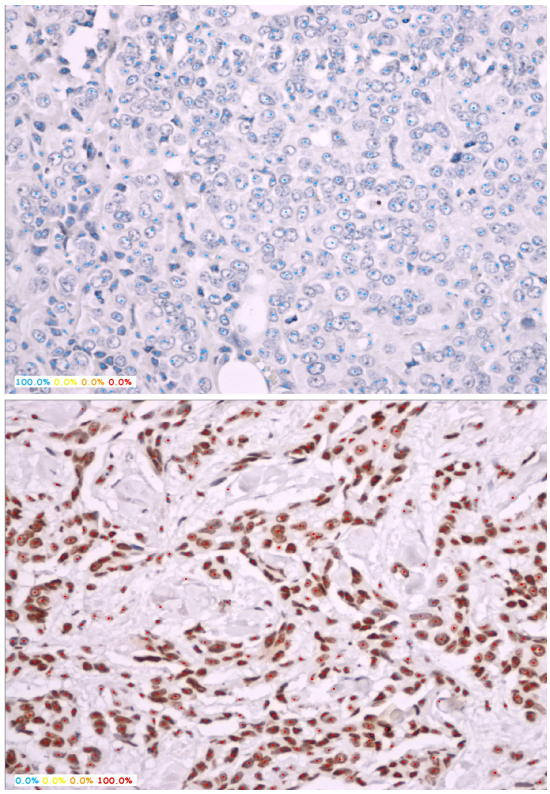


Figure 3.1. Automated IHC scoring in breast cancer tumors samples. a) low expression score (100% negative) and b) high expression score (100% positive).

Statistical analysis

For the statistical analysis SPSS version 25.0 was used. Associations between variables were assessed using Chi-square test. Multivariate analysis was performed using binary correlation. The log-rank test was used to compare survival between groups in Kaplan–Meier survival curves. All *P* values were two sided and $P<0.05$ was considered statistically significant.

Results

IHC H3K27me3 expression evaluation

For the N=160 cases the median H3K27me3 expression was 87.2% (range 3.3-99.9%). Regarding the pre-specified conditions, a median of 6 frames were analyzed/case (range 5-10 frames/case),

a total of 975 frames for the study and a median 3,414 cells analyzed/case (range 3.015-5.292 cells analyzed/case), a total of 546.249 cells were analyzed.

H3K27me3 expression and known clinicopathological prognostic markers

Commonly used clinicopathological prognostic elements were described for the general population of the study (Table 3.1).

Table 3.1. Clinicopathological variables description in the general cohort population.

Variables		N	%
Luminal subtypes			
	A-like	66	41.3
	B-like	94	58.8
Invasive Ductal Carcinoma		133	83.1
Grade			
	G1	21/149	14.1
	G2	76/149	51.0
	G3	52/149	34.9
pT Stage			
	pT1	56/149	37.6
	pT2	86/148	58.1
	pT3-p/cT4*	6/148	4.1
Pathological Nodal status			
	pN-	68/153	44.4
	pN+	85/153	55.6
pN+ ≥ 4 nodes		35/85	41.2
Did adjuvant Chemotherapy		63/100	63.0
Did adjuvant Radiotherapy		117/143	81.8
Did adjuvant endocrine therapy		128/132	97.0
Adjuvant Tamoxifen-only		93/128	72.7
Adjuvant Tamoxifen + Aromatase inhibitor		34/128	26.6
Adjuvant Tamoxifen plus Goserelin		1/128	0.8
Recurrence		45	28.1
	Early recurrence	22/44	50.0
	Endocrine-treatment resistant recurrence	24/42	57.1
	Systemic recurrence	33/43	76.7
Death		35	21.9

Notes: N=160 unless otherwise specified. *T3 and T4 cases analyzed conjointly due to low N (see text for further details).

H3K27me3 expression and recurrence and death risk

The H3K27me3 expression cut-off that obtained the best statistical significance in the general cohort was 85% for breast cancer recurrence (OR 1.914; 95% CI, 1.142-3.208, *P*=0.011) and death risk (OR 1.967; 95% CI, 1.067-3.624 *P*=0.026). Therefore, we considered a dichotomic variable (< 85% and ≥ 85% expression for low and high expression, respectively). By the Kaplan-Meier method (Figure 3.2) the log-rank was not statistically significant for death (*P*=0.086) but was statistically significant for recurrence (< 85% median 14.9 years vs ≥ 85% median not reached, *P*=0.010).

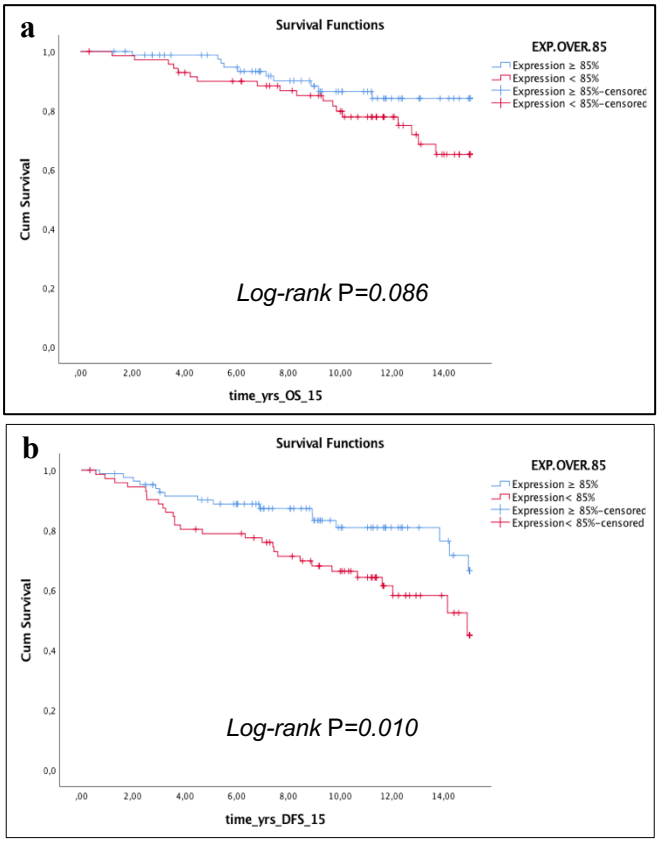


Figure 3.2. Kaplan–Meier analysis (censored at 15 years of follow-up) for a) overall survival and b) disease free survival, both according to H3K27me3 expression at 85% of expression cut-off.

Recurrence occurred in 45 patients (28.1%). The median H3K27me3 expression in this subgroup was 81.1% (range 8.7-99.7%). We calculated H3K27me3 expression <85% as a test for recurrence having a sensitivity of 62.2%, specificity of 60.0%, a positive predictive value of 37.8% and a negative predictive value of 80.2%.

There were no statistical association in univariate analysis for commonly used clinicopathological prognostic markers in this setting (Table 3.2), namely median age (*P*=0.557), luminal A/B-like subtypes (*P*=0.878), pathological tumor size (*P*=0.817) or nodal metastization status (*P*=0.262), histology (*P*=0.836), grade (*P*=0.069), adjuvant chemotherapy (*P*=0.424) and adjuvant Tamoxifen only/Tamoxifen and Aromatase inhibitor (*P*=0.576).

Table 3.2. Correlation of H3K27me3 expression at 85% cut-off with evaluated variables

Variables		H3K27me3 "low" expression < 85% N(%)	H3K27me3 "high" expression ≥ 85% N(%)	*P* value
N		74 (46.3%)	86 (53.7%)	
Median age				
	< 61 years old	37 (50%)	39 (45.3%)	0,557
	≥ 61 years old	37 (50%)	47 (54.7%)	
Luminal subtype				
	Luminal A-like	31 (41.9%)	35 (40.7%)	0.878
	Luminal B-like	43 (58.1%)	51 (59.3%)	
Pathological tumor size				
	≤ 2 cm (pT1)	26/71 (36.6%)	29/77 (37.7%)	0.817
	> 2 cm (pT2-4)	45/71 (63.4%)	48/77 (62.3%)	
Pathological nodal status				
	N0	29/73 (39.7%)	39/80 (48.8%)	0.262
	N+ (N1-3)	44/73 (60.3%)	41/80 (51.2%)	
Grade				
	1 or 2	39/68 (57.4%)	58/81 (71.6%)	0.069
	3	29/68 (42.6%)	23/81 (28.4%)	
Histology				
	Ductal invasive carcinoma	62 (83.8%)	71 (82.6%)	0.836
	Non-ductal invasive carcinoma	12 (16.2%)	15 (17.4%)	
Adjuvant chemotherapy				
	Chemotherapy	29/43 (67.4%)	34/57 (59.6%)	0.424
	No chemotherapy	14/43 (32.6%)	23/57 (40.4%)	
Adjuvant endocrine therapy				
	Tamoxifen only	45/60 (75%)	48/68 (70.6%)	0.576
	Tamoxifen + Aromatase inhibitor	15/60(25%)	19/68 (27.9%)	
Recurrence status				0.011
	Recurrence	28/74 (37.8%)	17/86 (19.8%)	
	No recurrence	46 (62.2%)	69 (80.2%)	
Death by breast cancer				0.026
	Death	22/74 (29.7%)	13/86 (15.1%)	
	No death	52/74 (70.3%)	73/86 (84.9%)	

A multivariate logistic regression was performed, and the cut-off was independently significant for recurrence (Table 3.3).

Table 3.3. Multivariate analysis of H3K27me3 expression for Recurrence, adjusted for common clinicopathological marks.

		Score	df	Sig.
Variables	AGE.MORE.61(1)	3,947	1	,047
	Luminal.A(1)	,264	1	,607
	CDI(1)	1,653	1	,199
	G3.COD(1)	2,677	1	,102
	T1.COD(1)	,451	1	,502
	T2.COD(1)	1,338	1	,247
	T3.T4.COD(1)	1,066	1	,302
	pN.POS(1)	,451	1	,502
	QT.Adjv(1)	1,077	1	,299
	RT.Adjv(1)	3,679	1	,055
	HT.Adjv(1)	,349	1	,554
	DEATH(1)	58,868	1	,000
	EXP.LESS.85(1)	5,566	1	,018

Death occurred in 35 patients (21.9%), which represents 77.8% (35/45) of patients that recurred. The median H3K27me3 expression was 78.1% (range 8.7-99.7%). We also calculated H3K27me3 expression <85% as a test for death having a sensitivity of 62.9%, specificity of 57.0%, a positive predictive value of 28.6% and a negative predictive value of 84.9%.

In the younger subpopulation - below the median of age of 61 years old – with N=76, the associations (chi-square test) between H3K27me3 expression 85% cut-off became stronger for recurrence (P=0.003) and death (P=0.009), and when using Kaplan-Meier method (recurrence log-rank p=0.006; death log-rank P=0.019).

H3K27me3 expression and endocrine-treatment resistance recurrence

Endocrine-treatment resistance was associated to increased death risk (OR 2.190; 95% CI, 1.249-3.842, P< 0.001) and early recurrence (OR 13.333; 95% CI, 1.982-89.695, P< 0.001).

There was no association between the 85% H3K27me3 expression cut-off and early recurrence (P=1), systemic recurrence (P=0.199) or endocrine-treatment recurrence (P=0.685).

Associated to increased risk of endocrine-treatment resistance recurrence was Luminal B-like subtype (OR 1.905; 95% CI, 1.063-3.425, P=0.009) and conversely lower risk in non-G3 (OR 0.457; 95% CI, 0.224-0.932, P=0.028).

Discussion

The results seem to expand on previous reports, being consistent in a sense that lower H3K27me3 expression appears to be associated to a poorer prognosis in estrogen receptor positive breast cancer. We could not find any cut-off value that would be statically significant for survival in the unselected cohort, although it was reached in the younger subpopulation (below the median). We elaborate on factors that may explain these results as compared with previously published results: populations may be intrinsically distinct (due to their diverse geographic location), the median age of the population (our population's median was higher), using the current 1% cut-off for estrogen receptor positivity (while other teams may have used different cut-off), different methods used to evaluate H3K27me3 expression and cut-off for H3K27me3 expression or score systems, the positive control used, among other potential aspects. About 20% of recurrences did not resulted in death (either loco-regional recurrences with potential multimodal curative treatments or long survivals/responders to palliative treatment) which can account for a statistically less strong relation with death.

A direct comparison between previously published results is difficult since each team used different IHC scoring methods, from the simplest (using the median or a specific cut-off value) to the more complex (using a scoring system). We intended to introduce software aided IHC assessment in order to increase reproducibility of results as a more suitable and practical mechanism for the heavy work loaded Pathologist.

A clinical limitation was the exclusion of more locally advanced cancers that were candidates to neoadjuvant treatment (since H3K27me3 expression could be affected by drug exposure), triple negative (we wanted to focus on luminal cases) or HER-2 positive cases (not performed at the time of the cohort beginning, therefore the cases were not treated according to nowadays guidelines) thus the general prognosis in our cohort is more favorable, although we feel it is nonetheless representative of the Luminal subtypes cases from clinical practice.

In a clinical practice perspective, if the results were prospectively validated, the H3K27me3 expression 85% cut-off could be used as a tool for treatment or follow-up intensity protocol decision (for example, since < 85% expression has increased risk for recurrence a patient could be candidate

to adjuvant chemotherapy followed by endocrine therapy vs endocrine therapy only). We would envision it as an additional element among already in use prognostic markers to determine adjuvant therapy intensity (such as Ki67, tumor size or nodal metastization, intrinsic subtype genetic testing among others).

A dedicated sub-analysis was previously reported[215] which focused on endocrine-treatment resistance recurrences but no statistically significant associations were found then or now, which could indicate that in the clinical setting H3K27me3 expression may not directly correlate with PI3K/AKT/mTOR signaling pathway as expected, at least in this clinical setting.

Conclusions

In this exploratory retrospective study that included 160 invasive breast cancer patients with a median 10-year plus follow-up an expression <85% of the epigenetic biomarker H3K27me3 was associated to statistically significant worse prognosis (higher recurrence risk) and statistical tendency to a decreased survival in luminal A/B-like HER2-negative breast cancer. These results expand and strengthen previous reports and encourage prospective validation, potential clinical use and targeted therapy. To our knowledge, our results are the first using computer aided IHC scoring for H3K27me3 expression. These results warrant further analysis, namely prospective validation, especially when an additional element can be of use in the era of individualized precision Oncology.

General discussion and considerations

The definition of endocrine-resistance breast cancer is clinical, characterized by a disease behavior (local and/or distal recurrence, progression) that occurs in strictly defined time-periods in relation to endocrine treatments, that aims to identify subsets of luminal breast cancers that are associated to increased aggressiveness, thus with worse prognosis, and limited response to endocrine therapy.

We can expect that these changes to be dynamic in time and in response to exposure to local and systemic treatments, i.e. the biomarkers may only reveal themselves or increase to detectable levels, or vice-versa, only after initiating treatments (and there are many endocrine therapies), for instance. For instance, as showed before, levels of the miR-30c-5p (p=0.0041), miR-30b-5p (p=0.0396) and miR-200b-3p (p=0.0293) were significantly downregulated in tumor tissues from endocrine-resistant BC compared to endocrine-sensitive tumors, upon disease recurrence and after or under endocrine treatment (Figure 2.2). It is rare to obtain same-patient primary tumor and metastasis samples (as not every patient is biopsied at metastization or many are lost to follow-up, for example) and it is a motivation to explore these mechanisms further.
Additionally, tumor heterogeneity and microenvironment may play significant roles, that are rarely taken into account, mostly due to the methodological complexity and costs involved.

Regarding the methylation essays findings, a lower promoter methylation of *PSAT1* (the gene encoding phosphoserine aminotransferase), translating into a decreased expression, was an independent predictor for worse DFS. The original report of the *PSAT1* gene[216] actually demonstrated that it was upregulated in a dose-dependent way by progesterone and more weakly by estradiol in rabbit endometrium, illustrating a complex regulation hormonal mechanism, that may be time and tissue specific. Strengthening this complexity, are the recent findings that *PSAT1* was one of the target genes whose tissue specific mRNA expression (hypomethylation) was most significant after exposure to estradiol-17β in embryos[217]. Of note, the use of *PSAT1* methylation to predict BC recurrence has been previously reported in early diagnosed luminal-type BC. Furthermore, a correlation between high *PSAT1* methylation levels, on the one hand, and low *PSAT1* mRNAs levels and better outcome, on the other, were described[33]. Interestingly, high *PSAT1* expression was associated with poor outcome in nasopharyngeal carcinoma, a non-hormonal tumor[142]. These data are in accordance with our findings.

FOXA1 has been known to correlate with *ESR1* expression in breast tumors cell cultures – downregulation of *FOXA1* suppressed ERα binding to *TFF1* promoter which subsequently prevented hormone-induced reentry into the cell cycle[218]. We found that high *FOXA1* methylation levels independently predicted shorter DSS, a clinical finding that, to best of our knowledge, has not been reported, thus far. Remarkably, *FOXA1* expression was previously shown to associate with good prognosis and response to endocrine therapy in BC patients[116,117], and, thus, promoter methylation is the most likely mechanism underlying *FOXA1* downregulation in BC.

On the other hand, our results also suggest a panel of miRNAs might be feasible to test in primary tumor tissues to assess the likelihood of recurrence and resistance to ET in newly diagnosed luminal BC. Our cohort displayed higher miR-182-5p and miR-200b-3p levels compared to normal breast, whereas miR-30b-5p, miR-30c-5p, miR-182-5p and miR-200b-3p downregulation associated with decreased DMFS, which has potential clinical utility. Additionally, decreased expression of miR-200f, and related members, might be associated with EMT initiation, which is a known factor of aggressiveness (tissue evasion and metastasis).

Even in striking different populations, lower H3K27me3 expression appears to be associated to a poorer prognosis in estrogen receptor positive breast cancer. In our pre-endocrine treatment exposure cohort, with the longest follow-up in literature regarding clinical endpoints, the epigenetic biomarker H3K27me3 was associated to statistically significant worse prognosis (higher recurrence risk) and statistical tendency to a decreased survival in luminal A/B-like HER2-negative breast cancer. And yet, no association between the H3K27me3 expression and endocrine-treatment recurrence (P=0,685) was identified, despite being the association between PI3K and H3K27me3 that led us to investigate it in the first place – then why?

Some clues may reside in recent findings. In the last years, there has been significant development in the endocrine-resistant breast cancer treatment. The first endocrine-resistant combination with exemestane and mammalian target of rapamycin (mTOR) inhibitor everolimus was published in 2012, and it improved progression-free survival in patients albeit increased, and sometimes limiting, toxicity[219]. The cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors palbociclib, ribociclib and abemaciclib[220] in combination with endocrine therapy, have also demonstrated prolongation in progression-free survival and overall survival results among patients with hormone-receptor–

positive HER2-negative advanced breast cancer, in first and further lines of treatment, and are believed to overcome endocrine resistance.

Again, these mechanisms have been explored due to the association to PI3K/AKT/mTOR pathway. But for instance, in the PALOMA-3 trial the molecular pathway related to PI3K was specifically evaluated, in a present/absent fashion, but there was no significant difference between subgroups, as both benefited from the treatment vs placebo[221,222]. This underscores the limitations of using PI3K as a surrogate of endocrine-resistance or even as biomarker as probably many escape mechanisms and intermediaries are still unknown. Its importance is evident as it was one of the only three genes (TP53, PIK3CA and GATA3) whose somatic mutations incidence occurred over 10% across all breast cancers in TCGAN[8].

Interestingly, these drugs, specifically designed to target endocrine treatment resistance mechanisms, showed clinical benefit in first line endocrine-treatment naïve patients, such as ribociclib in MONALEESA-2 trial[223], and considering that not all patients were primary endocrine treatment resistant, maybe these mechanisms are operating sooner than expected. So far, no biomarker has been uncovered in the CD4/6 inhibitors setting.

Limitations/protocol proposal

Choosing luminal or luminal-like breast cancer as an area of research may be very limiting, and frustrating.

First, in order to obtain biological and statistically robust data, large number of patients are needed, not only to overcome patient heterogeneity, but because it is the most representative subtype of breast cancer (over 70% of breast cancers), so that the effect of confounding factors is less pronounced.

Secondly, most patients with localized breast cancer that are adequately treated, fortunately, may expect long disease-free intervals and overall survival, but that reduces immensely the number of events to correlate with biomarkers. Some of these events can happen decades apart from the initial diagnosis. There are also current practice protocols in which a patient is discharged after 5 years of follow-up, what greatly limits the follow-up, not to mention the patients that are lost to follow-up.

In a more practical perspective, when research protocols are integrated in the everyday clinical practice of health institutions, such as IPO Porto, the circuits for signaling key moments for biological sampling can be quite straining and time-consuming.
We developed a methodology to aggregate the date of clinical appointments, but it is naturally dependent on the availability of the researcher and based on schedules that may suffer changes.
We envisioned a protocol to allow informatically signaling of participating patients so that the lab technicians could identify the patients, and collect, store and transport the adequate biological samples to the Lab.

Future directions

1.

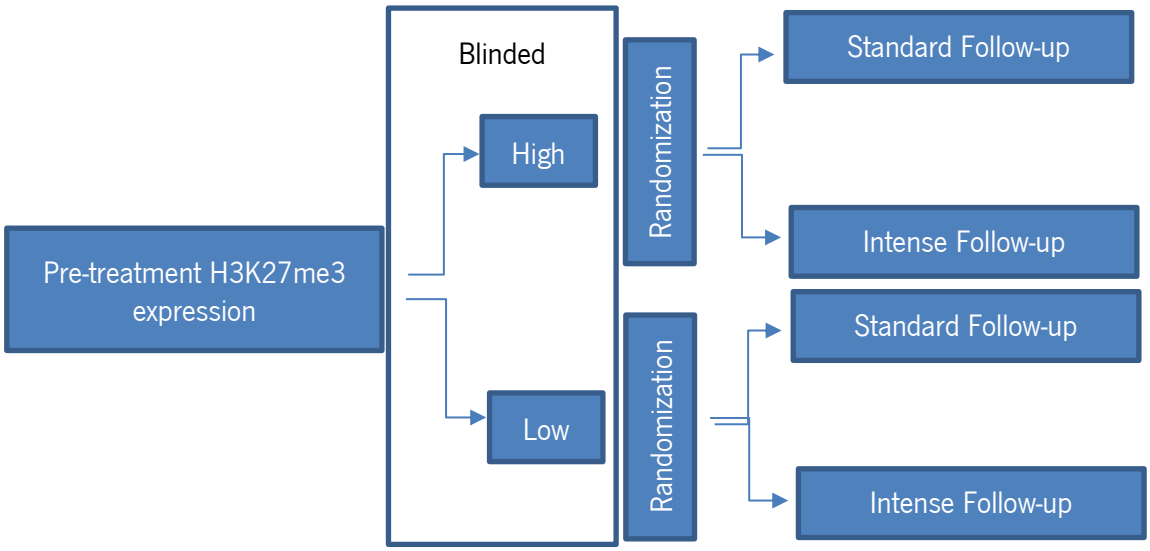
We described that high *FOXA1* methylation levels were associated to lower gene expression, and hence worse prognosis, associated to endocrine treatment resistance. We could test this interaction by reversing *FOXA1* methylation levels[224], and potentially reestablish endocrine sensitivity, if successful, anticipating a combined treatment modality as the ultimate goal.

2.

Considering the potential for biomarker of miR-30c-5p, which was downregulated in endocrine-resistant BC patients and independently predicted better ERFS in luminal B BC patients, we are presented with two challenges: increase our pool of luminal A-like patients and test prospectively the usefulness of the biomarker. Efforts are under way to test prospectively in liquid biopsies, the least invasive method of assessing disease status.

3.

We believe there is enough data to pursue a clinical trial regarding the impact of H3K27me3 in a prospective mode, as (lower) expression was associated with disease behavior. We could envision a four-arm trial, with the following scheme:



Scheme depicting protocol proposal.

The hypothesis is that lower expression might benefit from intense follow-up protocol (clinical visits according to year of follow-up; annual breast ultra-sound and mammogram plus annual bone scintigraphy plus annual CT scan), and conversely, high expression being associated to better prognosis, a standard protocol (clinical visits according to year of follow-up; annual breast ultra-sound and mammogram) would be enough (and ethically acceptable since it corresponds to current clinical practice). The expression of H3K27me3 should be blinded to investigators, so that there is no selection bias. Population: > 18 years old consenting female patients after curative intent treatment (incl. chemotherapy and/or endocrine therapy and/or radiotherapy). Randomization 1:1. Stratification by age (≤ 60 vs > 60), subtype (Luminal A-like vs Luminal B-like), disease stage (II vs III) and grade (G1-2 vs G3). No HER2-positive or neoadjuvant patients allowed. The primary endpoints are: disease-free survival (DFS) and Overall Survival (OS).

Population enrichment should be expected, namely including only stage II or III patients (in order to have more events). A long follow-up time would be necessary (at least 10 years).

Conclusions

We intended to explore the potential of epigenetic biomarkers, that despite becoming increasingly mainstream, their practical application is often limited by inaccurate or unstandardized methodologies. We wanted to focus especially in the area of endocrine-resistant breast cancer that is epidemiologically significant, and yet lacks diagnostic or monitorization markers (namely non-invasive) – thus an area of unmet needs. Additionally, our ultimate goal was to identify potential targets to evaluate prospectively and using less invasive ways of obtaining biological samples, such as blood or urine. We explored different epigenetic and unrelated epigenetics markers and concluded that the clinical definition of endocrine-resistance, although practical, may be biologically insufficient. The mechanisms of endocrine-resistance remain elusive, what instead of considering a flaw, may be interpreted as a challenge to continue researching.

Upon completing the PhD program, we believe we have made the field advance further, especially considering the individualized oncology paradigm, going a little step further in potentially contributing to the lives of our patients, and this is just the beginning of the epigenetics in the history of breast cancer.

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Annexes

(published articles)

Mário Fontes-Sousa, Maria Amorim, Sofia Salta, Susana Palma de Sousa, Rui Henrique, Carmen Jerónimo. **Predicting resistance to endocrine therapy in breast cancer: it's time for epigenetic biomarkers**. *Oncology Reports*. 2019; 41:1431-1438.

Sofia Salta, Sandra P. Nunes, Mário Fontes-Sousa, Paula Lopes, Micaela Freitas, Margarida Caldas, Luís Antunes, Fernando Castro, Pedro Antunes, Susana Palma de Sousa, Rui Henrique, Carmen Jerónimo. **A DNA methylation-based test for breast cancer detection in circulating cell-free DNA**. J Clin Med. 2018 Nov; 7(11): 420.

(published poster abstract, international meeting)

Mário Fontes Sousa, Silvana Lobo, Sofia Salta, Paula Lopes, João Lobo, Susana Sousa, Rui Henrique, Carmen Jerónimo: "**Epigenetic biomarkers in breast cancer: preliminary results from H3K27m3 assessment in endocrine-treatment resistant breast cancers**" *Annals of Oncology* (2017) 28 (suppl_5)

Oral communication (international) – Certificate of presentation

- Mário Fontes Sousa: "**Epigenetic markers for Endocrine-treatment resistant breast cancer cell niche: towards an individual and targeted approach**" presented at *StratCan Interactive Summer School*, organization Karolinska Institutet, 14-17/06/2016; Stockholm, Sweden.

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Predicting resistance to endocrine therapy in breast cancer: It's time for epigenetic biomarkers (Review)

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Abstract. Notwithstanding the marked progress in breast cancer (BC) management, it still constitutes the most common malignancy in women and a major cause of morbidity and mortality, thus remaining a major health issue worldwide. Most BC cases are hormone receptor (HR) positive (luminal A or B molecular subtypes) and endocrine treatment (ET) is an important therapeutic modality at all disease stages. Nevertheless, despite substantial improvements in BC patient outcome, effectiveness of ET is limited, as up to 40% of patients eventually relapse or progress and endocrine resistant BC has a less favorable prognosis and constitutes a therapeutic challenge. The biological mechanisms underlying endocrine resistance are, however, still poorly understood. In this review, we focused on data regarding the main epigenetic mechanisms associated with the development of endocrine treated-resistant BC described so far, including alterations in DNA methylation, non-coding RNAs, chromatin remodeling, post-translational histone modifications and histone variants. Notably, specific epigenetic alterations have been characterized in this subset of breast tumors and may be of clinical value for individualized patient management in the future.

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Key words: breast cancer, epigenetic biomarkers, endocrine therapy, drug resistance, patient management

Contents

1. Introduction

Most breast cancers (BC), over 2/3 of cases, express estrogen (ER) and progesterone (PR) receptors (1). This is extremely important since these are used as biomarkers for subtype classification, with implications in choice of treatment and prognosis in BC patients (2). Notably, endocrine therapies (ET) have been successfully used for treating ER positive BC patients with significant impact in patient outcome. Several endocrine drugs are approved for BC treatment, most notably tamoxifen, toremifene, anastrozole, letrozole, exemestane and fulvestrant, which may be used in different clinical contexts, such as chemoprophylaxis, neoadjuvant, adjuvant and palliative treatments. However, the effectiveness of ET is limited as up to 40% of patients may experience disease recurrence while on ET adjuvant treatment (1,3). Moreover, in the metastatic setting, acquired resistance to ET is virtually an universal feature, and is clinically defined in accordance to the 3rd ESO-ESMO International Consensus Guidelines (4) and many efforts have been made to understand the mechanisms involved in acquisition of acquired resistance to ET. These, however, remain mostly elusive and no biomarkers have been validated in this setting despite intense drug development and approval.

Epigenetics may be defined as mechanisms that regulate cell fate specifications, while the DNA remains unchanged (5). Some of these mechanisms include DNA methylation, non-coding RNAs, chromatin remodeling and histone post-translational modifications or variants. Collectively, these components constitute the epigenome machinery whose role is to define

which information is available for transcription and for translation (5). DNA methylation is performed by specific enzymes, the DNA methyltransferases (DNMTs) that introduce a methyl group at the 5' position of a cytosine ring inside CpG dinucleotides (6). Globally, promoter methylation of genes is associated with transcription inhibition (6). Furthermore, the N-terminal tails of histones may undergo post-translation modifications that subsequently impact the chromatin structure (7). The most well-studied histone post-translation modifications are histone acetylation and histone methylation. Histone acetylation is associated with gene expression and is carried out by histone acetyltransferases (HATs), while histone deacetylation is accomplished by histone deacetylases (HDACs) (7). Histone methylation, which depending on the residue and the number of methyl groups may lead either to transcription repression or activation (8), is catalyzed by histone methyltransferases (HMTs), while histone demethylation is performed by histone demethylases (HDMs) (7). In addition to post-translational histone modifications, histone variants that can replace canonical histones are an additional level of epigenetic complexity, and contribute to the shaping of the chromatin structure.

Non-coding RNAs (ncRNAs) comprise a hidden layer of internal signals that control various levels of gene expression (9). Among these, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are the most frequently reported in BC. lncRNAs are ncRNA molecules usually longer than 200 nucleotides that do not fit into known classes of small or structural RNAs (9) and may act as protein-DNA or protein-protein scaffolds, miRNA sponges, protein decoys, or regulators of translation (10). miRNAs are endogenous, small non-coding single-stranded RNAs with ~22 nucleotides in length, that exert a finely tuned regulation of gene expression at the post-transcriptional level (11) by binding to mRNA targets, inducing its cleavage or repressing its translation (11).

Over the last few years, convincing data has suggested that altered epigenetic regulation may be involved in tumor initiation, progression and cancer resistance to therapy, including endocrine resistance, particularly in BC. For instance, ER expression is currently one of the foremost predictive biomarkers of response to ET, and altered expression of ER may be due to hypermethylation of CpG islands within its promoter, increased histone deacetylase activity in the ESR1 promoter or translational repression by miRNAs (12). Since ER was found to be deleted in only 15-20% of endocrine-resistant BC, several epigenetic mechanisms may be involved in the development of endocrine treatment-resistance (3), and some of these are depicted in Fig. 1.

Our objective was to review the published evidence regarding epigenetic mechanisms associated to ET resistance in BC, as it may be considered an emerging subject and worth special focus.

2. Evidence acquisition

For the selection of the most relevant bibliography, we conducted a PubMed® search using the terms 'endocrine resistance', 'breast cancer' and 'epigenetic mechanisms'. Reference lists from key articles were also searched for additional relevant data. The criteria for article selection were: written in English, central theme based on ET resistance on BC and epigenetic mechanisms. Original studies were selected based on the detail of analysis, mechanistic support of data, novelty, and potential clinical usefulness of the findings. Chemotherapy/radiotherapy-resistance, HER2-enriched subtype or 'triple negative' BC citations were excluded for being outside the scope of this review.

DNA methylation. DNA methylation is one of the most common epigenetic changes and has been reported in multiple tumors, including BC (9,13). This epigenetic alteration is inherently stable and has been proposed as a promising cancer biomarker in multiple cancers since it can be sampled from less invasive sources such as liquid biopsies (plasma or urine) (13-15). Thus, the role of DNA methylation as a predictor of ET resistance is a field of growing interest and has become the focus of several research teams (16-18) since it may improve BC patients' risk stratification.

Notably, Stone *et al* reported that in endocrine treated-resistant cell lines, DNA hypermethylation occurs predominantly at estrogen-responsive enhancers, leading to reduced ER binding and subsequently to expression downregulation. Furthermore, luminal subtype BC patients with relapsed disease exhibited significantly higher methylation levels at all enhancer loci studied (19). By comparing anti-estrogen-resistant cell lines with the parental sensitive cell line, DNA methylation of the promoter region of genes was also suggested to play a role in the emergence of endocrine resistance (17,20) (Table I). Multicenter studies, including several cohorts of BC patients were able to confirm these findings. Specifically, PITX2 methylation levels were consistently identified as a valuable biomarker to predict outcome in low-risk BC patients (ER-positive, node-negative) treated with surgery followed by adjuvant tamoxifen (21,22). Nevertheless, multiple validations are still required before the implementation of these markers in the clinical setting (Table I). Thus, to date, no clinical trials have assessed the clinical relevance of these candidate biomarkers.

Non-coding RNAs. As previously mentioned, decreased ER expression may be due to post-transcription regulation of miRNAs, including that of miR-221/222, whose overexpression has been associated with resistance to tamoxifen (23,24) and fulvestrant (25). Conversely, miR-342-3p levels were revealed to be positively correlated with ER mRNA expression in human BC and associated with tamoxifen sensitivity (26,27). miRNAs that regulate growth, survival, apoptosis, epithelial-mesenchymal transition (EMT) and metastasis of BC cells may be implicated in loss of responsiveness to ET. In particular, PTEN downregulation due to specific miRNAs, permitting abnormal Pi3K/Akt pathway activation, promote estrogen-independent growth and survival of BC cells leading to endocrine treatment resistance (28,29).

Several clinical trials are currently ongoing to evaluate the role of miRNAs as predictive biomarkers in BC. Specifically, trials such as NCT01231386 and NCT01722851, aim to identify circulating miRNAs aiding at the identification of biomarkers of early response to neoadjuvant therapy, including ET, which may be used as potential targets for personalized therapies. Conversely, the NCT01612871 trial was set to explore a panel of circulating miRNAs that could aid to monitor the disease status of the patient while on adjuvant ET (30-32).

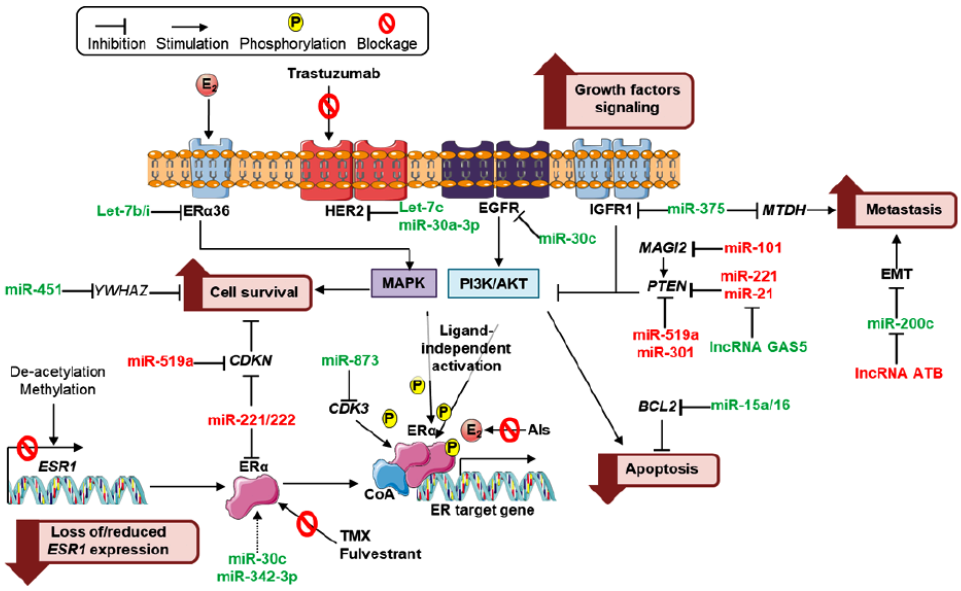

Figure 1. NcRNAs and their established targets involved in endocrine resistance. The ncRNAs and their targets involved in several mechanisms associated with endocrine resistance, along with their functional implication (in pink boxes), including loss of/reduced ESR1 expression, alternative growth-factor signaling inducing downstream signaling, including PI3K/Akt and MAPK signaling pathways, dysregulation of cell survival and apoptosis pathways, and increased metastasis. NcRNAs that confer sensitivity and resistance to endocrine therapies are depicted in green and red, respectively. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; EGFR, epidermal growth factor receptor; IGFR1, insulin-like growth factor 1 receptor; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ; MTDH, metadherin; MAGI2, membrane-associated guanylate kinase inverted 2; PTEN, phosphatase and tensin homolog; EMT, epithelial-mesenchymal transition; CDKN, cyclin-dependent kinase inhibitor; CDK3, cyclin dependent kinase 3; BRCAL2, B-cell CLL/lymphoma 2; PI3K/AKT, phosphoinositide 3-kinase/protein kinase B; ESR1, estrogen receptor 1; TMX, tamoxifen; AIs-aromatase inhibitors; E2, estradiol; miR, microRNA.

Table I. DNA methylation of the promoter region of genes as predictive biomarkers to different modalities of endocrine therapies along with their role and the biological samples used in each study.

Biomarker	Role	Agent	Samples	Ref.
PTEN	Hypermethylation is associated with resistance	TMX	Cell lines	(47)
PTGER4	Hypomethylation is associated with resistance	EDT		(48)
CDK10	Hypermethylation is associated with shorter PFS and OS	TMX	Cell lines and	(49)
HOXC10	Hypermethylation is associated with resistance	EDT, AIs and TMX	tumor tissues	(50)
ESR1 CYP1B1	High methylation levels are associated with a better outcome	TMX	Tumor tissues	(16)
ID4	Hypomethylation is associated with resistance			(51)
NAT1	Hypermethylation is associated with resistance			(52)
PITX2	Hypermethylation is associated with worse outcome and shorter MFS			(21,22,53)
PR	Hypermethylation is associated with resistance			(54)
PSAT1	Hypermethylation is associated with good clinical benefit			(55)

PFS, progression-free survival; OS, overall survival; MFS, metastasis-free survival; TMX, tamoxifen; AIs, aromatase inhibitors; EDT, estrogen deprivation therapy; PTEN, phosphatase and tensin homolog; PTGER4, prostaglandin E receptor 4; CDK10, cyclin dependent kinase 10; HOXC10, homeobox C10; BRCA1, BRCA1 DNA repair associated; ESR1, estrogen receptor 1; CYP1B1, cytochrome P450 family 1 subfamily B member 1; ID4, inhibitor of DNA binding 4 HLH protein; NAT1, N-acetyltransferase 1; PITX2, paired like homeodomain 2; PR, progesterone receptor; PSAT1, phosphoserine aminotransferase 1.

Table II. Non-coding RNAs involved in response (sensitivity/resistance) to different modalities of endocrine therapies along with their putative targets/mechanism and the biological samples used in each study.

ET	Role	miRNA	Putative target	Agent	Samples	Refs.
AntiE	Sensitivity	miR-375	*MTDH*	TMX	Cell lines	(56)
		miR-873	*CDK3*			(57)
		miR-320a	*ARPP19, ESRRG*			(58)
		Let-7b/i	*ESR1* (ER-α36 variant)			(59)
		miR-451	*YWHAZ*			(60)
		miR-17/20	*CCND1*			(61)
		miR-148a	*ALCAM*			(62)
		miR-152				(63)
		miR-200c/b	*ZEB1/2*	TMX and FULV		(64)
		miR-15a/16	*BRCAL2*	TMX	Cell lines and xenografts	(26)
		miR-342-3p	*BMP7, GEMIN4*		Cell lines and tumor tissues	(65)
		miR-26a	*EZH2*		Tumor tissues	(66)
		miR-30c	*EGFR*			(67)
		miR-10a	-			(68)
		miR-126				(35)
	Resistance	miR-10b	*HDAC4*	TMX	Cell lines	(29)
		lncRNA DSCAM-AS1	Binding to the hnRNPL protein	Tumor tissue and cell lines		(34)
		miR-519a	*CDKN1A, PTEN, RB1*			(23,25,69)
		lncRNA BRCAAR4	-			(28)
		miR-221/222	*ESR1, CDKN1B, CTNNB1*	TMX and FULV		(70)
		miR-301	*FOXF2, PTEN, BBRCA3iso2, COL2A1*	TMX	Tumor tissue, cell lines and xenografts	(71)
		miR-155	*SOCS6*			(72)
		miR-210	*EFNA3, E2F3, RAD52, FGFRL1, MET*		Tumor tissue	(73)
AIs	Sensitivity	Let-7f	*CYP19A1*	LET	Cell lines	(74)
		miR-125b	*ERBB2*	LET and ANA	Tumor tissues and cell lines	(75)
		let-7c				
	Resistance	miR-128a	*TGFBR1*	LET	Cell lines	
		miR-181a	*BRCAL2L11*		Cell lines, xenografts and tumor tissue	

miR, microRNA; lncRNA, long non-coding RNA; ET, endocrine therapies; AntiE, anti estrogen; AIs, aromatase inhibitors; ANA, anastrozole; FULV, fulvestrant; DSCAM-AS1, DSCAM antisense RNA 1; BRCAAR4, breast cancer anti-estrogen resistance 4; MTDH, metadherin; CDK, cyclin-dependent kinase; ARPP19, cAMP-regulated phosphoprotein 19; ESRRG, estrogen related receptor gamma; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ; CCND1, cyclin D1; ALCAM, activated leukocyte cell adhesion molecule; ZEB, zinc finger E-box-binding homeobox; BRCAL-2, B-cell lymphoma 2; BMP7, bone morphogenetic protein 7; GEMIN4, gem (nuclear organelle)-associated protein 4; EZH2, enhancer of zeste homolog 2; EGFR, epidermal growth factor receptor; HDAC4, histone deacetylase 4; HnRNPL, heterogeneous nuclear ribonucleoprotein L; CDKN, cyclin-dependent kinase inhibitor ; PTEN, phosphatase and tensin homolog; RB1, retinoblastoma 1; ESR1, estrogen receptor 1; CTNNB1, catenin β 1; FOXF2, forkhead box F2; BBRCA3iso-2, BRCAL2 binding component 3 isoform 2; COL2A1, collagen type II alpha 1; SOCS, suppressor of cytokine signaling; EFNA3, ephrin A3; E2F3, E2F transcription factor 3; RAD52, RAD52 homolog DNA repair protein; FGFRL1, fibroblast growth factor receptor-like 1; MET, hepatocyte growth factor receptor; CYP19A1, cytochrome P450 family 19 subfamily A member 1; ERBB2, erb-b2 receptor tyrosine kinase 2; TGFBR1, transforming growth factor β-receptor 1; BRCAL2L11, BRCAL2 like 11; ZNF217, zinc finger protein 217.

Table III. Chromatin remodeling, post-translational histone modifications and histone variants involved in response (sensitivity/resistance) to endocrine therapies along with their putative epigenetic mechanism and role in response.

Biomarker	Epigenetic mechanism	Role	Samples	Refs.
H3K27me3 profiles	Post-translational histone modification	H3K27me3 profile predicts the treatment outcomes for first-line AIs	Tumor tissues	(38)
PBX1	Chromatin remodeling	Resistance to ET	Cell lines and tumor tissues	(46)
HDAC6	Post-translational histone modification	Sensitivity to TMX by deacetylation of alpha-tubulin		(76,77)
HIST1H2BE	Histone variant	Overexpressed in AI-resistant tumors/cell lines compared to AI-sensitive tumors/cell lines		(45)
NSD2	Post-translational histone modification	Histone H3K36 methyltransferase that confers resistance to TAM by upregulating key glucose metabolic enzyme genes		(37)
H3K27me3 demethylation	Post-translational histone modification	Resistance to ET due to BrCal-2 expression	Cell lines	(78)
H2A.Z	Histone variant	Increased H2A.Z expression promotes cellular proliferation, namely when E2 levels are low and during TMX treatment		(44)

PBX1, PBX homeobox 1; HDAC6, histone deacetylase 6; HIST1H2BE, histone cluster 1 H2B family member E; NSD2, nuclear receptor binding SET domain protein 2; H2A.Z, H2A histone family member Z; ET, endrocrine therapies; TMX, tamoxifen; AIs, aromatase inhibitors.

lncRNAs have also been associated with endocrine treatment resistance. Particularly, lncRNAs, breast cancer anti-estrogen resistance 4 (BRCAAR4) overexpression (33,34) and DSCAM antisense RNA 1 (DSCAM-AS1) (35), which contains an ER promoter binding motif, have been revealed to predict tamoxifen resistance in primary BC (Table II and Fig. 1).

Chromatin remodeling, post-translational histone modifications and histone variants. Histone post-translation modifications induce chromatin landscape changes that subsequently favor ER repression, thus promoting other signaling pathways that could lead to endocrine resistance, as exemplified by Magnani *et al* that revealed how the genome's accessibility is altered in drug-resistant vs. drug-responsive BC cells (36). Recently, expression of the H3K36 methyltransferase NSD2 was found to be higher in tamoxifen-resistant BC cell lines, associated with disease recurrence and worse survival (37). Furthermore, H3K37me3 profiles enabled the identification of patients with poor outcome after aromatase inhibitor (AI) treatment (38).

Furthermore, it was recently demonstrated that transcription repression performed by ER co-repressors confer tamoxifen sensitivity through recruitment of HDACs to DNA (39). This evidence suggests that loss of ER co-repressors may sensitize BC cells to the cytotoxic effects of HDACs inhibitors (HDACi). Notably, some clinical trials have demonstrated that HDACi appears to re-establish sensitivity to anti-estrogens in a subset of endocrine treated-resistant tumors (40,41). In addition, the ENCORE-301, a randomized phase II trial (41) tested entinostat, an oral HDACi, in the endocrine-resistance, more specifically AI in post-menopausal women. The results revealed modest improvement in PFS but much greater improvement in overall survival (OS)-median OS improved to 28.1 months in the experimental arm vs. 19.8 months (HR, 0.59; 95% CI, 0.36 to 0.97; P=0.036). Ongoing clinical trials are further testing entinostat in monotherapy or in combination. Moreover, in custom-generated tamoxifen resistant cell lines, treatment with HDACi re-established sensitivity to tamoxifen through significant Bcl-2 downregulation, growth arrest and apoptosis (42).

Histone variants, such as H2A.Z, an H2A variant, have been shown to be intimately linked to estrogen signaling (43). Notably, a study has already provided a link (yet uncharacterized) between H2A.Z and endocrine resistance by revealing that H2A.Z overexpression led to increased estrogen-independent proliferation (44). Furthermore, another study demonstrated that the histone HIST1H2BE, an H2B variant, was overexpressed not only in endocrine-resistant cell lines, but also in AI-treated tumors from patients which relapsed compared to those that did not (45).

Furthermore, an emerging class of transcription factors named 'pioneer factors', appear to be key players in shaping chromatin structure through binding to chromatin prior to transcription factors, making it accessible for transcription factors, together with histone post-translation modifications and histone variants [68-70]. PBX1 is an example of this class-its expression levels have been associated with reduced metastasis-free survival in ER-positive BC patients (46). Furthermore, a gene expression signature based on NOTCH-PBX1 activity was found to discriminate BC patients that are responsive to ET from those which are not. Notably, PBX1 knockdown was sufficient to arrest ER-resistant BC cell growth (36).

These and other chromatin remodeling complexes associated with endocrine resistance are summarized in Table III along with their putative role and the biological samples in which they were characterized.

3. Conclusion

Notwithstanding the prevalence of endocrine treatment resistance in BC, predictive and diagnostic biomarkers in this setting are markedly lacking in clinical practice. In this review, we summarized emerging evidence that epigenetic mechanisms may prove useful for this purpose. These would perform as non-invasive predictive biomarkers of treatment-resistance, providing affordable and sequential monitoring during the course of treatment. The concept of early detection (preclinical) of therapy resistance is compelling, as it could assist clinicians in choosing the most appropriate individualized therapeutic strategy.

Furthermore, some epigenetic modifications in addition to conveying information concerning prediction of response, are also appealingly targetable, in particular due to their reversible nature. The clinical usefulness of these findings, however, is still elusive, mostly due to lack of standardization in methodology, limiting reproducibility.

Promising results have been arising in clinically meaningful trials, such as ENCORE-301. A useful approach would be the integration of the candidate biomarkers into a panel, enabling its validation in a clinical trial setting. Hopefully, this will be accomplished in the near future.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

MFS, SPDS, RH and CJ conceived and designed the review. MFS, MA, SS performed the literature search and wrote the manuscript. SPDS, RH and CJ reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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A DNA Methylation-Based Test for Breast Cancer Detection in Circulating Cell-Free DNA

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Abstract: Background: Breast cancer (BrC) is the most frequent neoplasm in women. New biomarkers, including aberrant DNA methylation, may improve BrC management. Herein, we evaluated the detection and prognostic performance of seven genes' promoter methylation (*APC*, *BRCA1*, *CCND2*, *FOXA1*, *PSAT1*, *RASSF1A* and *SCGB3A1*). Methods: Methylation levels were assessed in primary BrC tissues by quantitative methylation-specific polymerase chain reaction (QMSP) and in circulating cell-free DNA (ccfDNA) by multiplex QMSP from two independent cohorts of patients (Cohort #1, $n = 137$; and Cohort #2, $n = 44$). Receiver operating characteristic (ROC) curves were constructed, and log-rank test and Cox regression were performed to assess the prognostic value of genes' methylation levels. Results: The gene-panel *APC*, *FOXA1*, *RASSF1A*, *SCGB3A1* discriminated normal from cancerous tissue with high accuracy (95.55%). In multivariable analysis, high *PSAT1*-methylation levels [>percentile 75 (P75)] associated with longer disease-free survival, whereas higher *FOXA1*-methylation levels (>P75) associated with shorter disease-specific survival. The best performing panel in ccfDNA (*APC*, *FOXA1* and *RASSF1A*) disclosed a sensitivity, specificity and accuracy over 70%. Conclusions: This approach enables BrC accurate diagnosis and prognostic stratification in tissue samples, and allows for early detection in liquid biopsies, thus suggesting a putative value for patient management.

Keywords: breast cancer; DNA methylation; epigenetic biomarker; Cell-free DNA; liquid biopsy; diagnosis; prognosis

1. Introduction

Breast cancer (BrC) is the most common and lethal cancer in women worldwide, corresponding to 25% of all cancers in females [1]. Implementation of mammography-based BrC screening increase the proportion of cancers detected at an early-stage, contributing to a decrease in BrC-related mortality [2]. Nevertheless, this screening strategy is hampered by frequent false positive results, leading to overdiagnosis. Furthermore, its usefulness in women with dense breast tissue remains controversial [3,4]. Although grade, stage, histological, and molecular subtype are currently used to risk-stratify BrC patients, divergent outcomes and therapeutic responses are common [5]. Furthermore, currently used prognostic and predictive biomarkers, such as hormone receptor or *Erb-b2 receptor tyrosine kinase* 2 (ERBB2) status have a limited power to predict recurrence and therapeutic response [6]. Hence, despite all improvements in early detection, patients' stratification and treatment, BrC remains the foremost cause of cancer-related mortality among women, mostly due to disease recurrence and/or metastasis development [1]. In recent years, several biomarkers for early diagnosis have been proposed. Yet, despite their less invasive nature [7–9], improved tumor characterization [10–13] or better patient stratification [9,14] have been proposed, but with limited success.

Because aberrant DNA methylation is considered a cancer-associated event, characterization of tumor-specific methylome has become the focus of multiple studies [15]. Interestingly, aberrant promoter methylation of several tumor suppressor genes was found in BrC precursor lesions, indicating that DNA methylation is an early event in breast carcinogenesis [16–19]. Moreover, DNA methylation has been proposed as a valuable cancer detection and prognosis biomarker owing to its link with tissue-specific gene silencing [9,20–23]. Tumor-specific DNA methylation may also be detected in circulating cell-free DNA (ccfDNA) from liquid biopsies [24], and its potential for early cancer detection was already reported [23,25–27], representing a minimal-invasive test [28]. Herein, we aimed to define a DNA methylation-based test to improve or complement early detection strategies and to enable better BrC patients' prognostic stratification. Thus, methylation levels of seven gene promoters [*Adenomatosis polyposis coli (APC), BRCA1, DNA repair associated (BRCA1), Cyclin D2 (CCND2), Fork-head box A1 (FOXA1), Phosphoserine Aminotransferase 1 (PSAT1), Ras association domain family 1 isoform A (RASSF1A)* and *Secretoglobin family 3A member 1 (SCGB3A1)*] previously reported as dysregulated in BrC and conveying diagnostic and/or prognostic information [7–9,14,29] were firstly assessed in tissue for confirmation of cancer-specificity and prognostic significance. Then, the best performing gene panel was tested in plasma ccfDNA to determine its BrC detection performance.

2. Experimental Section

2.1. Patients and Samples Collection

Two independent cohorts of BrC patients were included in this study. Cohort #1 comprised 137 patients, primarily submitted to surgery, from 1996 to 2001, at the Portuguese Oncology Institute of Porto (IPO Porto), with frozen tissue available. For control purposes, normal breast tissue (NBr) was collected from reduction mammoplasty of contralateral breast of BrC in patients without BrC hereditary syndrome. After surgical resection and examination, samples were immediately frozen at −80 °C. Relevant clinical and pathological data was retrieved from the patients' clinical charts. Five µm frozen sections were cut and stained by hematoxilin-eosin for histological evaluation by an experienced pathologist.

Cohort #2 was composed of 44 BrC patients, primarily diagnosed between 2015 and 2017 at IPO Porto, which voluntarily provided blood samples prior any treatment. For control purposes, blood samples were also obtained from 39 asymptomatic controls (AC). The blood samples were collected in two EDTA tubes and centrifuged at 2000 rpm for 10 min at 4 °C for plasma separation. Plasma was immediately frozen at −80 °C until further use. Relevant clinical data was collected from clinical records.

This study was approved by the institutional review board (Comissão de Ética para a Saúde—CES 120/2015) of IPO Porto, Portugal. All patients and controls enrolled had signed an informed consent.

2.2. Immunohistochemistry

Immunohistochemistry (IHC) allowed for identification of BrC molecular subtype of each case in Cohort #1, using corresponding formalin-fixed paraffin-embedded tissue. Commercially available antibodies for Estrogen Receptor (ER) (Clone 6F11, mouse, Leica, Newcastle, UK), Progesterone Receptor (PR) (Clone 16, mouse, Leica, Newcastle, UK), ERBB2 (Clone 4B5, rabbit, Roche, Tucson, AZ, USA) and Ki67 (Clone MIB-1, mouse, Dako, Glostrup, Denmark) were used. IHC was carried out in BenchMark ULTRA (Ventana, Roche, Tucson, AZ, USA) using ultraView Universal DAB Detection Kit (Ventana, Roche, Tucson, AZ, USA) according to manufacturer's instructions.

IHC staining was evaluated by an experienced pathologist according to College of American Pathologists' recommendations. Each case was categorized according to European Society for Medical Oncology (ESMO) guidelines [6]. Cutoff values were set for Ki67 (high proliferative rate if $\geq$15% positive cells) and PR (high expression if $\geq$25% positive cells).

2.3. DNA Extraction

Genomic DNA was extracted from tumor and normal tissues by the phenol–chlorophorm method at pH 8, as previously described [30]. Samples were first submitted to overnight digestion in a bath at 55°C, using buffer solution SE (75 mM NaCl; 25 mM EDTA), SDS 10% and proteinase K, 20 mg/mL (Sigma-Aldrich®, Schnelldorf, Germany). After digestion, extraction was performed with phenol/chloroform (Sigma-Aldrich®, Schnelldorf, Germany, Merck, Darmstadt, Germany) followed by precipitation with 100% ethanol.

CcfDNA was extracted from 2 mL of plasma using QIAamp MinElute ccfDNA (Qiagen, Hilden, Germany), according to manufacturers' recommendations. The ccfDNA was eluted in 20 µL of ultra-clean water (Qiagen, Hilden, Germany).

2.4. Bisulfite Treatment and Whole Genome Amplification (WGA)

Bisulfite conversion was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA), according to manufacturer's instructions. One µg of DNA obtained from fresh frozen sections was used. Modified DNA was eluted with 60 µL of sterile distilled water. In plasma samples, 20 µL of ccfDNA was used for bisulfite modification. Modified ccfDNA was eluted in 10 µL of sterile distilled water. For control purposes, 1 µg of CpGenome™ Universal Methylated DNA (Millipore, Temecula, CA, USA) was also modified, according to the method described above and eluted in 20 µL of M-elution buffer. All samples were stored at −80 °C until further use. Whole-genome amplification of sodium bisulfite modified ccfDNA was carried out using the EpiTect Whole Bisulfitome Kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations. The amplified ccfDNA final volume was 65 µL.

2.5. Quantitative Methylation-Specific Polymerase Chain Reaction (QMSP)

Modified DNA was used as template for QMSP. Overall, seven gene promoters (*APC*, *BRCA1*, *CCND2*, *FOXA1*, *PSAT1*, *RASSF1A* and *SCGB3A1*) were assessed in BrC tissue samples. The primers used specifically amplify methylated bisulfite converted complementary sequences and are listed in Supplementary File 1 (Table S1). *β-actin* (*ACTβ*) was used as reference gene to normalize for DNA input in each sample [9]. Reactions were performed in 96-well plates using Applied Biosystems 7500 RealTime System (Applied Biosystems, Perkin Elmer, CA, USA) using 2 µL of modified DNA and 5 µL of 2X KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA). All the samples were run in triplicates and the melting curves were obtained for each case/gene. Owing to the limited amount of ccfDNA plasma samples, three gene promoters were selected (*APC*, *FOXA1*, *RASSF1A*) in addition to the reference gene (*ACTβ*) for assessment of methylation using

multiplex QMSP with TaqMan probes having different fluorescent reporters and Xpert Fast Probe (GRISP, Porto, Portugal), whereas *SCGB3A1* methylation levels were assessed in a separated QMSP reaction (Supplementary File 1—Table S2).

Modified CpGenome™ Universal Methylated DNA was used in each plate to generate a standard curve, allowing for quantification, as well as to ascertain polymerase chain reaction (PCR) efficiency. All plates disclosed efficiency values between 90–100%. For each gene, relative methylation levels in each sample were determined by the ratio of the mean quantity obtained by QMSP analysis for each gene and the respective value of the internal reference gene (*ACTβ*), multiplied by 1000 for easier tabulation (methylation level = target gene/reference gene × 1000).

2.6. Statistical Analysis

The frequency, median and interquartile range of promoter methylation levels of normal tissue/control samples and plasma samples were determined. Non-parametric tests were performed to determine statistical significance in all the comparisons made. Specifically, Kruskall-Wallis test was used for comparisons between three or more groups, whereas Mann-Whitney U test was used for comparisons between two groups.

For each gene, receiving operating characteristic (ROC) curves were built to assess respective performance as tumor biomarker. Moreover, specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), accuracy were determined. For this purpose, the cut-off established was the highest value obtained by the ROC curve analysis [sensitivity + (1 − specificity)]. To categorize samples as methylated or unmethylated, a cut-off value was chosen based on Youden's J index obtained by the ROC curve analysis for each gene [31,32]. For combination of markers, the cases were considered positive if at least one of the individual markers was positive. Logistic regression models were built in order to evaluate the potential of confounding factors as age in our BrC detection model.

Spearman nonparametric correlation test was used to assess the association of methylation levels and age. Disease-specific survival curves and disease-free survival curves (Kaplan–Meier with log rank test) were computed for standard clinicopathological variables and for categorized methylation status. A Cox-regression model comprising all significant variables (multivariable model) was computed to assess the relative contribution of each variable to the follow-up status.

Two-tailed *p*-values were derived from statistical tests, using a computer assisted program (SPSS Version 20.0, Chicago, IL, USA), and results were considered statistically significant at $p < 0.05$, with Bonferroni's correction for multiple tests, when applicable. Graphics were assembled using GraphPad 6 Prism (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Clinical and Pathological Data of Tissue Cohort

Relevant clinical and pathological data are presented in Supplementary File 1 (Tables S3 and S4). Although patients' age did not differ between the two cohorts, a significant difference was observed between BrC patients and controls age (Cohort #1: $p = 0.007$, Cohort #2: $p = 0.001$).

3.2. Assessment of BrC and NBr Tissue Samples Methylation Levels

To assess cancer-specificity, promoters' methylation levels of *APC*, *BRCA1*, *CCND2*, *FOXA1*, *PSAT1*, *RASSF1A* and *SCGB3A1* were evaluated in Cohort #1 (BrC and NBr tissue samples). Overall, BrC samples displayed higher *APC*, *CCND2*, *FOXA1*, *PSAT1*, *RASSF1A*, and *SCGB3A1* methylation levels than NrB samples ($p < 0.001$ for all genes, Supplementary File 1—Table S5), whereas no differences were found for *BRCA1*, which was not further tested (Supplementary File 1—Table S5).

Subsequently, ROC curve analysis was performed, and an empirical cutoff value was determined for each gene (*APC*: 16.99, *CCND2*: 0.4171 for, *FOXA1*: 21.57, *PSAT1*: 48.05, *RASSF1A*: 114.5 and *SCGB3A1*: 67.18). All genes displayed an Area under the Curve (AUC) superior to 0.70 (Supplementary

File 1—Table S6). *APC* and *SCGB3A1* disclosed 100% specificity for cancer detection, whereas *PSAT1* showed the highest sensitivity (91.97%). *RASSF1A* demonstrated the best individual performance, with 78.83% sensitivity and 96.43% specificity (Table 1). Several gene combinations were tested, and the best detection performance was achieved for the panel comprising *APC*, *FOXA1*, *RASSF1A* and *SCGB3A1*, displaying 97.81% sensitivity, 78.57% specificity and 94.50% accuracy (Table 1, Figure 1). Due to age's difference between patients and controls, a multivariable model was constructed using logistic regression with the most informative genes and age. In this model, age did not show a significant impact in BrC detection ($p = 0.2227$). Moreover, biomarker performance was carried out restricted to BrC patient's with similar age to controls ($p = 0.136$, Mann-Whitney for age). Similar results were obtained in biomarker performance (Supplementary File 1—Table S7).

Table 1. Performance of promoter gene methylation as biomarkers for detection of breast cancer (Brc) in tissue samples.

Genes	Sensitivity %	Specificity %	PPV [a] %	NPV [b] %	Accuracy %
APC	51.09	100.0	100.0	29.47	59.39
CCND2	72.26	92.86	98.02	40.63	75.76
FOXA1	62.04	82.14	94.44	30.67	65.45
PSAT1	91.24	50.00	89.93	53.85	84.24
RASSF1A	78.83	96.43	99.08	48.21	81.82
SCGB3A1	64.96	100.0	100.0	36.84	70.91
APC/FOXA1 RASSF1A/SCGB3A1	97.81	78.57	95.71	88.00	94.55

[a] PPV—Positive Predictive Value; [b] NPV—Negative Predictive Value.

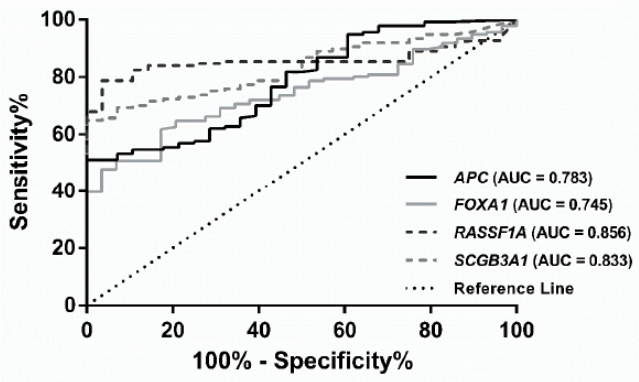

Figure 1. Receiver operating characteristic (ROC) curve of the four-gene panel (*APC*, *FOXA1*, *RASSF1A* and *SCGB3A1*) in Breast Cancer tissues from Cohort #1.

3.3. Association between Promoter Methylation Levels, Molecular Subtypes and Standard Clinicopathological Parameters in Cohort #1

No significant differences in promoter methylation levels were apparent according to molecular subtype (Supplementary File 1—Figure S1), tumor grade, pathological stage or ERBB2 status in tissue samples. Nevertheless, in BrC patients, but not in controls, a significant correlation was found between *CCND2* and *RASSF1A* methylation levels and age (R = 0.194, $p = 0.023$ and R = 0.223, $p = 0.009$, respectively). Additionally, a significant association was found between histological subtype and *APC* and *SCGB3A1* methylation levels: special subtype carcinomas disclosed the lowest *SCGB3A1* methylation levels in comparison to all the other histological subtypes ($p = 0.016$) and lower *APC* methylation levels comparing with invasive lobular carcinomas ($p = 0.0293$).

Additionally, *FOXA1* and *RASSF1A* methylation levels associated with hormone receptor status. ER+ and PR+ BrC displayed significantly lower *FOXA1* methylation levels than ER− and PR− BrC ($p = 0.0084$) or ER+ BrC ($p = 0.0319$). Contrarily, ER+ and PR+ BrC showed higher *RASSF1A* methylation levels than ER+ BrC ($p = 0.0017$). No statistical differences were observed for the remainder genes and for Cohort #2.

3.4. Survival Analysis

Survival analysis was only carried out for Cohort #1, due to the short-time of follow-up of Cohort #2. In the former Cohort (#1), 10 years of follow-up was considered for analysis. During this period, 37 patients (27.0%) had deceased, 24 of which were due to BrC (17.5% of all cases). At the time of the last follow-up, eight patients (5.8%) were alive with cancer and 92 patients (67.2%) were alive with no evidence of cancer.

Clinicopathological features were grouped according to: Grade (G1 & G2 vs. G3), pathological tumor size and extension (pT) stage (pT1, pT2 and pT3 & pT4), pathological regional lymph node status (pN) stage (N0 & N1 vs. N2 & N3) and stage (I, II and III & IV).

Higher tumor grade and pN stage and low *PSAT1* methylation levels categorized by P75significantly associated with worse disease-free survival (DFS) in Cox regression univariable analysis (Table 2). Nonetheless, in multivariable analysis, however, only *PSAT1* methylation levels and pN stage remained independent predictors of DFS (Table 2).

Table 2. Cox regression models assessing the potential of clinical and epigenetic variables in the prediction of disease-free survival for 122 patients with BrC and disease-specific survival for 127 patients with BrC.

Disease-Free Survival	Variable	HR [a]	CI [b] (95%)	*p* Value
Univariable	Grade			
	G1	1		
	G2 [c] & G3	2.054	1.029–4.098	0.041
	pN Stage			
	N0 [d] & N1	1		
	N2 & N3	3.894	1.940–7.812	<0.001
	PSAT1			
	>P75 [e]	1		
	≤P75	3.707	1.133–12.127	0.030
Multivariable	Grade			
	G1	1		
	G2 & G3	1.490	0.717–3.096	0.286
	pN Stage			
	N0 & N1	1		
	N2 & N3	4.345	2.114–8.930	<0.001
	PSAT1			
	>P75 [e]	1		
	≤P75	3.613	1.077–12.123	0.038
Disease-Specific Survival	**Variable**	**HR [a]**	**CI [b] (95%)**	***p* Value**
Univariable	Grade			
	G1	1		
	G2 & G3	2.725	1.155–6.428	0.022
	pN Stage			
	N0 & N1	1		
	N2 & N3	4.061	1.814–9.089	0.001
	FOXA1			
	≤P75 [f]	1		
	>P75	2.678	1.200–5.978	0.016

Table 2. *Cont.*

Disease-Free Survival	Variable	HR [a]	CI [b] (95%)	*p* Value
Multivariable	Grade			
	G1	1		
	G2 & G3	2.005	0.082–4.866	0.124
	pN Stage			
	N0 & N1	1		
	N2 & N3	4.855	1.981–10.611	<0.001
	FOXA1			
	≤P75 [f]	1		
	>P75	2.710	1.161–6.324	0.021

[a] HR—Hazard Ratio; [b] CI—Confidence Interval; [c] G—Grade; [d] N—Regional lymph node status; [e] P75—Percentile 75 of methylation levels of *PSAT1*; [f] P75—Percentile 75 of methylation levels of *FOXA1*.

Concerning disease-specific survival (DSS), in univariable model, pN stage and grade significantly associated with prognosis. Moreover, BrC patients with high *FOXA1* promoter methylation (above P75) levels disclosed shorter DSS (Table 2). In the Cox regression multivariable model, only *FOXA1* methylation levels and pN stage retained significance for DSS prediction (Table 2).

3.5. Biomarker Detection Performance in ccfDNA Liquid Biopsies (Cohort #2)

The 4-gene panel (*APC*, *FOXA1*, *RASSF1A*, and *SCGB3A1*) identified in Cohort #1 was tested in ccfDNA extracted from plasma samples of Cohort #2. *APC*, *FOXA1* and *RASSF1A* promoter methylation levels significantly differed between BrC patients and AC ($p = 0.008$, $p < 0.001$ and $p = 0.017$, respectively), whereas no significant differences were found for *SCGB3A1* ($p = 0.127$) (Supplementary File 1—Table S8). Thus, *SCGB3A1* was not further analyzed. An empirical cutoff value was determined for each gene using ROC curve analysis (*APC*: 3.446, *FOXA1*: 64.38 and *RASSF1A*: 30.00). *FOXA1* disclosed the best individual performance, with 68.18% sensitivity and 82.05% specificity (Table 3). Nevertheless, the three-gene panel achieved 81.82% sensitivity and 76.92% specificity (Table 3, Figure 2). Similar to Cohort #1, a biomarker performance analysis restricted by the maximum age of the controls was performed ($p = 0.777$, Mann-Whitney for age). The biomarker performance was similar (Supplementary File 1—Table S9).

Table 3. Performance of promoter gene methylation as biomarkers for detection of BrC in plasmas samples.

Genes	Sensitivity %	Specificity %	PPV [a] %	NPV [b] %	Accuracy %
APC	27.27	94.87	85.71	53.62	59.04
FOXA1	68.18	82.05	81.08	69.57	74.70
RASSF1A	13.64	100.0	100.0	50.65	54.22
APC/FOXA1/RASSF1A	81.82	76.92	80.00	78.95	79.52

[a] PPV—Positive Predictive Value; [b] NPV—Negative Predictive Value.

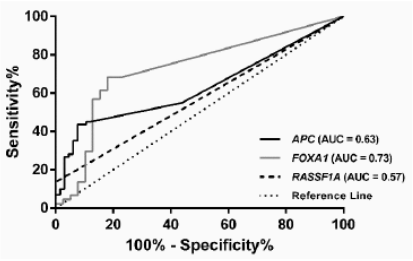

Figure 2. Receiver operating characteristic (ROC) curve of the three-gene panel (*APC*, *FOXA1* and *RASSF1A*) in plasma samples from breast cancer patients from Cohort #2.

4. Discussion

BrC remains the major cause of cancer-related death among women worldwide. Mammographic-based screening has contributed to a 28–45% reduction in BrC mortality [4,33–35], disclosing 70% sensitivity, and 92% specificity for BrC detection [3]. Owing to its limitations, the need for novel detection methods, with improved accuracy and allowing for stratification of BrC aggressiveness has been emphasized [35]. In recent years, the methylome has emerged as the basis for diagnostic and prognostic biomarkers, which might be used in DNA extracted from liquid biopsies [36,37]. Considering published studies on gene promoter methylation in BrC, we aimed to define the best gene panel for detection and prognosis in tissue samples, as well as BrC detection in ccfDNA.

From the seven most promising candidates, six (*APC*, *CCND2*, *FOXA1*, *PSAT1*, *RASSF1A* and *SCGB3A1*) confirmed its cancer-specificity, discriminating normal from cancerous tissues, although with variable performance, paralleling previous observations from our team and others [8,9,38]. Interestingly, a panel combining *APC*, *FOXA1*, *RASSF1A* and *SCGB3A1* disclosed the highest accuracy for BrC detection (94%). *APC*, *RASSF1A* and *SCGB3A1* promoter methylation have been previously tested in a diagnostic setting of fine-needle aspiration biopsy samples [8,9], whereas *FOXA1* expression has been associated with BrC subtype and prognosis [39,40], but not diagnosis. This result compares well with other gene promoter methylation panels that have been reported, disclosing 60–80% sensitivity and 78–100% specificity, and differences in performance are most likely related to biological sample type (tissue vs. bodily fluids) and methylation assessment methods [41].

Since a major goal of this study was to define a panel for BrC detection, ideally its performance should be homogenous regardless of molecular subtype. Thus, we used IHC for tumor subtyping, although acknowledging its limitations in triple negative breast cancer (TNBC)/basal-type classification and luminal A vs. luminal B discrimination [42–44]. Interestingly, no association was found between gene promoter methylation and BrC molecular subtype, suggesting that the gene panel might be effective across molecular subtypes. Some studies have associated DNA methylation and specific molecular subtypes, but these have used a similar proportion of all subtypes or have only analyzed a specific subtype [13,45,46], or even used different methods [10,11,47,48]. Our results, however, are based on a consecutive series of cases, which were not selected according to subtype, and, thus, ERBB2-like and TNBC tumor subtypes are, naturally, in a smaller proportion, which might limit statistical analysis. Nevertheless, *APC* and *SCGB3A1* promoter methylation levels associated with specific histological subtypes, confirming previous observations [49]. Interestingly, *FOXA1* and *RASSF1A* promoter methylation levels associated with hormone receptor status. Although the reason for these associations is unclear, similar results for *RASSF1A* have been reported [50,51]. On the other hand, the higher *FOXA1* promoter methylation observed in hormone receptor negative BrC is in accordance with reported *FOXA1* hypermethylation in basal BrC cell lines [29,52].

Because liquid biopsies represent a promising method for minimally-invasive early cancer detection [24,28], we tested the selected gene panel in ccfDNA. Interestingly, three genes retained diagnostic significance (*APC*, *FOXA1* and *RASSF1A*), whereas *SCGB3A1* did not discriminate BrC patients from controls. These results are in accordance with another study [53], and might be due to differences in sample number and methylation assessment methods [54]. Moreover, the frequency of gene methylation in Cohort #2 was similar to that previous reported in ccfDNA (Table 4) [25,54–63].

Nonetheless, the three gene-panel identified BrC with sensitivity, specificity and accuracy higher than 75%. Thus, this panel disclosed a better combination of sensitivity and specificity than most published studies using plasma or serum samples (Table 5), excepting those of Skvortosova et al. (three-gene panel in plasma) and Kim et al. (four-gene panel in serum) [25,54–63]. Nevertheless, the same authors tested a very limited set of samples (20 BrC, 15 fibroadenomas and 10 healthy donors). Importantly, we used a 4-color multiplex assay that, when compared with the most widely reported two-color multiplex assays represents a faster method and requires less amounts of DNA, thus facilitating its use in clinical routine [36,37,64–66]. Hence, this gene-panel may constitute an

appealing alternative to conventional diagnostic methods, due to its less-invasive characteristics and to also detect also women with a dense breast.

Table 4. Frequency of positive cases [*n*(%)] for methylation levels of cancer-related genes in ccfDNA.

Genes/Panel	Controls (Healthy Donors)		Patients		References
	n	%	*n*	%	
HIC-1/RARβ2/RASSF1A [a]	0/10	0%	18/20	90%	[60]
APC	0/38	0%	8/47	17%	[55]
GSTP1	0/38	0%	12/47	26%	
RARβ2	3/38	8%	12/47	26%	
RASSF1A	2/38	5%	15/47	32%	
APC/GSTP1/RARβ2/RASSF1A	5/38	13%	29/47	62%	
ATM	0/14	0%	13/50	26%	[58]
RASSF1A	0/14	0%	7/50	14%	
ATM/RASSF1A	0/14	0%	18/50	36%	
RARβ2	8/125	6%	103/119	87%	[54]
RASSF1A	6/125	5%	39/119	33%	
SCGB3A1	0/125	0%	36/119	30%	
Twist	10/125	8%	65/119	55%	
RARβ2/RASSF1A/SCGB3A1/Twist	23/125	18%	117/119	98%	
ITIH5	7/135	6%	19/138	14%	[25]
DKK3	2/135	2%	41/138	30%	
RASSF1A	25/135	26%	64/138	47%	
ITIH5/DKK3/RASSF1A	42/135	31%	92/138	67%	
CDH1	0/25	0%	24/50	48%	[57]
RASSF1A	0/25	0%	32/50	64%	
CDH1/RASSF1A	0/25	0%	38/50	76%	
SFN	143/245	58%	197/268	74%	[59]
P16	41/245	17%	60/268	33%	
hMLH1	35/245	14%	75/268	28%	
HOXD13	6/245	2%	37/268	14%	
PCDHGB7	116/245	48%	149/268	56%	
RASSF1A	25/245	10%	46/248	17%	
SFN/P16/hMLH1/HOXD13/PCDHGB7/RASSF1A [b]	68/245	28%	213/268	80%	
ESR1	35/74	47%	80/106	75%	[56]
14-3-3-σ	28/74	38%	69/106	65%	
ESR1/14-3-3-σ [b]	33/74	45%	86/106	81%	
GSTP1	2/87	2%	4/101	4%	[62]
RARβ2	0/87	0%	7/101	7%	
RASSF1A	4/87	5%	12/101	12%	
GSTP1/RARβ2/RASSF1A	6/87	7%	22/101	22%	
DAPK1	0/12	0%	23/26	88%	[63]
RASSF1A	1/12	8%	18/26	69%	
DAPK1/RASSF1A	1/12	8%	25/26	96%	
APC	1/19	5%	23/79	30%	[61]
ESR1	2/19	11%	16/79	20%	
RASSF1A	0/19	0%	28/79	35%	
APC/ESR1/RASSF1A	3/19	16%	42/79	53%	
APC	2/39	5%	12/44	27%	—
FOXA1	7/39	18%	30/44	68%	
RASSF1A	0/39	0%	6/44	14%	
APC/FOXA1/RASSF1A	9/39	23%	36/44	82%	

[a] No information about single gene methylation; [b] The cut-off used in the panel was different the one used in the single gene analysis.

Table 5. Comparison of sensitivity and specificity of previously published panels with values obtained.

Panels	Sensitivity (%)	Specificity (%)	Specimen Type	Methods	References
HIC-1/RARβ2/RASSF1A	90	100	Plasma	MSP [a]	[60]
APC/GSTP1/RARβ2/RASSF1A	62	87	Plasma	QMSP [b]	[55]
ATM/RASSF1A	36	100	Plasma	QMSP [b]	[58]
RARβ2/RASSF1A/SCGB3A1/ Twist	98.3	81.6	Serum	Two-steps QMSP [b]	[54]
ITIH5/DKK3/RASSF1A	67	72	Serum	QMSP [b]	[25]
CDH1/RASSF1A	76	90	Serum	MSP [a]	[57]
SFN/P16/hMLH1/HOXD13/PCDHGB7/RASSF1A	79.6	72.4	Serum	QMSP [b]	[59]
ESR1/14-3-3-σ	81	55	Serum	QMSP [b]	[56]
GSTP1/RARβ2/RASSF1A	22	93	Serum	One-step MSP [a]	[62]
DAPK1/RASSF1A	96	71	Serum	MSP [a]	[63]
APC/ESR1/RASSF1A	53	84	Serum	QMSP [b]	[61]
APC/FOXA1/RASSF1A	81.82	76.92	Plasma	Multiplex QMSP [b]	—

[a] MSP—Methylation-Specific Polimerase Chain Reaction; [b] QMSP—Quantitative Methylation-Specific Polimerase Chain Reaction.

Although BrC displays high mortality and recurrence rate, clinical course is heterogeneous and perfecting disease prognostication might improve patient management. Interestingly, lower *PSAT1* promoter methylation independently predicted for worse DFS. The potential of *PSAT1* methylation to predict BrC recurrence has been previously reported in early diagnosed luminal-type BrC. Futhermore, a correlation between high *PSAT1* methylation levels, on the one hand, and low *PSAT1* mRNAs levels and better outcome, on the other, were disclosed [14]. Interestingly, high *PSAT1* expression were associated with poor outcome in nasopharyngeal carcinoma [67]. These data are in accordance with our findings. Furthermore, high *FOXA1* methylation levels independently predicted shorter DSS, a finding that, to best of our knowledge, has not been reported, thus far. Remarkably, *FOXA1* expression was previously shown to associate with good prognosis and response to endocrine therapy in BrC patients [39,40], and, thus, promoter methylation is the most likely mechanism underlying *FOXA1* downregulation in BrC. In Cohort #1, *RASSF1A* methylation levels did not show prognostic value, which is in accordance with some previous publications [68–70]. Nonetheless, other studies have found *RASSF1A* hypermethylation as a poor prognosis marker in BrC, associating with shorter DFS and DSS [9,22,71]. This discrepancy might be due to differences in sample type and methodologies. Because a meta-analysis suggested that *RASSF1A* methylation is, indeed, associated with worse DFS and DSS [72], additional studies are needed to definitively establish the prognostic value of *RASSF1A* promoter methylation in BrC.

The retrospective nature of Cohort #1, the limited sample size of Cohort #2 and the age differences between BrC patients and controls in both series constitute the main limitations of our study. Nonetheless, it should be emphasized that the use of a multiplex assay for a three-gene panel that is able to accurately detect BrC in ccfDNA, regardless of tumor subtype, constitutes a step forward in this field and allow for a swifter translation into routine clinical practice. Indeed, owing to its characteristics, this panel might not only be useful for BrC detection, but also for disease monitoring which deserves further exploration.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/7/11/420/s1, Supplementary File 1 which includes: Table S1: Primers sequences and QMSP conditions for each gene studied in tissues samples; Table S2: Primers and probe sequences for each gene studied in plasma samples; Table S3: Clinical—pathological data of normal breast tissue, and (NBr) breast cancer (BrC) patient's (Cohort #1); Table S4: Clinical and pathological data of asymptomatic controls (AC) and Breast Cancer (BrC) patients (Cohort #2); Table S5: Frequency of positive cases [*n*(%)] and distribution of methylation levels of cancer-related genes in tissues from Cohort #1 [gene/ACTB × 1000 median (Interquartile range (IQR))]; Table S6: Area Under the Curve (AUC) of Receiver Operating Characteristic (ROC) Curve for each gene in tissues from Cohort#1; Table S7: Performance of promoter gene methylation as biomarkers for detection of BrC in tissue samples from patients with age below 70 years (controls $n = 27$ and tumors $n = 108$); Table S8: Frequency of positive cases [*n*(%)] for methylation levels of cancer-related genes in ccfDNA of Cohort #2.; Table S9: Performance of promoter gene methylation as biomarkers for detection of BrC in plasmas samples from patients with age below 66 years (controls $n = 39$ and tumors $n = 25$);

Figure S1: Boxplots of *APC* (a), *BRCA1* (b), *CCND2* (c), *FOXA1* (d), *PSAT1* (e), *RASSF1A* (f) and *SCGB3A1* (g) methylation levels in the breast cancer molecular subtypes and normal breast tissues.

Author Contributions: S.S. and S.P.N. performed fresh-frozen sections, DNA extraction and QMSP, analyzed data and drafted the manuscript. M.C. and R.H. assisted in the histopathological evaluation of tissue samples and IHC evaluation. P.L. performed the FFPE sections for IHC for molecular subtype determination. M.F. assisted in molecular analyses. L.A. assisted in the statistical analyses. S.P.d.S. and M.F.-S. collected clinical follow-up data. F.C. and P.A. assisted in patients' enrolment for plasma cohort. R.H. and C.J. designed and supervised the study and revised the manuscript. All the authors read and approved the final manuscript.

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significantly associated with increased risk of death (p = 0.024). An intermediate H3K27me3 expression (60-70% pos.) was observed in non-DC BC (CI 95% 1.42-13.92, p = 0.04).

Conclusions: In this preliminary retrospective cohort, H3K27m3 positivity was not found to be a pre-treatment endocrine resistance biomarker. However, statistical trends were observed between H3K27m3 expression and increased cancer-related death risk. Thus, further studies with extended cohort of patients are warranted.

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136P Epigenetic biomarkers in breast cancer: Preliminary results from H3K27m3 assessment in endocrine-treatment resistance

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Background: Breast cancer (BC) is an important cause of morbidity and mortality. Most BC are hormone-receptor positive and can be treated with endocrine treatment (ET), until resistance or toxicity is developed. Specifically, H3K27m3, an epigenetic marker of gene repression, has been associated with prognosis in ET resistant BC. Herein, we aim to further understand its potential role as predictive marker of ET resistance before exposure to treatment.

Methods: A cohort of BC patients diagnosed between 1995 and 2002 at our institution were enrolled after informed consent. Expression of H3K27me3 was determined by immunohistochemistry (IHC) in formalin fixed paraffin embedded tissues. GenASIs™ software was used to assess cell-positivity using a custom profile from positive control. Pre-specified conditions: ≥ 5 frames ≥ 3000 cells analyzed/case. SPSS v24 was used for statistical purposes.

Results: A total of 102 cases were assessed for H3K27me3 immunoexpression. Median of 6 frames/case (range 5-10) and 3311 cells/case (range 3017-5292) were obtained. Of the total cases analyzed, a median of 80% of cells showed positivity (range 9-100%). Using a negative/positive 50% cut-off, 81% were considered positive (pos.). The analyzed cohort displayed a median age of 60 years (33-82 years), 89% were classified as ductal carcinoma (DC) and 38% were grade 3. Concerning IHC subtyping, 43% classified as Luminal A-like, whereas 57% Luminal B-like. Around 66% of the cases evaluated for H3K27me3 expression were treated with adjuvant Tamoxifen exclusively. BC cases with H3K27me3 50-60% positivity were associated with higher cancer-related death (CI 95% 1.10-34.38, p = 0.05), although not reaching significance. Grade 3 BC

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Poster 136-P

Epigenetic biomarkers in breast cancer: preliminary results from H3K27m3 assessment in endocrine-treatment resistance

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INTRODUCTION

Breast cancer (BC) is an important cause of morbidity and mortality[1]. Most BC are hormone-receptor positive and endocrine treatment (ET) is indicated but resistance can develop[1]. Specifically, H3K27m3, a histone marker of gene repression, has been associated with prognosis in ET resistant BC (a PI3K/AKT/mTOR pathway surrogate)[2]. Herein, we aim to further understand its potential role as predictive marker of ET resistance before exposure to treatment.

METHODS

- **BC patients cohort diagnosed between 1995-2002 at our institution were enrolled after informed consent. Only stages I - III were included (AJCC 7th edition) – all samples were reviewed for immunohistochemistry (IHC), namely hormone receptor status. ET-Resistance as defined according to ABC3[ref.3]**
- **Expression of H3K27me3 determined by IHC. A positive control was established from colon cancer samples. GenASIs™ software was used to assess cell expression using a custom profile from positive control in Pathologist-identified areas of carcinoma (*in situ* carcinoma excluded). A grid pattern was used to lower the probability evaluation of repeated areas. Clinical data was blinded to pathologist and investigator using the software.**
- **Pre-specified conditions for each sample: ≥ 5 frames ≥ 3000 cells analyzed/case. SPSS v24 was used for statistical purposes.**

RESULTS

Table 1. Total cases and their analysis regarding medians frames/case, cells analysed/case and **H3K27me3** expression.

Total cases analysed	Median frames/case	Median cells analysed /case	Median H3K27me3 expression
102	6 (5-10)	3311 (3017-5292)	80.3% (9-100%)

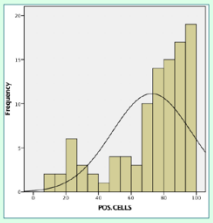

Figure 1. Histogram representing the frequencies (absolute number) and **H3K27m3** expression, by positivity percentage.

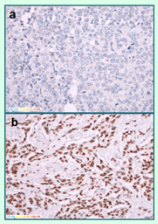

Figure 2. IHC **H3K27m3** expression in invasive breast cancer patients, evaluated by GenASIs™ software. a) Absence of expression (0%) and b) High expression (100%).

Table 2. Comparisons between ET-Sensitive and ET-Resistance recurrences. Notes: ≥ 4 Metastasized nodes at diagnosis; * means there is missing information – presented valid percentages; ᵀGrade 3 BC cases significantly associated with increased risk of death (p=0.024).

Variables N(%)	ET-Sensitive Recurrence (N=15)	ET-Resistant Recurrence (N=14)
Age > 60 years (median)	6 (40%)	**3 (21.4%)**
Luminal A-like / Luminal B-like	6 (40%) / 9 (60%)	**5 (35.7%)/ 9 (64.3%)**
Grade 3ᵀ	4/14* (28.6%)	**8 (57.1%)**
Invasive Ductal Carcinoma	11/13* (84.6%)	**12 (85.7%)**
≥ 4 Metastasized nodes	3/14* (20%)	**4 (28.6%)**
Adjuvant Tamoxifen (only)	6/11* (54.5%)	**7 (50%)**
Cancer-related death	6 (40%)	**10 (71.4%)**
Median H3K27me expression	77.10%	**76.80%**
Median cells analyzed	3291 (3152-4852)	**3368 (3060-4249)**

Table 3. Distibution of cases by **H3K27me3** expression according to 10 groups

Groups	1	2	3	4	5	6	7	8	9	10
Exp %	90-100	80-89	70-79	60-69	50-59	40-49	30-39	20-29	10-19	0-9
N	28	23	19	8	5	4	4	7	3	1
%	27.5	22.5	18.6	7.8	4.9	3.9	3.9	6.9	2.9	1.0

Increased risk cancer-related deaths (CI 95% 1.484 - 7.897, p=0.05)

Associated to ≥ 4 metastasized nodes (CI 95% 1.08-14.44, p=0.05) and Associated to non-IDC histology (CI 95% 1.42-13.92, p=0.04)

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CONCLUSIONS

In this preliminary retrospective cohort, with a 20-year follow-up, H3K27m3 expression was not found to be pre-treatment endocrine resistance biomarker. However, a statistical trend was observed between higher H3K27m3 expression and increased cancer-related death risk. Thus, further studies with prospective and extended cohort of patients are warranted.

Karolinska Institutet

StratCan

2016-06-22

Certificate

This is to certify that ***Mário Fontes e Sousa*** has attended the **StratCan Interactive Summer School** on "***Cancer cell niche***", June 14 – 17, 2016, at the conference hotel Yasuragi, Stockholm, Sweden.

The research school focused on the role of cancer cell niche including cancer stromal cells, immune cells and the factors secreted by cancer stromal cells in cancer initiation and progression and the underlying molecular mechanisms. The preclinical as well as clinical trials targeting cancer cell niche was also included.

At the summer school, ***Mário Fontes e Sousa*** presented his work entitled *Epigenetic markers for Endocrine-treatment resistant breast cancer cell niche: towards an individual and targeted approach'*. In addition, ***Mário Fontes e Sousa*** was selected as a chairman for one of 'short talk' sessions where he did fantastic job in introducing the presentation topics and leading the discussions after the presentations.

Organizers: Hong Qian, Ph.D. Hong Qian Stockholm

On behalf of Karolinska Institute Strategic Research Programme in Cancer (StratCan) and Center for Hematology and Regenerative medicine

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